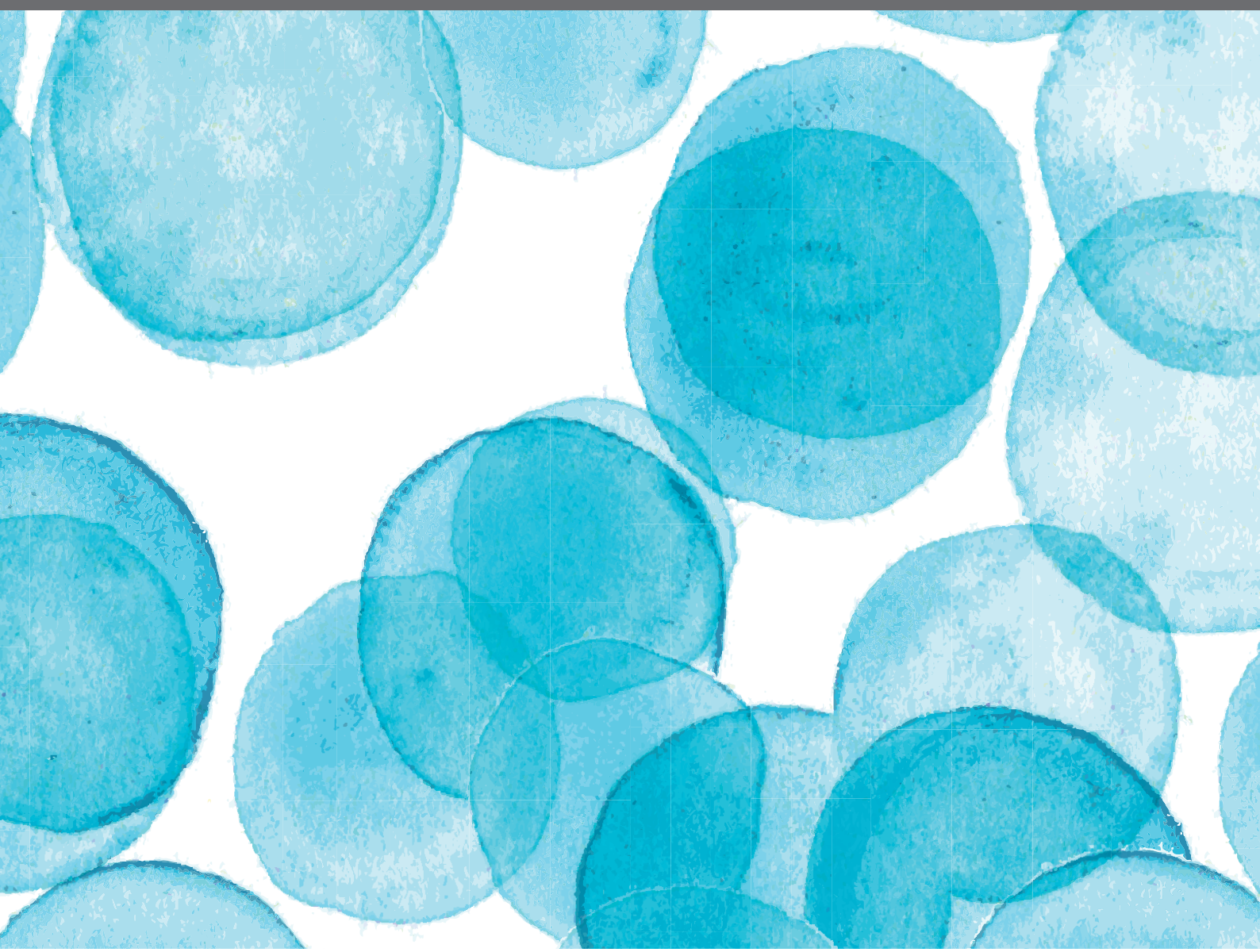
FOODBORNE PATHOGENS: HYGIENE AND SAFETY

EDITED BY: Maria Schirone, Pierina Visciano, Rosanna Tofalo and Giovanna Suzzi

PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

Frontiers Copyright Statement

© Copyright 2007-2019 Frontiers Media SA. All rights reserved.

All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.

The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.

Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.

Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.

As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.

All copyright, and all rights therein, are protected by national and international copyright laws.

The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.

ISSN 1664-8714
ISBN 978-2-88963-186-5
DOI 10.3389/978-2-88963-186-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

FOODBORNE PATHOGENS: HYGIENE AND SAFETY

Topic Editors:
Maria Schirone, University of Teramo, Italy
Pierina Visciano, University of Teramo, Italy
Rosanna Tofalo, University of Teramo, Italy
Giovanna Suzzi, University of Teramo, Italy

Citation: Schirone, M., Visciano, P., Tofalo, R., Suzzi, G., eds. (2019). Foodborne Pathogens: Hygiene and Safety. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88963-186-5

Table of Contents

08 ***Editorial: Foodborne Pathogens: Hygiene and Safety***
Maria Schirone, Pierina Visciano, Rosanna Tofalo and Giovanna Suzzi

12 **Listeria monocytogenes *10403S Arginine Repressor ArgR Finely Tunes Arginine Metabolism Regulation Under Acidic Conditions***
Changyong Cheng, Zhimei Dong, Xiao Han, Jing Sun, Hang Wang, Li Jiang, Yongchun Yang, Tiantian Ma, Zhongwei Chen, Jing Yu, Weihuan Fang and Houhui Song

24 ***MLVA for* Salmonella enterica *subsp.* enterica *Serovar Dublin: Development of a Method Suitable for Inter-Laboratory Surveillance and Application in the Context of a Raw Milk Cheese Outbreak in France in 2012***
Marie-Léone Vignaud, Emeline Cherchame, Muriel Marault, Emilie Chaing, Simon Le Hello, Valerie Michel, Nathalie Jourdan-Da Silva, Renaud Lailler, Anne Brisabois and Sabrina Cadel-Six

34 ***Antibacterial Action of Jineol Isolated From* Scolopendra subspinipes mutilans *Against Selected Foodborne Pathogens***
Vivek K. Bajpai, Shruti Shukla, Woon K. Paek, Jeongheui Lim, Pradeep Kumar and MinKyun Na

41 ***Turn up the Heat—Food and Clinical* Escherichia coli *Isolates Feature Two Transferrable Loci of Heat Resistance***
Erik J. Boll, Roger Marti, Henrik Hasman, Søren Overballe-Petersen, Marc Stegger, Kim Ng, Susanne Knøchel, Karen A. Krogfelt, Joerg Hummerjohann and Carsten Struve

55 **Helicobacter pullorum*: An Emerging Zoonotic Pathogen***
Sundus Javed, Farzana Gul, Kashaf Javed and Habib Bokhari

63 ***Biosynthesis of Silver Nanoparticles From* Protium serratum *and Investigation of Their Potential Impacts on Food Safety and Control***
Yugal K. Mohanta, Sujogya K. Panda, Akshaya K. Bastia and Tapan K. Mohanta

73 ***A Highlight for Non*-Escherichia coli *and Non*-Salmonella *sp.* Enterobacteriaceae *in Dairy Foods Contamination***
Angelo M. B. Amorim and Janaína dos Santos Nascimento

77 ***Detection of Mycobacteria by Culture and DNA-Based Methods in Animal-Derived Food Products Purchased at Spanish Supermarkets***
Iker A. Sevilla, Elena Molina, Maitane Tello, Natalia Elguezabal, Ramón A. Juste and Joseba M. Garrido

87 ***Prevalence and Antimicrobial Susceptibility of* Vibrio parahaemolyticus *Isolated From Short Mackerels (*Rastrelliger brachysoma*) in Malaysia***
Chia W. Tan, Tan T. H. Malcolm, Chee H. Kuan, Tze Y. Thung, Wei S. Chang, Yuet Y. Loo, Jayasekara M. K. J. K. Premarathne, Othman B. Ramzi, Mohd F. S. Norshafawatie, Nordin Yusralimuna, Yaya Rukayadi, Yoshitsugu Nakaguchi, Mitsuaki Nishibuchi and Son Radu

96 ***A Novel Approach to Predict the Growth of* Staphylococcus aureus *on Rice Cake***
Jun Wang, Shige Koseki, Mi-Ja Chung and Deog-Hwan Oh

106 *Comparative Genomic Characterization of the Highly Persistent and Potentially Virulent* Cronobacter sakazakii *ST83, CC65 Strain H322 and Other ST83 Strains*
Hannah R. Chase, Gopal R. Gopinath, Athmanya K. Eshwar, Andrea Stoller, Claudia Fricker-Feer, Jayanthi Gangiredla, Isha R. Patel, Hediye N. Cinar, HyeJin Jeong, ChaeYoon Lee, Flavia Negrete, Samantha Finkelstein, Roger Stephan, Ben D. Tall and Angelika Lehner

115 *Production of Lipopeptide Biosurfactant by a Marine* Nesterenkonia *sp. and its Application in Food Industry*
George S. Kiran, Sethu Priyadharsini, Arya Sajayan, Gopal B. Priyadharsini, Navya Poulose and Joseph Selvin

126 *Intrinsic and Extrinsic Aspects on* Campylobacter jejuni *Biofilms*
Roberta T. Melo, Eliane P. Mendonça, Guilherme P. Monteiro, Mariana C. Siqueira, Clara B. Pereira, Phelipe A. B. M. Peres, Heriberto Fernandez and Daise A. Rossi

141 *Modeling the Combined Effect of Pressure and Mild Heat on the Inactivation Kinetics of* Escherichia coli*,* Listeria innocua*, and* Staphylococcus aureus *in Black Tiger Shrimp (*Penaeus monodon*)*
Barjinder P. Kaur and P. Srinivasa Rao

154 *Colonisation of Meat by* Escherichia coli *O157:H7: Investigating Bacterial Tropism With Respect to the Different Types of Skeletal Muscles, Subtypes of Myofibres, and Postmortem Time*
Caroline Chagnot, Annie Venien, Sandra Renier, Nelly Caccia, Régine Talon, Thierry Astruc and Mickaël Desvaux

163 *Comparison of the Microbiological Quality and Safety Between Conventional and Organic Vegetables Sold in Malaysia*
Chee-Hao Kuan, Yaya Rukayadi, Siti H. Ahmad, Che W. J. Wan Mohamed Radzi, Tze-Young Thung, Jayasekara M. K. J. K. Premarathne, Wei-San Chang, Yuet-Ying Loo, Chia-Wanq Tan, Othman B. Ramzi, Siti N. Mohd Fadzil, Chee-Sian Kuan, Siok-Koon Yeo, Mitsuaki Nishibuchi and Son Radu

173 *Rapid Flow Cytometry Detection of a Single Viable* Escherichia coli *O157:H7 Cell in Raw Spinach Using a Simplified Sample Preparation Technique*
Anna J. Williams, Willie M. Cooper, Shawn Ramsaroop, Pierre Alusta, Dan A. Buzatu and Jon G. Wilkes

181 *Gene Expression Response of* Salmonella enterica *Serotype Enteritidis Phage Type 8 to Subinhibitory Concentrations of the Plant-Derived Compounds* Trans*-Cinnamaldehyde and Eugenol*
Anup Kollanoor Johny, Jonathan G. Frye, Annie Donoghue, Dan J. Donoghue, Steffen Porwollik, Michael McClelland and Kumar Venkitanarayanan

191 *Caco-2 Invasion by* Cronobacter sakazakii *and* Salmonella enterica *Exposed to Drying and Heat Treatments in Dried State in Milk Powder*
Emilie Lang, Stéphane Guyot, Pablo Alvarez-Martin, Jean-Marie Perrier-Cornet and Patrick Gervais

199 *DNA-Sequence Based Typing of the* Cronobacter *Genus Using MLST, CRISPR-*cas *Array and Capsular Profiling*
Pauline Ogrodzki and Stephen J. Forsythe

214 *Prevalence and Antibiotic Resistance of Non-typhoidal* Salmonella *Isolated From Raw Chicken Carcasses of Commercial Broilers and Spent Hens in Tai'an, China*
Song Li, Yufa Zhou and Zengmin Miao

220 *Combination of Microfluidic Loop-Mediated Isothermal Amplification With Gold Nanoparticles for Rapid Detection of* Salmonella *spp. in Food Samples*
Alejandro Garrido-Maestu, Sarah Azinheiro, Joana Carvalho, Sara Abalde-Cela, Enrique Carbó-Argibay, Lorena Diéguez, Marek Piotrowski, Yury V. Kolen'ko and Marta Prado

228 *Risk of Transmission of Antimicrobial Resistant* Escherichia coli *From Commercial Broiler and Free-Range Retail Chicken in India*
Arif Hussain, Sabiha Shaik, Amit Ranjan, Nishant Nandanwar, Sumeet K. Tiwari, Mohammad Majid, Ramani Baddam, Insaf A. Qureshi, Torsten Semmler, Lothar H. Wieler, Mohammad A. Islam, Dipshikha Chakravortty and Niyaz Ahmed

241 *Increased Adhesion of* Listeria monocytogenes *Strains to Abiotic Surfaces Under Cold Stress*
Bo-Hyung Lee, Michel Hébraud and Thierry Bernardi

251 *Characterization of Four Novel Bacteriophages Isolated From British Columbia for Control of Non-typhoidal* Salmonella in Vitro *and on Sprouting Alfalfa Seeds*
Karen Fong, Brett LaBossiere, Andrea I. M. Switt, Pascal Delaquis, Lawrence Goodridge, Roger C. Levesque, Michelle D. Danyluk and Siyun Wang

265 *Food Grade Pimenta Leaf Essential Oil Reduces the Attachment of* Salmonella enterica *Heidelberg (2011 Ground Turkey Outbreak Isolate) on to Turkey Skin*
Divek V. T. Nair and Anup Kollanoor Johny

280 *An Assessment of Different Genomic Approaches for Inferring Phylogeny of* Listeria monocytogenes
Clémentine Henri, Pimlapas Leekitcharoenphon, Heather A. Carleton, Nicolas Radomski, Rolf S. Kaas, Jean-François Mariet, Arnaud Felten, Frank M. Aarestrup, Peter Gerner Smidt, Sophie Roussel, Laurent Guillier, Michel-Yves Mistou and René S. Hendriksen

293 *Prevalence and Antibiotic Resistance Against Tetracycline in* Campylobacter jejuni *and* C. coli *in Cattle and Beef Meat From Selangor, Malaysia*
Jayasekara M. K. J. K. Premarathne, Aimi S. Anuar, Tze Young Thung, Dilan A. Satharasinghe, Nuzul Noorahya Jambari, Noor-Azira Abdul-Mutalib, John Tang Yew Huat, Dayang F. Basri, Yaya Rukayadi, Yoshitsugu Nakaguchi, Mitsuaki Nishibuchi and Son Radu

302 *Genetic Diversity, Antimicrobial Susceptibility, and Biofilm Formation of* Cronobacter *spp. Recovered From Spices and Cereals*
Yuanhong Li, Huan Yu, Hua Jiang, Yang Jiao, Yaodong Zhang and Jihong Shao

313 *Prevalence, Virulence Genes and Antimicrobial Resistance Profiles of* Salmonella *Serovars From Retail Beef in Selangor, Malaysia*
Tze Y. Thung, Son Radu, Nor A. Mahyudin, Yaya Rukayadi, Zunita Zakaria, Nurzafirah Mazlan, Boon H. Tan, Epeng Lee, Soo L. Yeoh, Yih Z. Chin, Chia W. Tan, Chee H. Kuan, Dayang F. Basri and Che W. J. Wan Mohamed Radzi

321 ***Comprehensive Proteomic Analysis of Lysine Acetylation in the Foodborne Pathogen* Trichinella spiralis**
Yong Yang, Mingwei Tong, Xue Bai, Xiaolei Liu, Xuepeng Cai, Xuenong Luo, Peihao Zhang, Wei Cai, Isabelle Vallée, Yonghua Zhou and Mingyuan Liu

336 ***Effect of Various Inoculum Levels of Multidrug-Resistant* Salmonella enterica *Serovar Heidelberg (2011 Ground Turkey Outbreak Isolate) on Cecal Colonization, Dissemination to Internal Organs, and Deposition in Skeletal Muscles of Commercial Turkeys After Experimental Oral Challenge***
Divek V. T. Nair, Jijo Vazhakkattu Thomas, Sally Noll, Robert Porter Jr. and Anup Kollanoor Johny

346 ***Determination of Lipophilic Marine Biotoxins in Mussels Harvested From the Adriatic Sea by LC-MS/MS***
Maria Schirone, Miriam Berti, Pierina Visciano, Francesco Chiumiento, Giacomo Migliorati, Rosanna Tofalo, Giovanna Suzzi, Federica Di Giacinto and Nicola Ferri

355 ***Phenotypic and Genotypic Characterization of* Klebsiella pneumoniae *Isolated From Retail Foods in China***
Shuhong Zhang, Guangzhu Yang, Qinghua Ye, Qingping Wu, Jumei Zhang and Yuanbin Huang

366 **Listeria monocytogenes *Sequence Types 121 and 14 Repeatedly Isolated Within One Year of Sampling in a Rabbit Meat Processing Plant: Persistence and Ecophysiology***
Frédérique Pasquali, Federica Palma, Laurent Guillier, Alex Lucchi, Alessandra De Cesare and Gerardo Manfreda

378 ***Novel Biocontrol Methods for* Listeria monocytogenes *Biofilms in Food Production Facilities***
Jessica A. Gray, P. Scott Chandry, Mandeep Kaur, Chawalit Kocharunchitt, John P. Bowman and Edward M. Fox

390 ***Complete Genomic Analysis of a* Salmonella enterica *Serovar Typhimurium Isolate Cultured From Ready-to-Eat Pork in China Carrying One Large Plasmid Containing* mcr-1**
Wei Wang, Zulqarnain Baloch, Mingyuan Zou, Yinping Dong, Zixin Peng, Yujie Hu, Jin Xu, Nafeesa Yasmeen, Fengqin Li and Séamus Fanning

399 ***Virulence Gene Sequencing Highlights Similarities and Differences in Sequences in* Listeria monocytogenes *Serotype 1/2a and 4b Strains of Clinical and Food Origin From 3 Different Geographic Locations***
Sofia V. Poimenidou, Marion Dalmasso, Konstantinos Papadimitriou, Edward M. Fox, Panagiotis N. Skandamis and Kieran Jordan

414 ***Enhanced Biofilm Formation by Ferrous and Ferric Iron Through Oxidative Stress in* Campylobacter jejuni**
Euna Oh, Katelyn J. Andrews and Byeonghwa Jeon

423 ***Detection of Foodborne Pathogens by Surface Enhanced Raman Spectroscopy***
Xihong Zhao, Mei Li and Zhenbo Xu

436 ***Prevalence and Characterization of* Staphylococcus aureus *Cultured From Raw Milk Taken From Dairy Cows With Mastitis in Beijing, China***
Wei Wang, Xiaohui Lin, Tao Jiang, Zixin Peng, Jin Xu, Lingxian Yi, Fengqin Li, Séamus Fanning and Zulqarnain Baloch

452 *Optimization of* E. coli *Inactivation by Benzalkonium Chloride Reveals the Importance of Quantifying the Inoculum Effect on Chemical Disinfection*
Míriam R. García and Marta L. Cabo

466 *Detection and Characterization of* Salmonella *Serotypes in the Production Chain of Two Pig Farms in Buenos Aires Province, Argentina*
Rocío Colello, María J. Ruiz, Valeria M. Padín, Ariel D. Rogé, Gerardo Leotta, Nora Lía Padola and Analía I. Etcheverría

474 *Characterizing the Antimicrobial Function of a Dairy-Originated Probiotic,* Propionibacterium freudenreichii*, Against Multidrug-Resistant* Salmonella enterica *Serovar Heidelberg in Turkey Poults*
Divek V. T. Nair and Anup Kollanoor Johny

487 *The Fimbrial Gene* z3276 *in Enterohemorrhagic* Escherichia coli *O157:H7 Contributes to Bacterial Pathogenicity*
Bicheng Zhang, Xiaohan Sun, Hongjie Fan, Kongwang He and Xuehan Zhang

496 *Combinational Inhibitory Action of* Hedychium spicatum *L. Essential Oil and* γ*-Radiation on Growth Rate and Mycotoxins Content of* Fusarium graminearum *in Maize: Response Surface Methodology*
Naveen K. Kalagatur, Jalarama R. Kamasani, Chandranayaka Siddaiah, Vijai K. Gupta, Kadirvelu Krishna and Venkataramana Mudili

511 Cronobacter sakazakii *and Microbiological Parameters in Dairy Formulas Associated With a Food Alert in Chile*
Julio Parra-Flores, Fabiola Cerda-Leal, Alejandra Contreras, Nicole Valenzuela-Riffo, Alejandra Rodríguez and Juan Aguirre

520 *Exploration of the Regulatory Mechanism of Secondary Metabolism by Comparative Transcriptomics in* Aspergillus flavus
Guangshan Yao, Yuewei Yue, Yishi Fu, Zhou Fang, Zhangling Xu, Genli Ma and Shihua Wang

frontiers in Microbiology

EDITORIAL
published: 27 August 2019
doi: 10.3389/fmicb.2019.01974

Editorial: Foodborne Pathogens: Hygiene and Safety

Maria Schirone, Pierina Visciano, Rosanna Tofalo and Giovanna Suzzi*

Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy

Keywords: food, microorganisms, virulence, illness, preservatives

Editorial on the Research Topic

Foodborne Pathogens: Hygiene and Safety

INTRODUCTION

The foodborne outbreaks occurred in last decades highlight the importance of the development and implementation of preventive measures and programs aiming at ensuring food safety on one hand and constituting a common basis for the hygienic production of food on the other hand. In particular, a *farm to fork approach* has been applied in all sectors of food production chain in order to improve hygiene and reduce all potential biological hazards. The food supply chain is very complex because of the differences in food composition and processing and this can result in emergence and re-emergence of foodborne pathogens. However, many factors related to an increase in foodborne illness have been reported, such as the change in eating habits and consumer preferences, increased international travels, change in food processing, production and distribution, pathogen adaptation to new environments, acquisition of virulence factors and antimicrobial drug resistance by microorganisms, advances in pathogen detection methods, inadequate sanitation and vector control measures, inadequate public health services, including consumer information (Smith and Fratamico, 2018). This Research Topic titled "Foodborne Pathogens: Hygiene and Safety" focuses on important food safety concerns such as the potential presence of pathogens in food as well as their toxins/metabolites, the resistance to antibiotics or sanitizers, and other virulence characteristics. It includes four reviews and 44 original research papers. The main foodborne pathogens studied herein are: *Campylobacter jejuni*, *Cronobacter sakazakii*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and *Staphyloccus aureus*, but some other researches deal with *Helicobacter pilori*, *Klebsiella pneumoniae*, *Vibrio parahaemolyticus*, mycobacteria, and molds as well. Studies on characterization and genetic typing of foodborne pathogens, detection methods and inactivation of these microorganisms by natural preservatives derived from plant sources, essential oils and biocontrol, and influence of probiotics are also reported.

PREVALENCE AND MONITORING OF PATHOGENS IN FOOD

Foodborne diseases represent one of the most important public health troubles worldwide. The potential of foodborne pathogens to cause illness or even death in consumers highlights the importance of such events and consequent need of their monitoring and prevention. Millions of cases of foodborne illnesses and/or chronic complications are reported in many countries every year (Heredia and García, 2018). Li S. et al. studied the prevalence and characteristics of Non-typhoidal *Salmonella* isolated from poultry meat (broilers and spent hens) from supermarkets in China. Three serotypes were identified in 40 *Salmonella* strains and *Salmonella* Enteritidis resulted as dominant. The antibiotic resistance was tested as well, showing the highest rates to ampicillin for the strains isolated from commercial broilers, and to nalidixic acid for those isolated from spent

OPEN ACCESS

Edited by:
Dario De Medici,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
David Rodriguez-Lazaro,
University of Burgos, Spain

****Correspondence:***
Maria Schirone
mschirone@unite.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *29 June 2019*
Accepted: *12 August 2019*
Published: *27 August 2019*

Citation:
Schirone M, Visciano P, Tofalo R and Suzzi G (2019) Editorial: Foodborne Pathogens: Hygiene and Safety. Front. Microbiol. 10:1974. doi: 10.3389/fmicb.2019.01974

hence. Thung et al. investigated the prevalence of *Salmonella* spp. in different beef meat samples from retail markets in Malaysia as well as the virulence genes and antimicrobial resistance. Eight different serovars were identified and *Salmonella* Agona was the predominant one. All 23 isolates were resistant at least to three antibiotics. Colello et al. determined the prevalence of *Salmonella* spp. in 764 samples collected from swine farms, slaughterhouses, boning rooms, and retail markets. The strains were classified into five serotypes (i.e., *Salmonella* Typhimurium, *Salmonella* Kentucky, *Salmonella* Brandenburg, *Salmonella* Livingstone, and *Salmonella* Agona) and showed different resistance to antibiotics.

The microbiological quality (mesophilic aerobic bacteria, total coliforms, yeasts, and molds) and safety level (*E. coli* O157:H7, Shiga toxin-producing *E. coli*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Listeria* spp., and *L. monocytogenes*) of organic and conventional vegetables from Malaysia were evaluated. *Salmonella* spp., *L. monocytogenes,* and *Listeria* spp. were the most representatives, with no trend between organically or conventionally grown vegetables (Kuan et al.). The presence of total and pathogenic *V. parahaemolyticus* strains was detected in short mackerel samples collected from different retail markets in Malaysia. The antimicrobial susceptibility profiles were also studied, showing a resistance to penicillin G and ampicillin (Tan et al.).

The genetic diversity as well as the antibiotic resistance and biofilm formation of *Cronobacter* spp. recovered from spices and cereals were studied by Li Y. et al. *Cronobacter sakazakii* was the most common species, and 62.5% of 40 *Cr. sakazakii* strains were non-biofilm producers. Parra-Flores et al. evaluated the presence of *Cr. sakazakii*, microbiological levels of aerobic plate count and *Enterobacteriaceae* in dairy product batches associated with a recent food alert in Chile.

Sevilla et al. investigated the presence of members of the genus *Mycobacterium* by culture and PCR-based methods in raw dairy and meat products purchased at different supermarkets in Spain. Mycobacterial DNA was detected in 23 out of 257 samples, corresponding to *Mycobacterium avium*, *Mycobacterium tuberculosis,* and other non-tuberculous mycobacteria.

Wang W et al. submitted two papers to this Research Topic, the first one concerned the complete genomic analysis of a *Salmonella* Typhimurium isolate from ready-to-eat pork samples in China, the second dealt with the prevalence of *S. aureus* among raw milk from dairy cows with clinical mastitis.

Lipophilic marine biotoxins belonging to okadaic acid, pectenotoxin, yessotoxin, and azaspiracid groups were determined in specimens of mussels collected along the coasts of the Central Adriatic Sea (Italy) by LC-MS/MS. The concentrations exceeded the maximum regulatory limits only for 11 out of 400 samples, and some samples showed a multi-toxin mixture contamination (Schirone et al.).

ANTIMICROBIAL RESISTANCE AND VIRULENCE FACTORS

Microbial interactions can show beneficial or detrimental effects that influence the fate of pathogenic species contaminating foods. The study of such interactions can provide a new knowledge about the different activities of the microorganisms from proliferation and metabolism to pathogenicity and virulence (Zilelidou and Skandamis, 2018).

Dairy products can host microorganisms belonging to *Enterobacteriaceae* family showing multidrug resistance to antibiotics and other virulence factors such as production of biofilm and synthesis of proteolytic and lipolytic enzymes responsible for spoilage. Their presence can be reduced or avoided through good hygiene conditions during processing and manufacturing, as well as storage and distribution (Amorim et al.). Chagnot et al. investigated the adhesion of *E. coli* O157:H7 to well-defined types of skeletal muscle and demonstrated that such microorganism mainly adhered to the extracellular matrix of muscle cells, with no significant differences among the different constituent myofibres, whereas the influence of post-mortem structural modifications of muscle tissues was substantial.

The adhesion capacity of 40 *C. jejuni* strains to abiotic surfaces was studied. All *C. jejuni* strains were shown to be capable of forming strong biofilms when Mueller Hinton medium was supplemented with chicken juice. However, the use of biocides was effective in controlling viable cells of strains in biofilm (Melo et al.). Oh et al. demonstrated that ferrous and ferric iron stimulated biofilm formation in *C. jejuni* through oxidative stress. Premarathne et al. determined the prevalence and antibiotic resistance of *Campylobacter* spp. in the beef food system in Malaysia. Most isolates were identified as *C. jejuni*, with a high percentage resistant to tetracycline and ampicillin.

The effect of cold stress on the adhesion to abiotic surfaces and biofilm formation of 22 *L. monocytogenes* strains from different serogroups and origins was studied by Lee et al. Such study demonstrated the increase of the adhesion capacity, whereas the cold-adapted cells remained in planktonic form. Pasquali et al. studied the persistence and physiological adaptation to food-processing environmental stress of *L. monocytogenes* strains from a rabbit meat processing plant. While some strains showed a resistance to sanitizers, some others were biofilm producers and these specific characteristics could contribute to their high prevalence. The nucleotide diversity of *L. monocytogenes* strains from human clinical cases—as well as food or food-related environments originating from three different geographical locations (i.e., Australia, Greece, and Ireland)—was studied by Poimenidou et al. The authors demonstrated that virulence genes showed different evolutionary pathways affected by the origin and serotype of the specific strain.

Lang et al. demonstrated that drying of milk powder increased the Caco-2 cell invasion capacity of two pathogens, i.e., *Salmonella enterica* and *Cr. sakazakii*, probably due to the activation of stress response transcriptional factors, and a subsequent heat treatment did not offset the loss of cultivability that was observed in the experimental design.

Javed et al. described the characteristics, prevalence, survival, and transmission, as well as pathogenesis and virulence determinants of *Helicobacter pullorum*. Such microorganism causes gastroenteritis in poultry, but it is also an emerging

zoonotic bacterium associated with enteric infections in humans with colitis, hepatitis, and recurrent diarrhea.

DETECTION METHODS APPLICABLE IN FOOD INDUSTRY

A microfluid system combining loop-mediated isothermal amplification with gold nanoparticles for rapid detection of *Salmonella* spp. in food samples was performed. Such method showed relative sensitivity, specificity and accuracy of 100% and could be used in the food industry as a simple, inexpensive and fast analytical approach (Garrido-Maestu et al.). A new standard operating procedure for multiple-locus variable number tandem repeat analysis (MLVA) of *Salmonella* Dublin was proposed by Vignaud et al. The MLVA scheme was applied to a foodborne outbreak occurred in France in 2012, in order to discriminate between epidemiologically related strains and sporadic case strains. Fong et al. characterized four *Salmonella* phages isolated from irrigation water, cattle feces, and sediment from irrigation ditches, based on their phenotypic and genotypic determinants, and assessed their infectivity against various *Salmonella* strains *in vitro*. Among them, the phage isolate SI1 was the most effective in control of *Salmonella* Enteritidis in sprouting alfalfa seeds artificially contaminated.

The study of Ogrodzki and Forsythe described the application of three genotyping methods (Multilocus Sequence Typing, MLST, capsular profiling of the *K*-antigen and colanic acid byosinthesis regions and CRISPR-*cas* array profiling) to discriminate different species belonging to *Cronobacter* genus. Chase et al. found a *Cr. sakazakii* isolate, H322, in a batch of powdered infant formula (PIF) and two other isolates showing indistinguishable Pulsed Field Gel Electrophoresis patterns with H322, during routine testing of these products ready for distribution. Therefore, whole genome sequencing, as well as microarray analysis, was applied to these strains, showing a phylogenetic relation among them. This study confirmed that the pathogen could persist within the PIF manufacturing facility for years.

Wang J. et al. developed a novel approach to predict the growth kinetics of *S. aureus* on rice cake under different environmental conditions. These probability models could be useful for food safety management and microbiological risk assessment of such pathogen.

Listeria monocytogenes encodes a functional ArgR, a transcriptional regulator with specific functions in arginine metabolism regulation and acid tolerance. Cheng et al. showed that a single ArgR regulator could have opposite regulatory effects on the arginine deiminase pathway in an arginine-independent and dependent manner under neutral and acidic conditions, respectively.

Henri et al. compared different genomic methods, i.e., MLST, Whole Genome Sequencing (WGS), and Single Nucleotide Polymorphism (SNP), used to cluster *L. monocytogenes* strains. This study revealed high concordance between MLST and SNP approaches for diagnostic laboratories responsible for outbreak detection and surveillance.

Williams et al. described a rapid flow cytometric method for determining *E. coli* O157:H7 contamination in raw spinach. This method could be used as a screening tool to detect such microorganism in food. The presence of two distinct *loci* of heat resistance on a plasmid encoding type three fimbriae and three bacteriocins, in 1 out 90 *E. coli* raw milk cheese strains, was investigated. Such plasmid was transferable to other *E. coli* strains including Shiga-toxin-producing strains, posing great concern in food production environments (Boll et al.). Hussain et al. evaluated the contamination with pathogenic and/or multiresistant *E. coli* among broiler free-range chicken specimens (ceca and meat). The isolates were characterized using both conventional typing and WGS and compared with human *E. coli* pathotypes. The results showed that the poultry *E. coli* strains shared closer genetic identity to human *E. coli*. Zhang B. et al. demonstrated that a specific genetic marker (named fimbrial gene *z3276*) of Enterohemorrhagic *E. coli* O157:H7 encoded multifunctional structures with properties contributing to host colonization and bacterial survival in the environment.

The regulatory mechanism of secondary metabolism by comparative transcriptomic in *Aspergillus flavus* was studied by Yao et al. Such approach allowed the authors to identify known and novel regulators required for aflatoxins biosynthesis.

Zhang S. et al. determined biotypes, serotypes, virulence genes, and antimicrobial resistance patterns of *K. pneumoniae* strains from retail foods in China. The authors reported that some strains from the same geographic area had a closer relationship and they showed high levels of resistance to ampicillin.

Yang et al. utilized a proteomic approach involving anti-acetyl lysine-based enrichment and highly sensitive mass spectrometry to identify the global acetylated proteome and investigate lysine acetylation in *Trichinella spiralis*.

Zhao et al. described the surface enhanced Raman spectroscopy as testing technology used for the detection of pathogenic bacteria in food. Such method can be considered fast, simple, specific, and sensitive.

PROMISING STRATEGIES FOR FOOD PRESERVATION

Preservation technologies are applied to extend the shelf-life, improve the hygienic quality, and ensure the safety of food. In food industry bacteriocins or other natural preservatives such as herbal extracts and essential oils are used as alternative to prevent the growth of both pathogenic and spoiling microorganisms (Martínez et al., 2019; Nazari et al., 2019).

Gray et al. described novel biocontrol methods such as bacteriophages, endolysins, bacteriocins, and plant derived products (essential oils) for the prevention of biofilm formation by *L. monocytogenes* in food production facilities. The inhibitory effect of *Hedychium spicatum* L. essential oil and radiation on production of deoxynivalenol and zearalenone by *Fusarium graminearum* in maize grains was studied by response surface methodology. The results showed a reduction of fungal growth rate as well as mycotoxin content (Kalagatur et al.).

Bajpai et al. described a significant antibacterial activity of a quinoline compound (jineol) isolated from the insect *Scolopendra subspinipes mutilans* against two selected foodborne pathogens (i.e. *E. coli* O157:H7 and *S. aureus* KCTC-1621). Such compound could be used as alternative means of antimicrobial in pharma and food industries.

The study of García and Cabo focused on the optimization of *E. coli* inactivation by a quaternary ammonium compound based on a mathematical model. The results showed that the optimal disinfectant dose increased exponentially with the initial bacterial concentration.

Different pressure-temperature combinations were applied to investigate the inactivation kinetics of *E. coli*, *Listeria innocua,* and *S. aureus* in black tiger shrimp. *Staphylococcus aureus* was the most baro-resistant species among the three bacteria. Such study could be used to predict non-linear survival curves of other microorganisms in foods (Kaur and Rao).

In their study, Kiran et al. isolated an actinobacterial strain from a marine sponge producing a lipopeptide that was demonstrated to be an effective emulsifier as well as good antioxidant and protective agent against *S. aureus*. The authors used this lipopeptide as food additive in muffin production with good results in organoleptic qualities of such food.

Kollanoor Johny et al. evaluated the antimicrobial effects of subinhibitory concentrations of two plant-derived compounds (i.e., *trans*-cinnamaldehyde and eugenol) on different genes of *S. enterica* serotype Enteritidis phage type 8 associated with virulence, colonization, motility, and invasion capability of such pathogen.

Mohanta et al. described the biological synthesis of silver nanoparticles using a cell-free aqueous leaf extract of plant *Protium serratum* and their antibacterial activity against some foodborne pathogens, i.e., *S. aureus*, *E. coli,* and *Pseudomonas aeruginosa*. The authors suggested the application of such nanoparticles in food packaging materials as well as disinfectant and cleaning agents.

Nair and Kollanoor Johny submitted two papers to the present Research Topic, the first study described the potential of pimenta leaf essential oil in reducing *Salmonella* Heidelberg attachment on to turkey skin during poultry processing, whereas the second work studied the antimicrobial function of a dairy-originated probiotic strain against multidrug resistant *Salmonella* Heidelberg in poults, i.e., young turkeys. The cecal colonization, dissemination to internal organs and potential for skeletal muscle deposition of multidrug resistant strains of *Salmonella* Heidelberg were studied by a challenge experimental design in poults and adult turkey hens. The results showed the highest recovery in the cecum followed by spleen, liver, thigh, and breast, and could be used to better control this microorganism at farm level and improve the safety of turkey products (Nair et al.).

CONCLUSIONS

The high number of studies collected in this Research Topic confirms the importance of foodborne pathogens as a global issue and provides a robust and up-to-date scientific advice. It has been highlighted how much important and essential is a rapid detection of foodborne pathogens by sensitive culture independent methods and by new technologies such as WGS or other biomarkers assay analysis. The outbreak investigations play also key roles in the prevention of foodborne pathogens growth and diffusion, such as their food vehicles and how the contamination can occur in the food supply chain. The positive results of this Research Topic suggest to collect additional and new data for the future on this topic "Foodborne pathogens: hygiene and safety."

AUTHOR CONTRIBUTIONS

MS and PV drafted the editorial. RT and GS contributed to editorial revision. All authors approved the final paper.

REFERENCES

Heredia, N., and García, S. (2018). Animals as sources of food-borne pathogens: a review. *Anim. Nutr.* 4, 250–255. doi: 10.1016/j.aninu.2018.04.006

Martínez, B., García, P., and Rodríguez, A. (2019). Swapping the roles of bacteriocins and bacteriophages in food biotechnology. *Curr. Opin. Biotechnol.* 56, 1–6. doi: 10.1016/j.copbio.2018.07.007

Nazari, M., Ghanbarzadeh, B., Kafil, H. S., Zeinali, M., and Hamishehkar, H. (2019). Garlic essential oil nanophytosomes as a natural food preservatives: its application in yogurt as food model. *Colloid Interface Sci. Commun.* 30:100176. doi: 10.1016/j.colcom.2019.100176

Smith, J. L., and Fratamico, P. M. (2018). Emerging and re-emerging foodborne pathogens. *Foodborne Pathog. Dis.* 15, 737–757. doi: 10.1089/fpd.2018.2493

Zilelidou, E. A., and Skandamis, P. N. (2018). Growth, detection and virulence of *Listeria monocytogenes* in the presence of other microorganisms: microbial interactions from species to strain level. *Int. J. Food Microbiol.* 277, 10–25. doi: 10.1016/j.ijfoodmicro.2018.04.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Schirone, Visciano, Tofalo and Suzzi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 January 2017
doi: 10.3389/fmicb.2017.00145

Listeria monocytogenes 10403S Arginine Repressor ArgR Finely Tunes Arginine Metabolism Regulation under Acidic Conditions

Changyong Cheng[1†], Zhimei Dong[1†], Xiao Han[1], Jing Sun[1], Hang Wang[1], Li Jiang[1], Yongchun Yang[1], Tiantian Ma[1], Zhongwei Chen[1], Jing Yu[1], Weihuan Fang[1,2] and Houhui Song[1]**

[1] College of Animal Science and Technology, China-Australia Joint-Laboratory for Animal Health Big Data Analytics, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection & Internet Technology, Zhejiang A&F University, Lin'an, China, [2] Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, Institute of Preventive Veterinary Medicine, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Maria Schirone,
University of Teramo, Italy

Reviewed by:
Angelica Reyes-Jara,
University of Chile, Chile
Rolf Dieter Joerger,
University of Delaware, USA

***Correspondence:**
Houhui Song
songhh@zafu.edu.cn
Weihuan Fang
whfang@zju.edu.cn

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 06 December 2016
Accepted: 19 January 2017
Published: 31 January 2017

Citation:
Cheng C, Dong Z, Han X, Sun J, Wang H, Jiang L, Yang Y, Ma T, Chen Z, Yu J, Fang W and Song H (2017) Listeria monocytogenes 10403S Arginine Repressor ArgR Finely Tunes Arginine Metabolism Regulation under Acidic Conditions. Front. Microbiol. 8:145. doi: 10.3389/fmicb.2017.00145

Listeria monocytogenes is able to colonize human and animal intestinal tracts and to subsequently cross the intestinal barrier, causing systemic infection. For successful establishment of infection, *L. monocytogenes* must survive the low pH environment of the stomach. *L. monocytogenes* encodes a functional ArgR, a transcriptional regulator belonging to the ArgR/AhrC arginine repressor family. We aimed at clarifying the specific functions of ArgR in arginine metabolism regulation, and more importantly, in acid tolerance of *L. monocytogenes*. We showed that ArgR in the presence of 10 mM arginine represses transcription and expression of the *argGH* and *argCJBDF* operons, indicating that *L. monocytogenes* ArgR plays the classical role of ArgR/AhrC family proteins in feedback inhibition of the arginine biosynthetic pathway. Notably, transcription and expression of *arcA* (encoding arginine deiminase) and *sigB* (encoding an alternative sigma factor B) were also markedly repressed by ArgR when bacteria were exposed to pH 5.5 in the absence of arginine. However, addition of arginine enabled ArgR to derepress the transcription and expression of these two genes. Electrophoretic mobility shift assays showed that ArgR binds to the putative ARG boxes in the promoter regions of *argC, argG, arcA,* and *sigB*. Reporter gene analysis with *gfp* under control of the *argG* promoter demonstrated that ArgR was able to activate the *argG* promoter. Unexpectedly, deletion of *argR* significantly increased bacterial survival in BHI medium adjusted to pH 3.5 with lactic acid. We conclude that this phenomenon is due to activation of *arcA* and *sigB*. Collectively, our results show that *L. monocytogenes* ArgR finely tunes arginine metabolism through negative transcriptional regulation of the arginine biosynthetic operons and of the catabolic *arcA* gene in an arginine-independent manner during lactic acid-induced acid stress. ArgR also appears to activate catabolism as well as *sigB* transcription by anti-repression in an arginine-dependent way.

Keywords: *Listeria monocytogenes*, arginine repressor, ArgR, regulation, acid tolerance

INTRODUCTION

Listeria monocytogenes is a foodborne bacterial pathogen capable of invasion and replication in phagocytic and non-phagocytic cells. This capacity allows it to cross protective epithelial barriers of the human body and cause severe infection with high mortality, especially in elderly populations, infants, immunocompromised individuals, and pregnant women (Cossart, 2011; Lebreton et al., 2015). The bacterium is resistant to acidic environments encountered during food processing, in acidic food, the stomach and phagosomes of macrophages (Cotter and Hill, 2003; Gray et al., 2006), and employs several mechanisms for pH homeostasis to survive or even proliferate in acidic conditions. *L. monocytogenes* utilizes the arginine deiminase (ADI) and agmatine deiminase (AgDI) systems to produce ammonia to neutralize intracellular protons by forming $NH4^+$ to elevate its cytoplasmic pH (Ryan et al., 2009; Chen et al., 2011; Cheng et al., 2013a,b). The general stress responsive alternative sigma factor sigma B (SigB or σB), which was first identified in *Bacillus subtilis* (Boylan et al., 1993), plays a pivotal role in response to environmental stresses in *Listeria* (Ferreira et al., 2001; Xia et al., 2016).

Arginine catabolism *via* the ADI pathway is widely distributed in bacteria enabling them to survive under harsh acidic environments and to evade host defenses (Marquis et al., 1987; Gruening et al., 2006; Lucas et al., 2007; Fulde et al., 2011; Xiong et al., 2015). The ADI pathway consists of three enzymes: ADI, ornithine carbamoyl-transferase and carbamate kinase, which are encoded by *arcA*, *arcB* and *arcC*, respectively. The actions of these three proteins convert arginine to ornithine, ammonia and carbon dioxide (Xiong et al., 2015). In many bacteria, the ADI pathway is regulated by an arginine repressor, ArgR, a hexameric protein that belongs to the ArgR/AhrC family of transcriptional regulators involved in regulation of arginine biosynthetic metabolism in a feedback manner (Fulde et al., 2011; Choi et al., 2012; Xiong et al., 2015). Classical biosynthesis of arginine from glutamate is composed of eight enzymatic steps (**Supplementary Figure S1A**). Five steps involving *N*-acetylated intermediates contribute to formation of ornithine, and three additional steps are required to convert ornithine into arginine *via* several biosynthetic enzymes encoded by *argABCDEFGH* (Cunin et al., 1986). In the recycling pathway as In bacilli and most other prokaryotes, the acetyl group of *N*-acetylornithine is effectively transferred to glutamate by an acetyltransferase (ArgJ), making *N*-acetylglutamate synthase (ArgA), and *N*-acetylornithinase (ArgE) of the linear pathway redundant (Lu, 2006). This situation is also found in *L. monocytogenes* (**Supplementary Figure S1B**). Generally, ArgR proteins regulate their target genes by binding to the operator sites (called ARG box), leading to repression of arginine biosynthetic genes and activation of catabolic genes in the presence of arginine. ArgR proteins also regulate various genes involved in arginine transport (Maghnouj et al., 1998; Caldara et al., 2006). ArgR-mediated regulation network has been shown to respond to various environmental stimuli, such as changes in concentration of arginine and other metabolites and fluctuations in pH, temperature, and oxygen tension (Dong et al., 2004; Gruening et al., 2006; Xiong et al., 2015).

Homologs of the ADI and the arginine biosynthesis pathway genes have been found in the sequenced genome of *L. monocytogenes* strain EGD-e by *in silico* analysis (Glaser et al., 2001). The ADI encoded by *arcA*, is a critical enzyme in the ADI system that triggers the first reaction. The molecular characteristics of the ADI system and its contributions to acid tolerance of *L. monocytogenes in vitro* have been studied (Ryan et al., 2009; Cheng et al., 2013b), but the underlying regulatory mechanisms have not been determined. Moreover, SigB is an important component that links survival to environmental stress and virulence in *L. monocytogenes* and is involved in the regulation of more than 150 genes (Palmer et al., 2011; Xia et al., 2016). Nevertheless, little is known about the regulation of transcription and expression of SigB in *L. monocytogenes*. Here, we report that *L. monocytogenes* ArgR is a negative regulator of the expression, not only of arginine biosynthesis genes (*argCJBD* and *argGH*) but also of ArcA, essential for arginine catabolism, and of SigB. Such regulation might occur by direct interaction with its ARG boxes in the promoter regions. Most notably, we determined that ArgR plays a unique role in acidic tolerance of *L. monocytogenes* by exerting a regulatory role on *arcA* and *sigB*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Listeria monocytogenes 10403S was used as the wild-type strain. *Escherichia coli* DH5α was employed for cloning experiments and as the host strain for plasmids pET30a(+; Merck, Darmstadt, Germany), pERL3 and pKSV7. *E. coli* Rosetta (DE3) was used for prokaryotic protein expression. *L. monocytogenes* was cultured in brain heart infusion (BHI) medium (Oxoid, Hampshire, England). DH5α and Rosetta (DE3) cells were grown at 37°C in Luria-Bertani broth (LB; Oxoid). Stock solutions of ampicillin (50 mg/ml), erythromycin (50 mg/ml), kanamycin (50 mg/ml), or chloramphenicol (50 mg/ml) were added to media when necessary. All chemicals were obtained from Sangon Biotech (Shanghai, China), Merck or Sigma-Aldrich (St. Louis, MO, USA) and were of the highest available purity.

Bioinformatics Analysis

Alignment of nucleotide and deduced amino acid sequences was performed with MUSCLE by using Geneious software (Edgar, 2004). The amino acid sequences of ArgR of *L. monocytogenes* 10403S strain and its homologs in other microbial species were obtained from the National Centre for Biotechnology Information database (NCBI). The known crystal structure of *B. subtilis* ArgR (PDB: 1F9N) was acquired from the Protein Data Bank (PDB). A putative model of *L. monocytogenes* ArgR was constructed using SWISS-MODEL Workspace (Arnold et al., 2006; Bordoli et al., 2009; Bordoli and Schwede, 2012). Promoters of genes of interest from the *L. monocytogenes* 10403S genome sequence were identified using the BPROM modules of the Softberry website[1]. This program gives output scores from

[1]http://www.softberry.com/

−1 to ∼25 to estimate the likelihood that a predicted promoter is functional and a higher score indicates that the prediction is more likely to be correct. ArgR binding sites composed of two palindrome sequences, known as ARG boxes, have been identified previously in several bacteria species (Larsen et al., 2005; Kloosterman and Kuipers, 2011; Perez-Redondo et al., 2012). Promoter/operator elements containing ARG box motifs were identified by searching the *L. monocytogenes* genome with a position weight matrix derived from known *E. coli* ArgR recognition elements (Chen et al., 1997), using the Virtual Footprint software program[2] (Munch et al., 2005).

Construction of Gene Deletion Mutant

The temperature-sensitive pKSV7 shuttle vector was used for generating mutations in *L. monocytogenes* 10403S. A homologous recombination strategy with the splicing by overlap extension (SOE) PCR procedure was used for in-frame deletion to construct gene deletion mutants (Monk et al., 2008). DNA fragments containing homologous arms upstream and downstream of the gene of interest were obtained via amplification of 10403S genomic DNA using the primer pairs listed in Supplementary Table S1. The obtained fragment was then cloned into pKSV7 and transformed into DH5α. After confirmation by sequencing, the recombinant vector containing the target gene deletion cassette was electroporated into the competent *L. monocytogenes* cells. Transformants were selected on BHI agar plates containing chloramphenicol (10 μg/ml). A single transformant was serially passaged at a non-permissive temperature (41°C) in BHI medium containing chloramphenicol to promote chromosomal integration. A single colony with chromosomal integration was successively passaged in BHI medium without chloramphenicol at a permissive temperature (30°C) to enable plasmid excision and curing (Camilli et al., 1993). Recombinants were identified as chloramphenicol-sensitive colonies, and mutagenesis was further confirmed by PCR and DNA sequencing. The single mutant strain was used in a second round of mutagenesis to construct double deletion mutants.

Complementation of *argR* Deletion Mutant

To complement the *L. monocytogenes* ΔArgR strain, the complete *argR* open reading frame (ORF) along with its promoter was amplified from genomic DNA using primer pairs listed in Supplementary Table S1. After digestion with appropriate enzymes, the PCR product was cloned into pERL3, a plasmid capable of replication in Gram positive bacteria. The resulting plasmid was then electroporated into the *L. monocytogenes* ΔArgR strain. Plasmid-containing cells were selected on BHI agar plates containing erythromycin (10 μg/ml). The complemented strain was designated as CΔArgR.

Expression and Purification of Recombinant Proteins

The recombinant proteins used in this study were expressed as fusion proteins to the N-terminal His-tag using pET30a(+) as the expression vector. Rosetta (DE3) was used as the expression host. The full-length ORF of the gene of interest from the 10403S genome was amplified with the primer pair listed in Supplementary Table S1 and inserted into the pET30a(+) vector, and finally transformed into Rosetta competent cells. *E. coli* cells harboring recombinant plasmids were grown in 250 mL LB supplemented with 50 μg/mL kanamycin at 37°C until cultures reached 1.2–1.4 at $OD_{600\ nm}$. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression of interest proteins for an additional 3–4 h at 30°C in the form of soluble protein. His-tagged fusion proteins were purified using nickel-chelated affinity column chromatography (Weishi-Bohui Chromtotech Co., Beijing, China). Specifically, IPTG-induced cell pellets were collected, re-suspended in 50 mM PBS (pH 7.4) and disrupted by sonication. After centrifugation at 12,000 g for 30 min, the soluble protein samples were collected and loaded onto a 1 ml pre-packed nickel-chelated agarose gel column according to the manufacturer's instructions. Finally, expression and purification of recombinant proteins were analyzed via 10% SDS-PAGE followed by Coomassie brilliant blue staining and protein concentration was quantified with the Bradford method.

Preparation of Polyclonal Antibodies against Recombinant Proteins

Rabbits were initially immunized via subcutaneous injection of 500 μg protein with an equal volume of Freund's complete adjuvant (Sigma). After 2 weeks, rabbits were given subcutaneous booster injections of 250 μg protein each in incomplete Freund's adjuvant (Sigma) three times at 10-day intervals. Rabbits were bled ∼10 days after the last injection.

Producing a Truncated ArgR by Site-Directed Mutagenesis

To identify the predicted active sites of ArgR, a double mutant (S42AR43A) was generated using the original vector template, pET30a-ArgR, and the QuikChange Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA, USA) with the primer pairs described in Supplementary Table S1. Template DNA was removed via digestion with *Dpn*I (TOYOBO, Osaka, Japan) for 2 h at 37°C. The mutant construct was sequenced to ensure that only the desired single mutations had been incorporated correctly. The corresponding mutant protein was designated $ArgR_{S42AR43A}$, and expressed and purified as described above.

Crosslinking Analysis

The purified N-terminal 6-histidine-tagged ArgR proteins were crosslinked with various amounts of glutaraldehyde (Sigma) in 50 mM HEPES (pH 8.0), containing 150 mM KCl and 1 mM L-arginine. The reaction mixture was incubated with or without 1% β-mercaptoethanol at room temperature for 2 h and the cross-linked ArgR complexes were analyzed by 10% SDS-PAGE, and stained with Coomassie Brilliant Blue.

[2]http://prodoric.tu-bs.de/

Electrophoretic Mobility Shift Assay (EMSA)

DNA binding of ArgR and its mutant $ArgR_{S42AR43A}$ was investigated *in vitro* by using electrophoretic mobility shift assay (EMSA). The DNA fragment of the promoter region of *argC, argG, arcA, or sigB* containing the putative ARG box was generated by PCR with the specific primer pairs (Supplementary Table S1). The DNA fragments were purified with a PCR Purification Kit (Sangon). Then 200 ng DNA was incubated with varying concentrations of purified recombinant ArgR or $ArgR_{S42AR43A}$ in binding buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5.0 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM EDTA, and 20% glycerol) for 30 min at room temperature. Protein-DNA complexes were separated electrophoretically on a native 5% polyacrylamide gel at 80 V with 0.5 x Tris-acetate-EDTA (TAE) buffer and visualized using ethidium bromide staining.

Construction of P_{argG} Fusing *gfp* Reporter Strains and Promoter Studies

For transcriptional fusion of the *argG* promoter (P_{argG}) to the GFP reporter protein, the fragment containing the promoter-operator region of the *argGH* operon was amplified with the primer pair listed in Supplementary Table S1 using genomic DNA from *L. monocytogenes* 10403S as template. In parallel, the promoterless *gfp* allele *gfp*mut3* was amplified from the *Listeria* shuttle vector pAMGFP3 using primers listed in Supplementary Table S1. The two fragments were fused by using overlapping PCR. The resulting fragment containing the promoter-*gfp* fusion was cloned into vector pERL3 to generate the reporter plasmid which was then electroporated into the wild-type 10403S or the ΔArgR strain. Transformants were selected by plating onto erythromycin-containing BHI agar plates. For promoter studies, *L. monocytogenes* was grown to stationary phase ($OD_{600\ nm} = 1.2$) in BHI broth at 37°C, and then exposed to acidic (pH 5.5) or neutral (pH 7.0) conditions for an additional 60 min. Bacteria in 1 mL of culture were harvested by centrifugation, the cell pellets were washed once with 10 mM PBS (pH 7.4) and resuspended in 1 mL of 10 mM PBS. One hundred microliters of the suspension was used for *gfp* measurements and fluorescence observation. For the former, relative fluorescence unites (RFU) were measured in a fluorescence reader (BioTek Synergy H1, Winooski, VT, USA) with excitation at 485 nm and emission at 535 nm. Relative fluorescence values were calculated by subtracting extinction from the PBS background. For the latter, fluorescence intensity was observed by using confocal laser scanning microscopy (FLV 1000; Olympus, Japan).

Survival in Acidic Conditions

Cells from stationary phase cultures of *L. monocytogenes* 10403S, mutants (ΔArgR, ΔSigB, and ΔArgRΔSigB) and complemented strain CΔArgR were harvested, washed in PBS and re-suspended in BHI (adjusted to pH 3.5 with 3M lactic acid). After 30, 60, 90, 120,160, or 200 min of incubation at 37°C, the surviving cells were plated onto BHI agar after appropriate dilutions. The plates were incubated at 37°C for 24 h and survival rates are reported as the mean of three independent experiments, which were performed in duplicate.

Real-Time Quantitative RT-PCR (qRT-PCR)

Listeria monocytogenes wild-type 10403S and its mutant strain ΔArgR were grown to the stationary phase ($OD_{600\ nm} = 1.2$) in BHI broth at 37°C, and then exposed to acidic (pH 5.5) and neutral (pH 7.0) conditions, respectively, for additional 1 h. Total RNA was extracted using the Column Bacterial total RNA Purification Kit (Sangon), according to the manufacturer's instructions, genomic DNA removed using DNase I (TaKara, Japan) and cDNA synthesized with reverse transcriptase (TOYOBO, Osaka, Japan). Real-time quantitative PCR was performed in a 20 μL reaction volume containing 200 ng cDNA, 10 μL SYBR quantitative PCR mix (TOYOBO), and 1 μL gene-specific primers (200 nM, Supplementary Table S1) to measure the transcriptional levels of *arcA, sigB, argC,* and *argG* using the Mx3000P PCR detection system (Agilent). The housekeeping gene, *gyrB,* was used as an internal control for normalization in each sample as previously described (Chen et al., 2011). Relative transcription levels were quantified using the $2^{-\Delta\Delta CT}$ method and shown as relative fold changes (Livak and Schmittgen, 2001). Triplicate assays were performed for each gene.

Preparation of Whole-Cell Lysates and Western Blot Analysis

Bacteria were grown in BHI broth to the stationary growth phase and lysates were prepared as described before (Ryan et al., 2009). Specifically, the stationary bacteria exposed to acidic (pH 5.5) and neutral (pH 7.0) conditions, respectively, for additional 1 h. Bacterial pellets were then re-suspended in 1 mL of extraction solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). One gram of glass beads (G8772, Sigma) was added and samples lysed using the homogenizer Precellys 24 (Bertin, Provence, France) at 6000 rpm for 30 s with intermittent cooling for 30 s (two cycles in total), followed by centrifugation at 12,000 g for 15 min. Supernatants were retained as cell-free extracts. Samples containing equal amounts of protein were subjected to 12% SDS-PAGE and the separated proteins were blotted onto 0.22 μm polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were blocked for 1 h with 5% skimmed milk, and incubated for 1 h with polyclonal antisera against recombinant recombinant ArgR, ArgG, SigB, or ArcA in 0.5% skimmed milk. Next, membranes probed with anti-interest protein were developed using HRP-conjugated goat anti-rabbit IgG (Santa Cruz, California, CA, USA) as the secondary antibody. Chemiluminescence was detected via a bio-imaging system (UVP EC3 Imaging System, UVP Inc., Upland, CA, USA), and the densities of the interest protein bands were normalized to the GAPDH signal and quantified using Quantity One software (Bio-Rad).

Statistical Analysis

All data comparisons were analyzed using the two-tailed Student *t*-test. Differences with *P*-values of 0.05 were considered

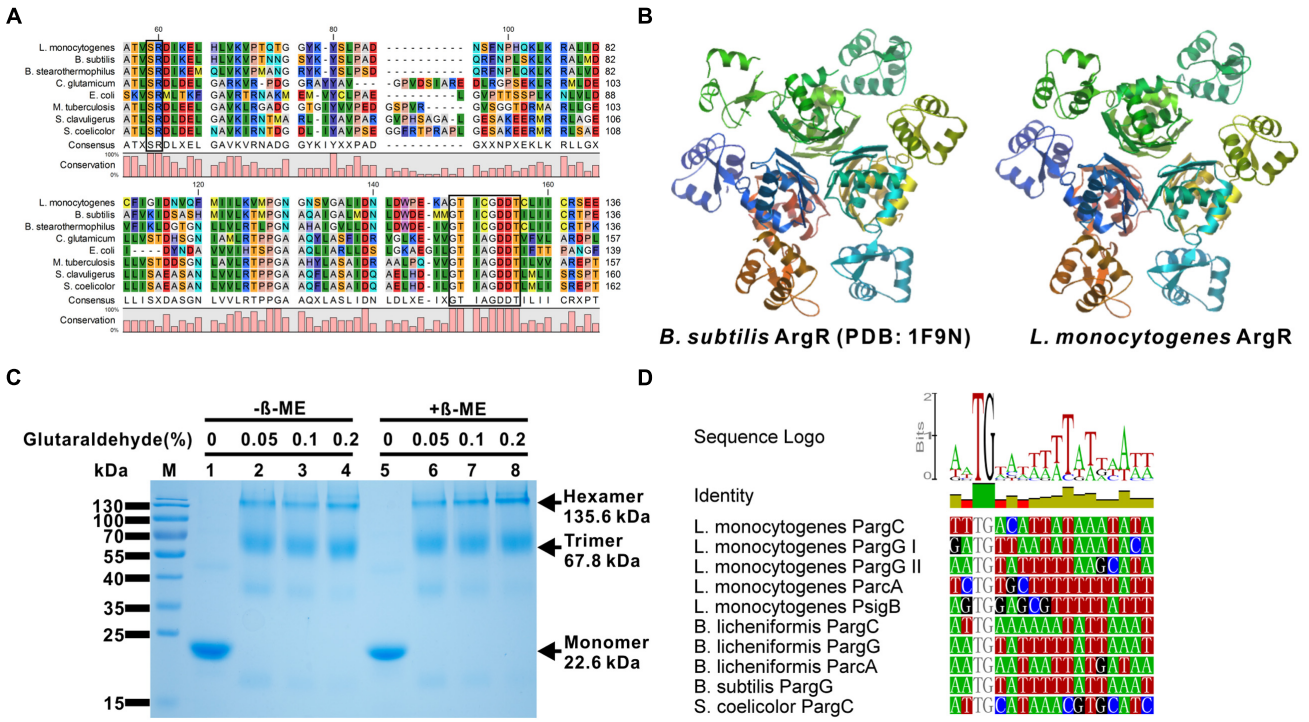


FIGURE 1 | *Listeria monocytogenes* ArgR protein is a member of ArgR/AhrC family transcriptional regulators. (A) Amino acid sequence alignment of *L. monocytogenes* ArgR against the members of the ArgR/AhrC family from *Bacillus subtilis, B. stearothermophilus*, *Corynebacterium glutamicum, Escherichia coli, Mycobacterium tuberculosis, Streptomyces clavuligerus,* and *S. coelicolor*. The two conserved motifs that are responsible for DNA and arginine binding are blackened. **(B)** Predicted tertiary fold of *L. monocytogenes* ArgR using the *B. subtilis* ArgR (PDB: 1F9N) as the template in the SWISS-MODEL Workspace. **(C)** SDS-PAGE analysis of glutaraldehyde crosslinking of *L. monocytogenes* ArgR for the identification of protein polymers. The monomeric, trimeric and hexameric proteins are indicated by arrows. **(D)** Promoter/operator elements containing binding sites of ArgR (ARG box) were identified by searching the *L. monocytogenes* genome with a position weight matrix derived from known ArgR recognition elements using the Virtual Footprint software program (as described in detail in the Materials and Methods). The identified potential consensus binding sites of ArgR in the promoter region from *L. monocytogenes* gene *argC*, *argG*, *arcA,* and *sigB* were further aligned with those from *B. subtilis*, *B. licheniformis,* and *S. coelicolor*.

statistically significant, and those with *P*-values of 0.01 were considered markedly statistically significant.

RESULTS

L. monocytogenes ArgR Is Predicted as a Typical Arginine Repressor

The best characterized ArgR homolog from Gram-positive bacteria is AhrC from *B. subtilis*, as its crystal structure was determined in 2002 (PDB ID: 1F9N; Garnett et al., 2007). To better characterize *L. monocytogenes* ArgR, the amino acid sequence of this protein was aligned with those from seven other bacterial species. The alignment showed sequence identities ranging from 22 to 65%, the highest percentage was exhibited between ArgR from *L. monocytogenes* and AhrC from *B. subtilis* (**Figure 1A**). Based on the amino acid sequence analysis, the *L. monocytogenes* ArgR monomer appears to possess two highly conserved motifs, the "SR" motif for DNA binding (residues 42–43) in the N-terminal region, and the "GTICGDDT" motif for arginine binding (residues 120–127) and oligomerization (residues 125–126) located in the C-terminal region (**Figure 1A**). Furthermore, we modeled *L. monocytogenes* ArgR in the SWISS-MODEL Workspace using the crystal structure of *B. subtilis* AhrC as the template. The predicted structure *L. monocytogenes* ArgR is of high similarity to that of AhrC. Specifically, the monomer ArgR also associates via its C-terminal (core) domain to form a hexamer, probably as a result of the face-to-face association of a pair of trimers (Dennis et al., 2002). The six C-terminal domains strictly follow the 32 non-crystallographic symmetry (NCS) and domains from each trimer locate above another one, giving the hexameric core a stacked configuration when viewed along the threefold axis. The DNA-binding domains (DBD) adopt slightly different positions around the periphery of the core and deviate from strict NCS (**Figure 1B**). The recombinant ArgR protein was expressed in *E. coli*, purified and subjected to crosslinking analysis using glutaraldehyde. The his-tagged ArgR protein had a molecular weight of about 23 kDa, and was able to form higher order multimeric complexes (mainly formed as the trimers and hexamers) in the presence of 0.05% glutaraldehyde (**Figure 1C**). These results suggest that ArgR of *L. monocytogenes* is a typical arginine repressor which might contribute to transcriptional regulation of arginine metabolism.

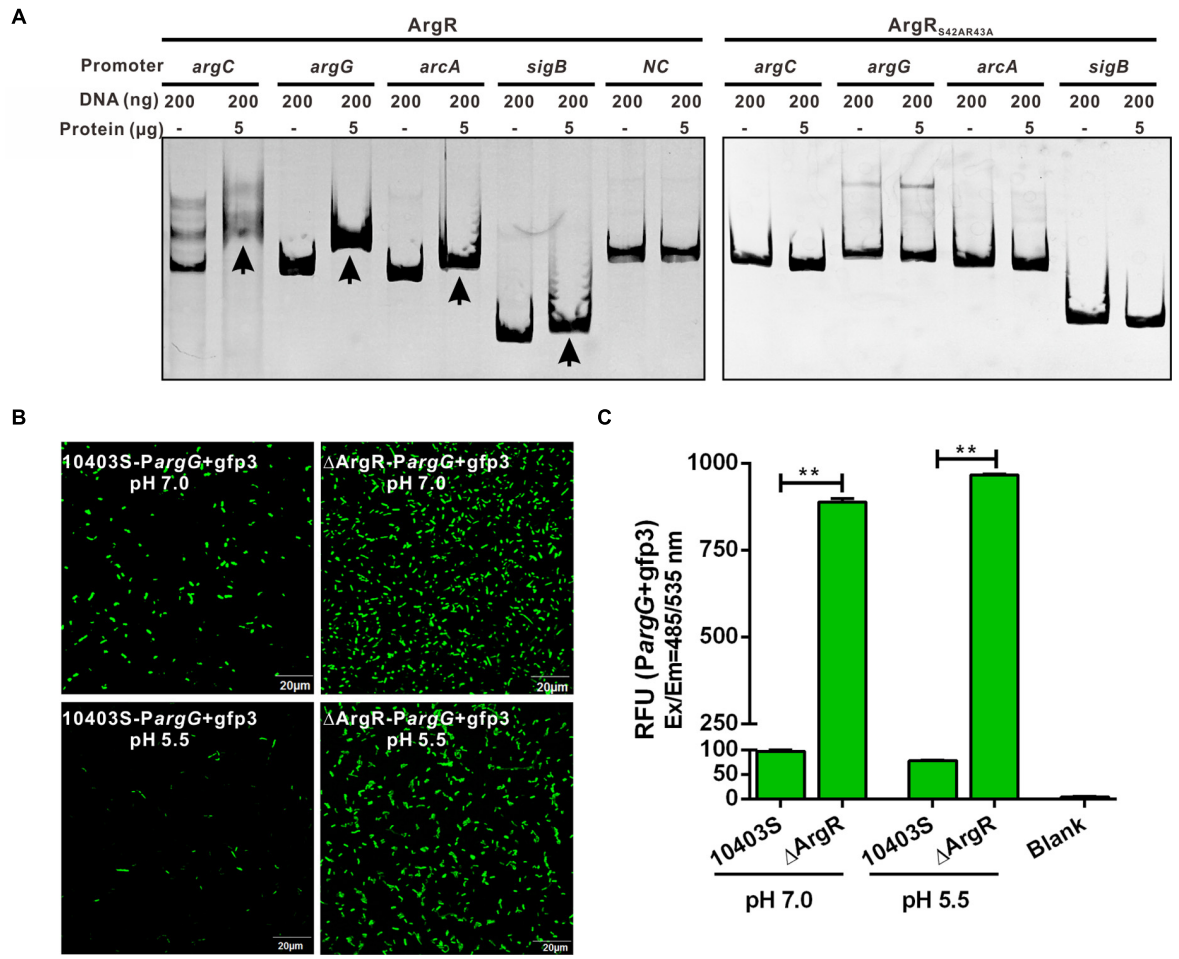


FIGURE 2 | (A) ArgR binds *in vitro* to the operating sites of *argC*, *argG*, *arcA* and *sigB,* and activates the *argG* operon promoter. Gel mobility shift assay (EMSA) experiments were employed to examine binding of the recombinant ArgR from *L. monocytogenes* (ArgR) and its amino acid mutant protein ($ArgR_{S42AR43A}$) to the *argC*, *argG*, *arcA,* and *sigB* promoter region DNA. The promoter fragments were obtained by PCR with primers specified in Supplementary Table S1, and incubated with recombinant proteins for 30 min at room temperature. Gel retardation by DNA–protein complexes was monitored after ethidium bromide staining. The housekeeping gene *gyrB* was used as a negative control (NC) for the EMSAs. Arrows indicate DNA-protein complexes. **(B,C)** ArgR activates the *argG* operon promoter. The fluorescence intensity was observed by confocal laser scanning microscopy of overnight grown *L. monocytogenes* wild-type and ArgR mutant strains carrying a *gfp3* reporter fused with the promoter of *argG*, and then stress-treated for an additional 1 h under pH 7.0 and 5.5 conditions **(B)**. Bars represent the relative fluorescence units (RFU) after subtracting the absolute values for the PBS control **(C)**. Data shown represents the Mean ± SD of three independent experiments, each performed in duplicate. $^{**}P < 0.01$ for comparisons between the wild-type and mutant strains.

ARG Boxes are Present in the Promoter Regions of *argCJBD*, *argGH*, *sigB,* and *arcA*

We further analyzed the sequences of the promoter regions of *argCJBD*, *argGH*, *sigB*, and *arcA* genes for possible ArgR binding sites using a virtual footprint promoter analysis program (see Materials and Methods; Munch et al., 2005). The ARG box consensus was described as TNTGAATWWWWATTCANW in *E. coli* (Maas, 1994), CATGAATAAAAATKCAAK in *B. subtilis* (Miller et al., 1997), and AWTGCATRWWYATGCAWT in *Streptomyces* (Rodriguez-Garcia et al., 1997; where W = A or T, K = G or T, R = A or G, Y = T or C, N = any base). Five putative ARG boxes were identified in each putative promoter region upstream of these five genes from *L. monocytogenes* on the basis of similarity with the *B. subtilis* (Makarova et al., 2001) and *B. licheniformis* (Maghnouj et al., 1998) and there were 1–3 bp mismatch with respect to the consensus sequence (**Figure 1D**).

ArgR Binds *In vitro* to the ARG Boxes of *argCJBD*, *argGH*, *sigB,* and *arcA* Promoters

To confirm that ArgR directly binds to the respective ARG boxes identified above, the EMSA was performed. The promoter regions containing the putative ARG boxes were generated and incubated with recombinant ArgR, and the protein-DNA complexes were assayed by native gel electrophoresis. **Figure 2A** shows that recombinant ArgR was able to bind to DNA oligonucleotides of each promoter of *argCJBD*, *argGH*, *sigB,* or

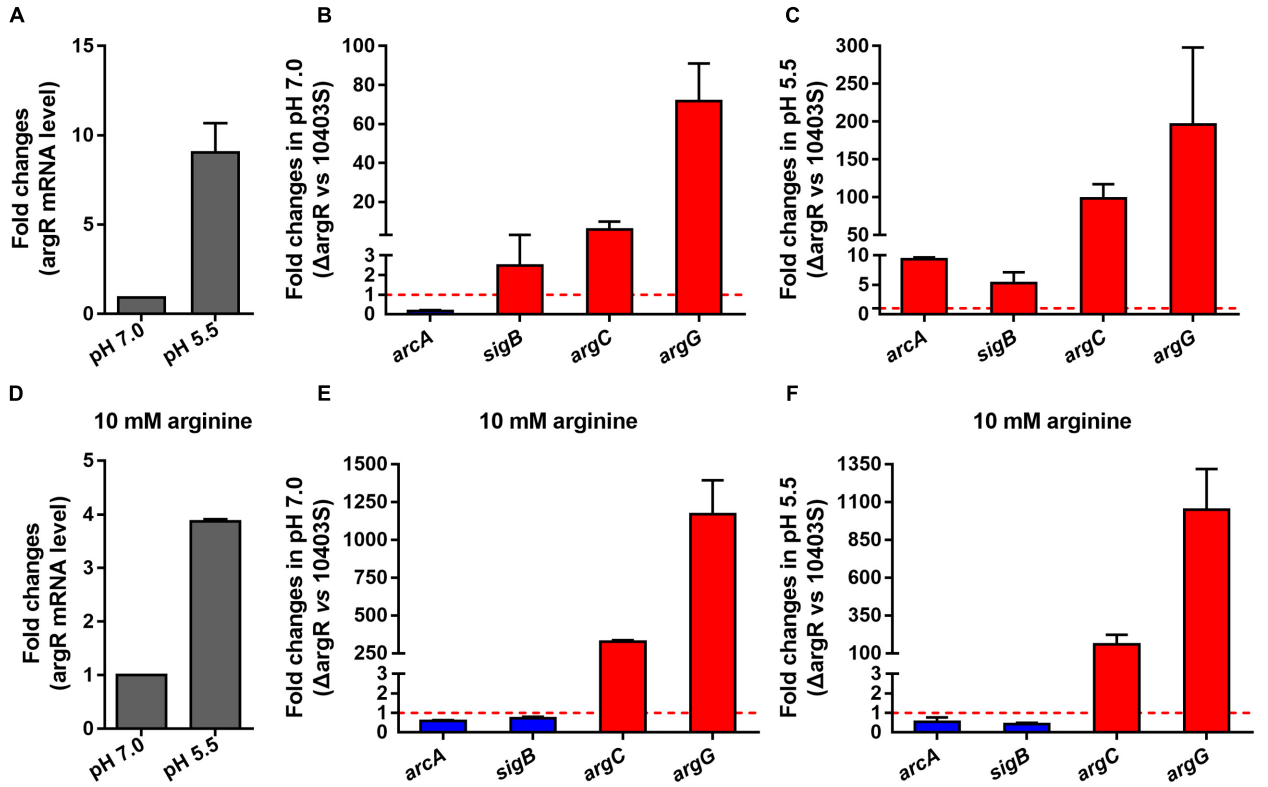


FIGURE 3 | ArgR regulates the transcription of *argC*, *argG*, *arcA,* and *sigB* using arginine as a cofactor. Relative quantification of *argR*, *arcA*, *sigB*, *argC,* and *argG* mRNA and protein expression levels in *L. monocytogenes* wild-type and ΔArgR mutant strains under different pH conditions (7.0 and 5.5) in the presence **(A–C)** or absence **(D–F)** of exogenous arginine (10 mM). Values are expressed as Mean ± SD. The dotted lines indicate the onefold change in transcription of the interest genes.

arcA. More significantly, ArgR shows stronger binding capacity to *argCJBD* and *argGH* promoters than to those of *sigB* and *arcA* under the experimental conditions we studied. These results indicate that *L. monocytogenes* ArgR contributes to overall regulation of the *argCJBD*, *argGH*, *sigB,* and *arcA* promoter activities although the degree of regulation could be different. More importantly, we found that ArgR protein completely lost the binding ability to ARG boxes when the two residues Ser42 and Arg43 (SR motif) were mutated to alanine (**Figure 2A**), strongly suggesting that these two sites are critical amino acids for ArgR-DNA binding.

ArgR Activates the *argG* Operon Promoter

To further analyze the regulatory function of ArgR in expression of its target genes, we cloned a DNA fragment covering the promoter-operator region of the *argGH* operon (as the representative gene cluster involved in arginine anabolism) into the *gfp* reporter vector which was transformed into ΔArgR mutant and its parent strain. Data show that GFP expression was significantly elevated in the ΔArgR mutant under neutral and acidic conditions while GFP was barely detectable in the wild-type strain (**Figures 2B,C**). Thus, ArgR is shown to repress the arginine biosynthetic pathway by interacting with the promoter of the *argGH* operon.

ArgR Regulates Transcription and Expression of *argC*, *argG*, *arcA,* and *sigB*

Since the putative ARG boxes are present in the promoter regions of *argC*, *argG*, *arcA,* and *sigB*, transcription of these genes might be regulated by ArgR. To find out if such regulation occurs, real-time quantitative PCR was performed using total RNA isolated from the wild-type strain *L. monocytogenes* 10403S and the *argR* deletion mutant ΔArgR in the presence (10 mM) or absence of arginine. We found that expression of *argR* was significantly induced in response to acidic pH at 5.5 regardless of arginine supplementation (**Figures 3A,D**). The transcriptional levels of two representative genes (*argC and argG)* involved in arginine anabolism were significantly increased in the ΔArgR mutant under neutral or acidic conditions (**Figures 3B,C**), and such effects were augmented by addition of exogenous arginine (**Figures 3E,F**). These findings indicate that *L. monocytogenes* ArgR plays a classical role of ArgR/AhrC family in feedback inhibition of the arginine biosynthetic pathway using arginine as a corepressor. Transcription of *arcA* was downregulated in the ΔArgR mutant under neutral pH (**Figure 3B**), which is consistent with findings from the previous study by Ryan et al. (2009),

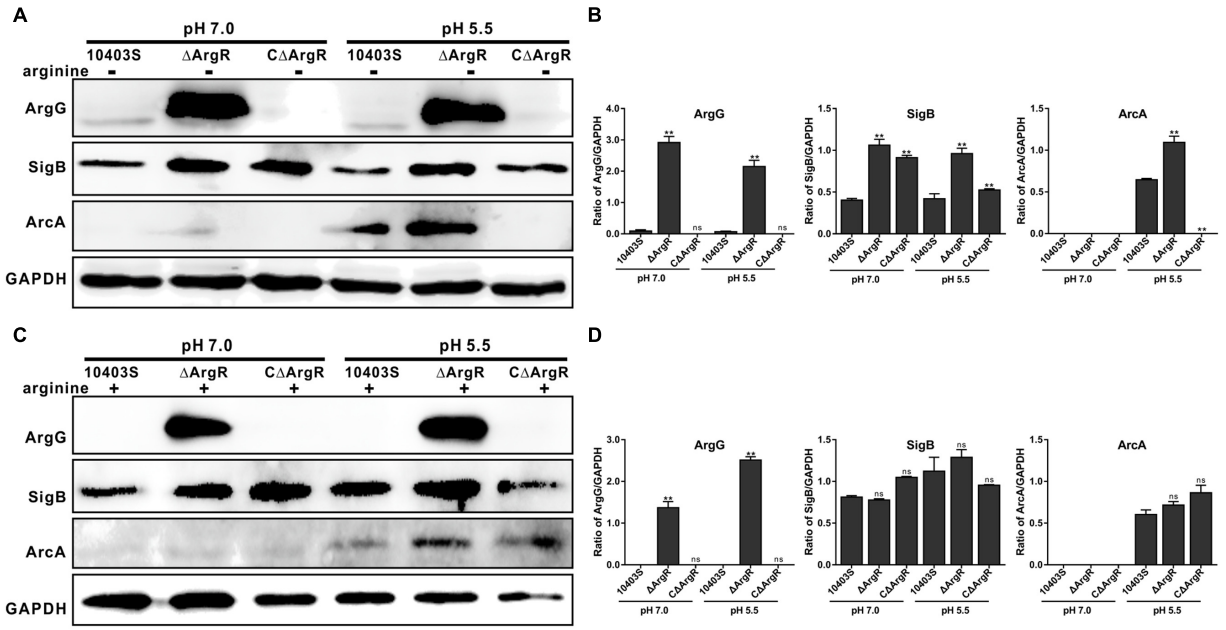


FIGURE 4 | ArgR regulates the expression of ArgG, SigB, and ArcA using arginine as a cofactor. Total bacterial cell-free protein was isolated 2 h after stress under different pH conditions (7.0 and 5.5) in the absence **(A)** or presence **(C)** of exogenous arginine (10 mM), and the protein expression levels of ArgG, SigB, and ArcA were then determined by Western blotting. GAPDH was used as an internal control. The results are indicated as of the gray scale ratio of each interest protein to GADPH **(B,D)**. Data shown represents the Mean ± SD of two independent experiments. $^{**}P < 0.01$; ns means no significance.

whereas *sigB* was slightly upregulated (**Figure 3B**). However, these two genes were markedly repressed by ArgR when bacterial cells were exposed to acidic pH in the absence of arginine (**Figure 3C**), but addition of arginine weakened the effect of ArgR on transcription of *arcA* and *sigB* regardless of pH conditions (**Figures 3E,F**). Therefore, *L. monocytogenes* ArgR appears to be a functional transcriptional regulator that modulates the expression of the *arc* operon positively and negatively under neutral and acidic pH conditions, respectively, by employing arginine as a cofactor.

Immuno-blotting was used to determine the relevance of ArgR to the expression of arginine metabolism operon proteins ArgG and ArcA as well as SigB under neutral and acidic pH (5.5) conditions. Expression of ArgG in ΔArgR strain was significantly higher under neutral and acidic environments than that of the wild-type strain. (**Figures 4A,B**). When exogenous arginine was added, expression of ArgG was not detected in the wild-type strain, but expression strongly increased when ArgR was absent (**Figures 4C,D**), further indicating that arginine cooperates with ArgR to repress the arginine biosynthetic pathway in *Listeria*. In addition, ArgR can regulate the expression of ArcA and SigB in an arginine-dependent and independent manner (**Figure 4**), which is consistent with the results from transcriptional analysis mentioned above.

Deletion of ArgR Enhances Survival of *L. monocytogenes* at Lethal Acidic pH

In order to investigate the contribution of ArgR to the survival of the bacterium at lethal pH values, acid tolerance experiments were carried out on the mutants in complex medium adjusted to a lethal pH of 3.5 using 3 M lactic acid. Data show that deletion of *argR* exhibited no significant difference in the rate of survival relative to the parent strain at the early time points (30 and 60 min; **Figure 5**). However, a notable increase in the number of surviving cells was observed for the ΔArgR from minutes 90 onward, as compared to those of the wild-type strain (**Figure 5**). Conversely, constitutive overexpression of ArgR compromised bacterial survival under the same pH conditions (**Figure 5**). It's worth noting that these data are contradictory to findings by Ryan et al. (2009) who reported that *L. monocytogenes* ΔArgR had defect in acid resistance at both sublethal and lethal pH levels (Ryan et al., 2009). Nonetheless, based on our findings for ArgR involved in regulations on *arcA* and *sigB*, we speculated that increasing of the acidic survival in the absence of ArgR was most probably due to the activation *arcA* and *sigB*. In addition, consistent with our previous studies (Cheng et al., 2015), survival of *L. monocytogenes* was markedly compromised in the absence of *sigB* in the lethal acidic conditions (**Figure 5**).

DISCUSSION

The current study demonstrates that *L. monocytogenes* deploys ArgR to control arginine metabolism by negative regulation of arginine metabolism associated genes *via* binding to the putative ARG box operators as previously described (Fulde et al., 2011; Xiong et al., 2015). Structure modeling and oligomerization analysis indicate that *L. monocytogenes* ArgR has features similar to those of arginine repressors from other bacteria species, in

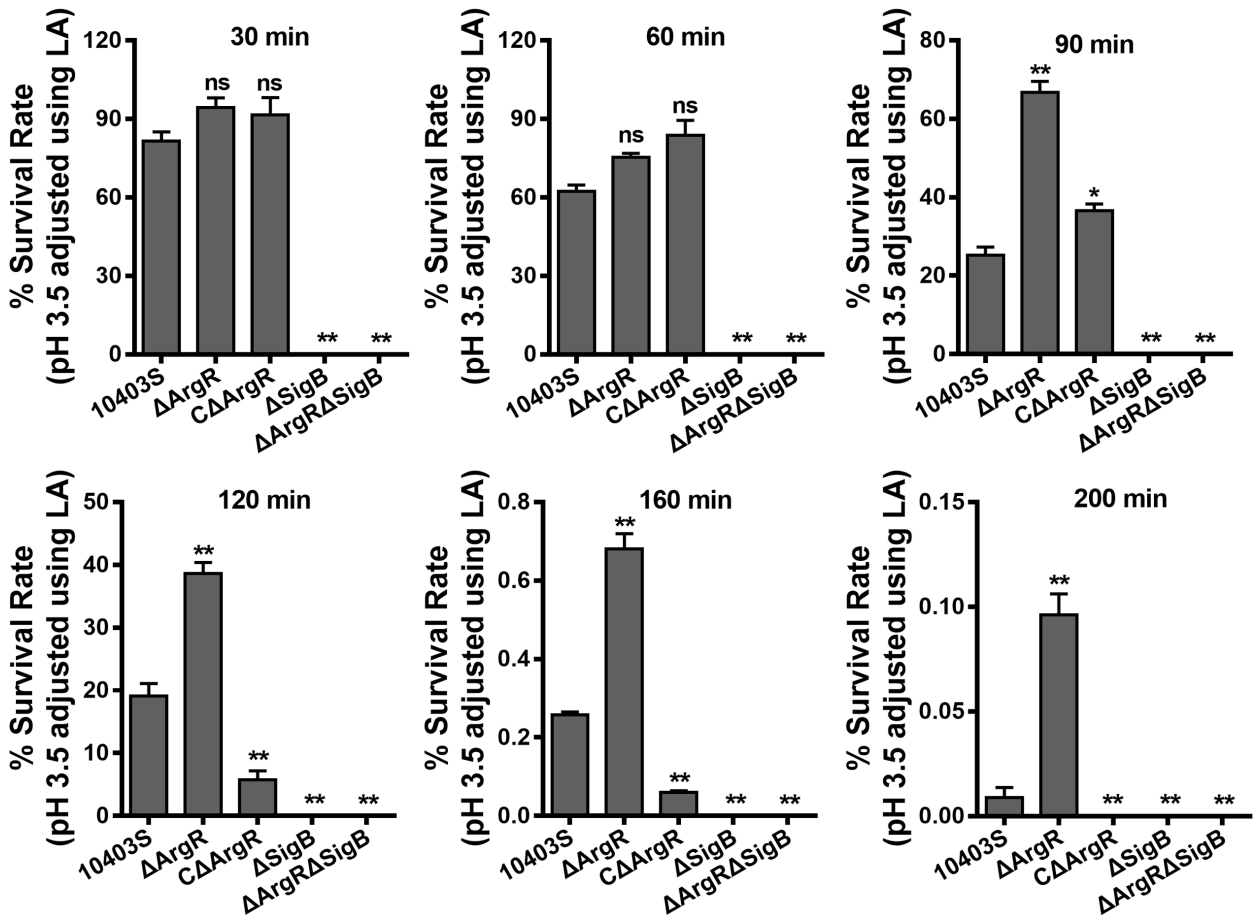


FIGURE 5 | Deletion of ArgR enhances survival of *L. monocytogenes* in the lethal acidic conditions. Overnight-grown *L. monocytogenes* wild-type and mutant strains were harvested, washed and then incubated in BHI broth (pre-adjusted to pH 3.5 using 3 M lactic acid, LA) at 37°C. Survivors were enumerated at regular intervals by plating serial dilutions on BHI plate. Data are expressed as Mean ± SD of recovery rate for each strain. $^{*}P < 0.05$; $^{**}P < 0.01$; ns means no significance.

particular with ArgR from *B. subtilis* (Dennis et al., 2002; Garnett et al., 2007). The N-terminal domain of ArgR is the DBD, whereas the C-terminal domain required for oligomerization and arginine binding (Sunnerhagen et al., 1997; Ni et al., 1999; Garnett et al., 2008). The ArgR protomers can form trimers and hexamers that are in equilibrium and their oligomerization state is manipulated by the presence of arginine corepressor that is bound in the space between ArgR trimers and link each pair of opposite trimers *via* their guanidinium groups, thereby providing additional stability as hexamer (Ni et al., 1999; Cherney et al., 2009).

As is the case in other bacteria species (Larsen et al., 2004; Nicoloff et al., 2004), we found that ArgR in *L. monocytogenes* also acts as a negative regulator of the arginine biosynthetic pathway by repression of *argCJBD* and *argGH* in the absence of ArgR, and such a regulatory effect was augmented under acidic conditions or in the presence of arginine. Generally, ArgR has been demonstrated to act as a positive regulator of *arcABC* operon expression in many bacteria species, which is essential for acid resistance (Griswold et al., 2004; Fulde et al., 2011; Xiong et al., 2015). ArcA and *sigB* were repressed by ArgR in the absence of extracellular arginine, while such effects were not seen when extracellular arginine was added. Notably, deletion of ArgR markedly enhanced the capacity of *L. monocytogenes* to survive in the lethal acid environments. However, Ryan et al. (2009) have previously noted that *L. monocytogenes* ΔArgR demonstrated a great defect in acid resistance at both sublethal and lethal pH levels. It's worth noting that we used the same acidic conditions (media adjusted to pH 3.5 using 3 M lactic acid) and bacterial growth status as reported by Ryan et al. (2009); however, these authors used *L. monocytogenes* LO28, a serotype 1/2c strain (Ryan et al., 2009). We here speculate that the capacity of *L. monocytogenes* to survive in acidic environments was attributable to the activation of *arcA* and *sigB* in the absence of ArgR. It is well known that in a number of bacterial species, catabolism of arginine *via* the ADI pathway has been demonstrated to play a critical role in an enhanced capacity to survive under acidic extracellular conditions (Xiong et al., 2014; Xu et al., 2016), and *L. monocytogenes* is likely no exception (Ryan et al., 2009; Cheng et al., 2013b). Besides, the alternative factor, SigB has been widely studied as it plays a key role in

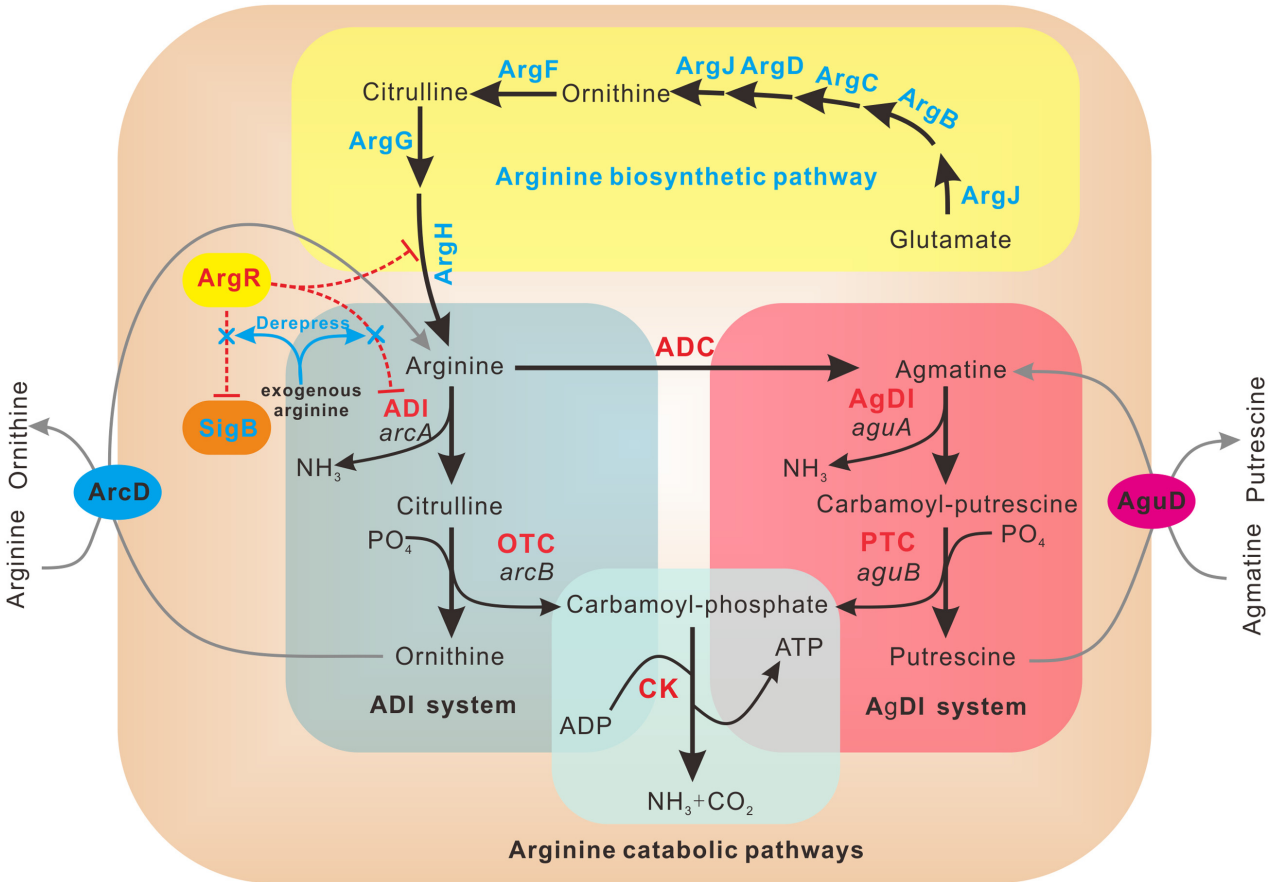


FIGURE 6 | Schematic representation of regulatory mechanism employed by ArgR in *L. monocytogenes*. *L. monocytogenes* ArgR plays a classical role of ArgR/AhrC family in feedback inhibitory of arginine biosynthetic pathway (highlighted in yellow) using arginine as a cofactor. ArgR unexpectedly represses the transcription and expression of *arcA* and *sigB* in the absence of exogenous arginine, preventing arginine degradation *via* the ADI and AgDI pathways (highlighted in light cyan and light red, respectively). Addition of arginine leads to derepression of *arcA* and *sigB*, allowing utilization of arginine as a nitrogen and energy source *via* the arginine degradation pathway.

L. monocytogenes survival under multiple environmental stress conditions, including elevated osmolarity, low pH and oxidative-stresses (Kim et al., 2004; Gahan and Hill, 2005).

This is the first study showing that a single ArgR regulator can have opposite regulatory effects on the ADI pathway in an arginine-independent and dependent manner under neutral and acidic conditions, respectively. However, the underlying molecular mechanisms are still unknown and warrant further study. In general, ArgR-type proteins act as a positive regulator of the ADI system and a negative regulator of the arginine biosynthetic pathway. However, there are two circumstances for unconventional ArgR regulatory mechanisms. One exists in bacteria that encode two ArgR homologs. For instance, the expression of arginine metabolism in *Lactococcus lactis* is controlled by the two homologous transcriptional regulators ArgR and AhrC. Specifically, ArgR binds to the promoter regions of both the arginine biosynthetic and catabolic operons in an arginine-independent manner. With both regulators present, addition of arginine leads to increased binding of ArgR-AhrC to the biosynthetic *argC* promoter but also to diminished binding to the catabolic *arcA* promoter (Larsen et al., 2005). The other circumstance is for the bacteria that contain one single ArgR homologous but two *arc* operons. Xiong et al. (2015) has demonstrated that arginine catabolism in *Laribacter hongkongensis* is finely regulated by manipulating the transcription of two *arc* operons. *L. hongkongensis* ArgR exhibited an opposite effect on transcription and expression of these two *arc* operons. In the presence of arginine, deletion of *argR* partially compromised the repressive effects that arginine had on *arcA1* expression; while it dramatically decreased the transcriptional levels of *arcA2* (Xiong et al., 2015).

Since *L. monocytogenes* encodes a single ArgR homolog and one *arc* operon, we speculate that ArgR maintains its functions as a unique transcriptional regulator with dual regulatory effects on ADI pathway and SigB under different environmental stresses. Based on the results presented here, we propose a model depicting the mechanisms of ArgR in arginine-meditated transcriptional regulation in *L. monocytogenes* (**Figure 6**). In the absence of arginine, ArgR shows higher affinity for *arc* operon promoter, and relatively lower affinity for *arg* operons

compared to that in the presence of arginine, consequently preventing arginine degradation *via* the ADI pathway and repressing arginine biosynthesis to a low extent. The addition of arginine shifts ArgR from the *arcA* promoter to the ARG box operators in the *arg* operons, which enhances repression of the arginine biosynthetic genes. Accordingly, the arginine catabolic *arc* operon is now derepressed, allowing catabolism of arginine as a nitrogen and energy source through the ADI arginine degradation pathway. Therefore, *L. monocytogenes* ArgR appears to have unusual roles in repression of arginine biosynthetic operon in an arginine-independent manner, and activation of catabolism by anti-repression in an arginine-dependent way.

AUTHOR CONTRIBUTIONS

CC, WF, and HS conceived the study. CC, JS, XH, ZD, HW, LJ, and TM carried out experiments. CC, YY, ZC, and JY analyzed data. CC, WF, and HS drafted the manuscript and all the authors contributed to preparing the final version of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (Nos. 31470179, 31502083, and 31402215), Zhejiang Provincial Natural Science Foundation (Nos. LY17C180001, LY15C010003, and LQ14C010007) and ZAFU talents starting program (Nos. 2014FR073). The funders had no role in design of the study or analysis and interpretation of the data.

ACKNOWLEDGMENTS

We specially thank Dr. Martin Wiedmann at Cornell University and Dr. Nancy Freitag at University of Illinois at Chicago for kindly providing the shuttle plasmids pKSV7 and pIMK2, respectively.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00145/full#supplementary-material

FIGURE S1 | The arginine biosynthetic pathway and organization of the gene cluster in *L. monocytogenes*. (A) The arginine biosynthetic pathway. Arginine is synthesized from glutamate in eight steps catalyzed by a series of enzymes encoded by *argABCDEFGH*. Five steps involving *N*-acetylated intermediates lead to ornithine, and three additional steps are required to convert ornithine into arginine. The synthesis of all enzymes is subject to repression by arginine, mediated by the repressor ArgR. **(B)** Genetic organization of the arginine biosynthesis pathway gene cluster in *L. monocytogenes* strain 10403S.

REFERENCES

Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195–201. doi: 10.1093/bioinformatics/bti770

Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J., and Schwede, T. (2009). Protein structure homology modeling using SWISS-MODEL workspace. *Nat. Protoc.* 4, 1–13. doi: 10.1038/nprot.2008.197

Bordoli, L., and Schwede, T. (2012). Automated protein structure modeling with SWISS-MODEL workspace and the protein model portal. *Methods Mol. Biol.* 857, 107–136. doi: 10.1007/978-1-61779-588-6_5

Boylan, S. A., Redfield, A. R., Brody, M. S., and Price, C. W. (1993). Stress-induced activation of the sigma B transcription factor of *Bacillus subtilis*. *J. Bacteriol.* 175, 7931–7937. doi: 10.1128/jb.175.24.7931-7937.1993

Caldara, M., Charlier, D., and Cunin, R. (2006). The arginine regulon of *Escherichia coli*: whole-system transcriptome analysis discovers new genes and provides an integrated view of arginine regulation. *Microbiology* 152, 3343–3354. doi: 10.1099/mic.0.29088-0

Camilli, A., Tilney, L. G., and Portnoy, D. A. (1993). Dual roles of plcA in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* 8, 143–157. doi: 10.1111/j.1365-2958.1993.tb01211.x

Chen, J., Cheng, C., Xia, Y., Zhao, H., Fang, C., Shan, Y., et al. (2011). Lmo0036, an ornithine and putrescine carbamoyltransferase in *Listeria monocytogenes*, participates in arginine deiminase and agmatine deiminase pathways and mediates acid tolerance. *Microbiology* 157(Pt 11), 3150–3161. doi: 10.1099/mic.0.049619-0

Chen, S. H., Merican, A. F., and Sherratt, D. J. (1997). DNA binding of *Escherichia coli* arginine repressor mutants altered in oligomeric state. *Mol. Microbiol.* 24, 1143–1156. doi: 10.1046/j.1365-2958.1997.4301791.x

Cheng, C., Chen, J., Fang, C., Xia, Y., Shan, Y., Liu, Y., et al. (2013a). *Listeria monocytogenes* aguA1, but not aguA2, encodes a functional agmatine deiminase: biochemical characterization of its catalytic properties and roles in acid tolerance. *J. Biol. Chem.* 288, 26606–26615. doi: 10.1074/jbc.M113.477380

Cheng, C., Chen, J., Shan, Y., Fang, C., Liu, Y., Xia, Y., et al. (2013b). *Listeria monocytogenes* ArcA contributes to acid tolerance. *J. Med. Microbiol.* 62(Pt 6), 813–821. doi: 10.1099/jmm.0.055145-0

Cheng, C., Yang, Y., Dong, Z., Wang, X., Fang, C., Yang, M., et al. (2015). *Listeria monocytogenes* varies among strains to maintain intracellular pH homeostasis under stresses by different acids as analyzed by a high-throughput microplate-based fluorometry. *Front. Microbiol.* 6:15. doi: 10.3389/fmicb.2015.00015

Cherney, L. T., Cherney, M. M., Garen, C. R., and James, M. N. (2009). The structure of the arginine repressor from Mycobacterium tuberculosis bound with its DNA operator and Co-repressor, L-arginine. *J. Mol. Biol.* 388, 85–97. doi: 10.1016/j.jmb.2009.02.053

Choi, Y., Choi, J., Groisman, E. A., Kang, D. H., Shin, D., and Ryu, S. (2012). Expression of STM4467-encoded arginine deiminase controlled by the STM4463 regulator contributes to *Salmonella enterica* serovar Typhimurium virulence. *Infect. Immun.* 80, 4291–4297. doi: 10.1128/IAI.00880-12

Cossart, P. (2011). Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19484–19491. doi: 10.1073/pnas.1112371108

Cotter, P. D., and Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* 67, 429–453. doi: 10.1128/MMBR.67.3.429-453.2003

Cunin, R., Glansdorff, N., Pierard, A., and Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* 50, 314–352.

Dennis, C. A., Glykos, N. M., Parsons, M. R., and Phillips, S. E. V. (2002). The structure of AhrC, the arginine repressor/activator protein from *Bacillus subtilis*. *Acta Crystallogr. D-Biol. Crystallogr.* 58, 421–430. doi: 10.1107/S0907444901021692

Dong, Y., Chen, Y. Y., and Burne, R. A. (2004). Control of expression of the arginine deiminase operon of *Streptococcus gordonii* by CcpA and Flp. *J. Bacteriol.* 186, 2511–2514. doi: 10.1128/JB.186.8.2511-2514.2004

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Ferreira, A., O'Byrne, C. P., and Boor, K. J. (2001). Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 67, 4454–4457. doi: 10.1128/AEM.67.10.4454-4457.2001

Fulde, M., Willenborg, J., de Greeff, A., Benga, L., Smith, H. E., Valentin-Weigand, P., et al. (2011). ArgR is an essential local transcriptional regulator of the arcABC operon in *Streptococcus suis* and is crucial for biological fitness in an acidic environment. *Microbiology* 157(Pt 2), 572–582. doi: 10.1099/mic.0.043067-0

Gahan, C. G., and Hill, C. (2005). Gastrointestinal phase of *Listeria monocytogenes* infection. *J. Appl. Microbiol.* 98, 1345–1353. doi: 10.1111/j.1365-2672.2005.02559.x

Garnett, J. A., Baumberg, S., Stockley, P. G., and Phillips, S. E. (2007). A high-resolution structure of the DNA-binding domain of AhrC, the arginine repressor/activator protein from *Bacillus subtilis*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 63(Pt 11), 914–917. doi: 10.1107/S1744309107048166

Garnett, J. A., Marincs, F., Baumberg, S., Stockley, P. G., and Phillips, S. E. (2008). Structure and function of the arginine repressor-operator complex from *Bacillus subtilis*. *J. Mol. Biol.* 379, 284–298. doi: 10.1016/j.jmb.2008.03.007

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., et al. (2001). Comparative genomics of *Listeria* species. *Science* 294, 849–852. doi: 10.1126/science.1063447

Gray, M. J., Freitag, N. E., and Boor, K. J. (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect. Immun.* 74, 2505–2512. doi: 10.1128/IAI.74.5.2505-2512.2006

Griswold, A., Chen, Y. Y., Snyder, J. A., and Burne, R. A. (2004). Characterization of the arginine deiminase operon of *Streptococcus* rattus FA-1. *Appl. Environ. Microbiol.* 70, 1321–1327. doi: 10.1128/AEM.70.3.1321-1327.2004

Gruening, P., Fulde, M., Valentin-Weigand, P., and Goethe, R. (2006). Structure, regulation, and putative function of the arginine deiminase system of *Streptococcus suis*. *J. Bacteriol.* 188, 361–369. doi: 10.1128/JB.188.2.361-369.2006

Kim, H., Boor, K. J., and Marquis, H. (2004). *Listeria monocytogenes* sigmaB contributes to invasion of human intestinal epithelial cells. *Infect. Immun.* 72, 7374–7378. doi: 10.1128/IAI.72.12.7374-7378.2004

Kloosterman, T. G., and Kuipers, O. P. (2011). Regulation of arginine acquisition and virulence gene expression in the human pathogen *Streptococcus* pneumoniae by transcription regulators ArgR1 and AhrC. *J. Biol. Chem.* 286, 44594–44605. doi: 10.1074/jbc.M111.295832

Larsen, R., Buist, G., Kuipers, O. P., and Kok, J. (2004). ArgR and AhrC are both required for regulation of arginine metabolism in *Lactococcus lactis*. *J. Bacteriol.* 186, 1147–1157. doi: 10.1128/JB.186.4.1147-1157.2004

Larsen, R., Kok, J., and Kuipers, O. P. (2005). Interaction between ArgR and AhrC controls regulation of arginine metabolism in *Lactococcus lactis*. *J. Biol. Chem.* 280, 19319–19330. doi: 10.1074/jbc.M413983200

Lebreton, A., Stavru, F., and Cossart, P. (2015). Organelle targeting during bacterial infection: insights from *Listeria*. *Trends Cell Biol.* 25, 330–338. doi: 10.1016/j.tcb.2015.01.003

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, C. D. (2006). Pathways and regulation of bacterial arginine metabolism and perspectives for obtaining arginine overproducing strains. *Appl. Microbiol. Biotechnol.* 70, 261–272. doi: 10.1007/s00253-005-0308-z

Lucas, P. M., Blancato, V. S., Claisse, O., Magni, C., Lolkema, J. S., and Lonvaud-Funel, A. (2007). Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. *Microbiology* 153(Pt 7), 2221–2230. doi: 10.1099/mic.0.2007/006320-0

Maas, W. K. (1994). The arginine repressor of *Escherichia coli*. *Microbiol. Rev.* 58, 631–640.

Maghnouj, A., de Sousa Cabral, T. F., Stalon, V., and Vander Wauven, C. (1998). The arcABDC gene cluster, encoding the arginine deiminase pathway of *Bacillus licheniformis*, and its activation by the arginine repressor argR. *J. Bacteriol.* 180, 6468–6475.

Makarova, K. S., Mironov, A. A., and Gelfand, M. S. (2001). Conservation of the binding site for the arginine repressor in all bacterial lineages. *Genome Biol.* 2:RESEARCH0013. doi: 10.1186/gb-2001-2-9-research0033

Marquis, R. E., Bender, G. R., Murray, D. R., and Wong, A. (1987). Arginine deiminase system and bacterial adaptation to acid environments. *Appl. Environ. Microbiol.* 53, 198–200.

Miller, C. M., Baumberg, S., and Stockley, P. G. (1997). Operator interactions by the *Bacillus subtilis* arginine repressor/activator, AhrC: novel positioning and DNA-mediated assembly of a transcriptional activator at catabolic sites. *Mol. Microbiol.* 26, 37–48.

Monk, I. R., Gahan, C. G., and Hill, C. (2008). Tools for functional postgenomic analysis of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 74, 3921–3934. doi: 10.1128/AEM.00314-08

Munch, R., Hiller, K., Grote, A., Scheer, M., Klein, J., Schobert, M., et al. (2005). Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* 21, 4187–4189. doi: 10.1093/bioinformatics/bti635

Ni, J., Sakanyan, V., Charlier, D., Glansdorff, N., and Van Duyne, G. D. (1999). Structure of the arginine repressor from *Bacillus stearothermophilus*. *Nat. Struct. Biol.* 6, 427–432. doi: 10.1038/8229

Nicoloff, H., Arsene-Ploetze, F., Malandain, C., Kleerebezem, M., and Bringel, F. (2004). Two arginine repressors regulate arginine biosynthesis in *Lactobacillus plantarum*. *J. Bacteriol.* 186, 6059–6069. doi: 10.1128/JB.186.18.6059-6069.2004

Palmer, M. E., Chaturongakul, S., Wiedmann, M., and Boor, K. J. (2011). The *Listeria monocytogenes* sigmaB regulon and its virulence-associated functions are inhibited by a small molecule. *MBio* 2:e241-11. doi: 10.1128/mBio.00241-11

Perez-Redondo, R., Rodriguez-Garcia, A., Botas, A., Santamarta, I., Martin, J. F., and Liras, P. (2012). ArgR of *Streptomyces coelicolor* is a versatile regulator. *PLoS ONE* 7:e32697. doi: 10.1371/journal.pone.0032697

Rodriguez-Garcia, A., Ludovice, M., Martin, J. F., and Liras, P. (1997). Arginine boxes and the argR gene in *Streptomyces clavuligerus*: evidence for a clear regulation of the arginine pathway. *Mol. Microbiol.* 25, 219–228. doi: 10.1046/j.1365-2958.1997.4511815.x

Ryan, S., Begley, M., Gahan, C. G., and Hill, C. (2009). Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. *Environ. Microbiol.* 11, 432–445. doi: 10.1111/j.1462-2920.2008.01782.x

Sunnerhagen, M., Nilges, M., Otting, G., and Carey, J. (1997). Solution structure of the DNA-binding domain and model for the complex of multifunctional hexameric arginine repressor with DNA. *Nat. Struct. Biol.* 4, 819–826. doi: 10.1038/nsb1097-819

Xia, Y., Xin, Y., Li, X., and Fang, W. (2016). To modulate survival under secondary stress sonditions, *Listeria monocytogenes* 10403S employs RsbX to downregulate sigmaB activity in the poststress recovery stage or stationary phase. *Appl. Environ. Microbiol.* 82, 1126–1135. doi: 10.1128/AEM.03218-15

Xiong, L., Teng, J. L., Watt, R. M., Kan, B., Lau, S. K., and Woo, P. C. (2014). Arginine deiminase pathway is far more important than urease for acid resistance and intracellular survival in *Laribacter hongkongensis*: a possible result of arc gene cassette duplication. *BMC Microbiol.* 14:42. doi: 10.1186/1471-2180-14-42

Xiong, L., Teng, J. L., Watt, R. M., Liu, C., Lau, S. K., and Woo, P. C. (2015). Molecular characterization of arginine deiminase pathway in *Laribacter hongkongensis* and unique regulation of arginine catabolism and anabolism by multiple environmental stresses. *Environ. Microbiol.* 17, 4469–4483. doi: 10.1111/1462-2920.12897

Xu, B., Yang, X., Zhang, P., Ma, Z., Lin, H., and Fan, H. (2016). The arginine deiminase system facilitates environmental adaptability of *Streptococcus equi* ssp. zooepidemicus through pH adjustment. *Res. Microbiol.* 167, 403–412. doi: 10.1016/j.resmic.2016.03.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Cheng, Dong, Han, Sun, Wang, Jiang, Yang, Ma, Chen, Yu, Fang and Song. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

METHODS
published: 27 February 2017
doi: 10.3389/fmicb.2017.00295

MLVA for *Salmonella enterica* subsp. *enterica* Serovar Dublin: Development of a Method Suitable for Inter-Laboratory Surveillance and Application in the Context of a Raw Milk Cheese Outbreak in France in 2012

*Marie-Léone Vignaud[1], Emeline Cherchame[1], Muriel Marault[1], Emilie Chaing[1], Simon Le Hello[2], Valerie Michel[3], Nathalie Jourdan-Da Silva[4], Renaud Lailler[1], Anne Brisabois[1] and Sabrina Cadel-Six[1]**

[1] Université PARIS-EST, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail, Laboratory for Food Safety, Maisons-Alfort, France, [2] French National Reference Center for E. coli, Shigella and Salmonella, Institut Pasteur, Paris, France, [3] Department of Dairy Products, Center of Expertise for the Food Industry, La Roche-sur-Foron, France, [4] French National Public Health Agency, Saint-Maurice, France

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Hong Du, Soochow University, China
Burkhard Malorny, Federal Institute for Risk Assessment, Germany

***Correspondence:**
Sabrina Cadel-Six
sabrina.cadelsix@anses.fr

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 December 2016*
Accepted: *13 February 2017*
Published: *27 February 2017*

Citation:
Vignaud M-L, Cherchame E, Marault M, Chaing E, Le Hello S, Michel V, Jourdan-Da Silva N, Lailler R, Brisabois A and Cadel-Six S (2017) MLVA for Salmonella enterica subsp. enterica Serovar Dublin: Development of a Method Suitable for Inter-Laboratory Surveillance and Application in the Context of a Raw Milk Cheese Outbreak in France in 2012. Front. Microbiol. 8:295. doi: 10.3389/fmicb.2017.00295

Salmonella enterica subspecies *enterica serovar* Dublin (*S*. Dublin) figures among the most frequently isolated *Salmonella* strains in humans in France. This serovar may affect production and animal health mainly in cattle herds with corresponding high economic losses. Given that the current gold standard method, pulsed-field gel electrophoresis (PFGE), provides insufficient discrimination for epidemiological investigations, we propose a standard operating procedure in this study for multiple-locus variable number tandem repeat analysis (MLVA) of *S.* Dublin, suitable for inter-laboratory surveillance. An *in silico* analysis on the genome of *S.* Dublin strains CT_02021853 was performed to identify appropriate microsatellite regions. Of 21 VNTR loci screened, six were selected and 401 epidemiologically unrelated and related strains, isolated from humans, food and animals were analyzed to assess performance criteria such as typeability, discriminatory power and epidemiological concordance. The MLVA scheme developed was applied to an outbreak involving *Saint-Nectaire* cheese for which investigations were conducted in France in 2012, making it possible to discriminate between epidemiologically related strains and sporadic case strains, while PFGE assigned only a single profile. The six loci selected were sequenced on a large set of strains to determine the sequence of the repeated units and flanking regions, and their stability was evaluated *in vivo* through the analysis of the strains investigated from humans, food and the farm environment during the outbreak. The six VNTR selected were found to be stable and the discriminatory power of the MLVA method developed was calculated to be 0.954 compared with that for PFGE, which was only 0.625. Twenty-four reference strains were selected from the 401 examined strains in order to represent most of the allele diversity observed

for each locus. This reference set can be used to harmonize MLVA results and allow data exchange between laboratories. This original MLVA protocol could be used easily and routinely for monitoring of serovar Dublin isolates and for conducting outbreak investigations.

Keywords: *Salmonella* Dublin, MLVA analysis, PFGE analysis, protocol for inter-laboratory surveillance, raw milk cheese, foodborne outbreak

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Dublin (*S.* Dublin) is one of the most frequently encountered *Salmonella* in cattle in the European Union. Data from the ANSES *Salmonella* Network (jointly with the National Reference Laboratory) show that this serovar contends with the Typhimurium serovar for the top spot in the ranking of the serovars most frequently isolated in cattle in France. Between 2002 and 2010, *S.* Dublin was outright the most common one (European Food Safety Authority [EFSA], 2015). Infected cattle may develop several clinical signs mainly characterized by i/ diarrhea, pneumonia and death in calves and adult cattle, and ii/ abortion and decreased milk yield in cows (Nielsen et al., 2013).

Beyond the significant economic losses caused in the bovine sector, *S.* Dublin is of concern to public health because it is potentially zoonotic and can cause gastro-intestinal disease and severe infection in humans (O'Leary, 2014). It can be transmitted to humans via meat and dairy products (Maguire et al., 1992). *Salmonella* Dublin outbreaks have occurred regularly in France in the last few years. Protracted difficulties are probably the result of several factors: persistence in the environment, e.g., in wet and dried feces (Findlay, 1972; Plym-Forshell and Ekesbo, 1996), persistence in cattle herds (Clegg et al., 1986; Boqvist and Vagsholm, 2005), a carrier state or prolonged shedding, or reinfection of susceptible animals (Centers for Disease Control [CDC], 1984; Nielsen, 2009). Persistently infected cows can shed the bacteria intermittently in their feces for prolonged periods without ever showing signs of disease, making control of infection particularly difficult at the breeding level (Nielsen et al., 2013).

In France, Dublin varied between the 20th to the 9th position ($n = 45$ to 120 clinical isolates) of the most frequently isolated serovars in humans between 2000 and 2013, with a peak observed in 2012 (Weill and Le Hello, 2013). During the same period, *S.* Dublin contamination in the bovine sector recorded by the *Salmonella* Network increased, with a peak observed in 2013. The relative frequency of *S.* Dublin detected by the *Salmonella* Network in 2010 for cattle and dairy products was 10.4 and 57.6%, respectively, compared to 37.7 and 5.8% for *S.* Typhimurium (Inventaire du Réseau Salmonella, 2010; Lailler et al., 2012). The increased contamination in humans, and in the animal and food sectors, demonstrates the need for a method that can monitor *S.* Dublin strains alongside outbreak investigations. PFGE, considered as the 'gold standard' among molecular typing methods, is routinely used for monitoring and surveillance, as well as investigation of outbreaks. Nevertheless, the discriminatory power for *S.* Dublin is low and does not enable investigation and tracking of the source of contamination during foodborne outbreaks (FBOs; Liebana et al., 2002). A study conducted by the *Salmonella* Network on a large panel of Dublin strains highlighted the genetic homogeneity of this serovar (Kerouanton et al., 1996). Moreover, the ANSES *Salmonella* Network's PFGE database shows that 84% of the *S.* Dublin strains collected since 2003 were assigned to the same profile (SDUBXB0003 for 153/183 strains).

Several methods have already been proposed in the past as an alternative to PFGE, including multilocus enzyme electrophoresis (MLEE; Beltran et al., 1988), ribotyping (Chowdry et al., 1993), restriction fragment length polymorphism analysis (Kerouanton et al., 1996), restriction enzyme fragmentation pattern (REFP) analysis (Olsen and Skov, 1994), various PCR techniques, IS200 typing, and quantitative evaluation of fatty acid methyl esters (FAME; McDonough et al., 1999). Nevertheless, among all these methods, either the discriminatory power was insufficient or the process was laborious, time-consuming and expensive. More recently, MLVA was proposed for subtyping *Salmonella* subsp. *enterica* strains (Ramisse et al., 2004). This method was shown to have better performance than historical methods by displaying higher discriminatory power for *Salmonella* serovars such as Typhimurium and Enteritidis (Lindstedt et al., 2004; Hopkins et al., 2011). Moreover, this method proved very useful in investigating FBOs and facilitated the analysis since it requires no specific technical expertise (Wattiau et al., 2011 review).

In this study, we developed a new MLVA protocol for high discriminatory typing of *S.* Dublin in accordance with the guidelines published by Nadon et al. (2013) on the development and application of MLVA methods as tools for inter-laboratory surveillance. A reference set of strains and a scheme for harmonization of results are also proposed to allow data exchange between laboratories. In order to assess the discriminatory power of the MLVA protocol developed here and the stability of tandem repeats (TRs), we used a set of 401 strains isolated between 1929 and 2015 from three different collections (*Salmonella* Network, National Reference Centre for *Salmonella* and Centre of Expertise for the Food Industry). In particular, this panel of strains comprised human and food isolates recovered in the framework of a FBO investigation that occurred in 2012 in France.

In August 2012, the French National Public Health Agency (SpFrance) detected an unusual increase in cases of human *S.* Dublin infection. Epidemiological investigations highlighted an association between cases and the consumption of raw milk cheese (Saint-Nectaire). Two producers of cheese were identified

Abbreviations: DI, discriminatory index; MLVA, multiple-locus variable number tandem repeat analysis; PFGE, pulsed-field gel electrophoresis; RU, repeated unit; VNTR, variable number tandem-repeat.

as potential sources of contamination. Enhanced self-monitoring on the two farms was implemented and further epidemiological and microbiological investigations were conducted. Fifteen FBOs were recorded corresponding to more than 100 cases due to *S*. Dublin (Giron et al., 2013). On 4 September 2012, a withdrawal/recall of contaminated batches of Saint-Nectaire cheese was ordered by the General Directorate for Food (regional office) and of the 7580 kg of cheese produced by the two farms, 1335 kg (17%) were immediately withdrawn from sale.

Salmonella Dublin strains isolated from the two different cheese producers suspected to be implicated in this FBO and the clinical strains from the patients were analyzed with the MLVA protocol proposed in this study. Since 2012, this MLVA Dublin protocol is used routinely in our laboratory in order to analyze strains of *S*. Dublin for monitoring and surveillance, investigation of outbreaks, and official controls. Indeed, there is still concern in this regard because new cases of *S*. Dublin associated with raw milk cheeses were reported over the summer months of 2016.

MATERIALS AND METHODS

Bacterial Strains

A total of 401 *S*. Dublin strains were used for the development of the MLVA scheme. Among them, 109 strains were isolated from animals, 198 from food products and 94 from humans (see **Supplementary Table S1**). The strains isolated from animals and food sources were collected by the *Salmonella* Network of ANSES and the Centre of Expertise for the Food Industry (Actalia); the clinical strains came from the National Reference Centre for *Salmonella* at Institut Pasteur in Paris. This panel included 251 epidemiologically unrelated and 150 epidemiologically related strains isolated along the cheese production chain. Among these, 13 strains were recovered from the Saint-Nectaire samples during the 2012 outbreak investigations. All strains were identified as belonging to serovar *Salmonella* Dublin according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

Pulsed-Field Gel Electrophoresis

Within the panel of 401 strains used for the development of the MLVA scheme, 51 *Salmonella* Dublin strains, isolated from 2002 to 2011, were PFGE subtyped according to a standardized protocol (PulseNet, 2013, EU) with some modifications in the composition of the cell lysis buffer and the concentration of the enzyme *Xba*I (Sigma–Aldrich, France). The cell lysis buffer was 1M Tris: 250 mM EDTA, pH 8.0 + 10% sarkosyl, and the restriction enzyme was five times less concentrated. This analysis of PFGE patterns was performed using BioNumerics® software v.6.6 (Applied Maths, Belgium) and comparison of patterns was carried out by building a dendrogram (Dice coefficients, the UPGMA method and position tolerance set at 1%). Each strain profile was assigned to a PFGE pattern corresponding to a unique pattern. Designation of each PFGE profile was done using a unique nomenclature, e.g., SDUBXB0001.

Procedure for the Multiple-Locus Variable Number Tandem Repeat Analysis

DNA Extraction

Strains were cultured overnight at 37°C on tryptone soya yeast extract agar plate. The extraction was performed with Instagene Matrix (Biorad, France) according to the manufacturer's instructions for Gram-negative organisms. The clarified supernatant was stored at −20°C. The DNA concentration was measured with Nanodrop ND-1000 (Labtech, France). The DNA concentration of the samples was normalized at a final concentration of 50 ng/μL.

Variable Number Tandem Repeat (VNTR) Selection

Twenty-one VNTR markers published from 2003 to 2009 (Kruy et al., 2011, review) to discriminate *S. enterica* subspecies were selected (see **Supplementary Table S2**) and blasted on the genomic sequence of *S*. Dublin strains CT_02021853 (accession No. NC_011205.1/CP001144.1) obtained from http://www.ncbi.nlm.nih.gov/genome/. The presence of TRs was verified using free access TRs Finder software by Benson (1999). The *in silico* analysis performed on the genome of *S*. Dublin strains CT_02021853 revealed that eight loci, on the 21 searched, presented microsatellite sequences. Among these eight loci, six were tested by PCR on 51 *S*. Dublin genomes from the *Salmonella* Network collection to check for the presence of microsatellites and variability of RUs. Finally, all six VNTRs were selected for the development of the MLVA procedure (**Table 1**). The primers for 2 of these VNTRs, STTR3, and SE-2 (developed for Typhimurium and Enteritidis, respectively), were adjusted to the sequence of the genome of *S*. Dublin strains CT_02021853.

PCR Multiplex Amplification

The six selected VNTR regions were targeted in two multiplex assays: M1 (STTR5, STTR7, STTR3) and M2 (SENTR1, SENTR3, SE-2), using the Qiagen multiplex Kit (Qiagen, Germany). The primers were pooled in two premixes, one for M1 the other for M2. The concentrations for each primer were as follow: STTR5 and STTR7: 1.5 μM, STTR3: 3 μM, SENTR1: 2.5 μM, SENTR3: 10 μM and SE-2: 7 μM. M1 and M2 were carried out with a final volume of 25 μL and 15 μL, respectively. Per reaction: 0.9 μL of pre-mix primer for M1, 1.85 μL for M2, and 2 μL of DNA were added. Multiplex PCRs were run on a Verity® thermocycler (Applied Biosystems, France) with different and specific cycling conditions; for M1 reactions:15 min at 95°C, then 25 cycles of 30 s at 95°C, 90 s at 60°C, 90 s at 72°C and ending with a hold at 72°C for 10 min; for M2 reactions: 15 min at 95°C, then 28 cycles of 30 s at 95°C, 90 s at 55°C, 90 s at 72°C and ending with a hold at 72°C for 10 min.

The M1 PCR solution was then diluted to 1/20 and the M2 PCR solution to 1/30 with RNase-free, molecular biology-grade water. Finally, 1 μL of each solution was pooled with 1 μL of 600 Liz internal size marker (Applied Biosystems, France) and 13 μL of formamide for M1 and 0.5 μL of 1200 Liz internal size

TABLE 1 | Variable number tandem repeats and primers selected for the MLVA *S*. Dublin scheme.

Locus	Dye	Primers (5′–3′)[a]	Sequence length (bp)[b]	Offset[c] (bp)	RU[d] (bp)	RU sequence (bp)
STTR3	PET	CCCCCTA**C**GCCCGATAATGG TGACGCCGTTGCTGAAGGTAATAA	409	66	33	GCGGCGATGACAATGTGATCCCGCCCGACGATA
STTR5	VIC	ATGGCGAGGCGAGCAGCAGT GGTCAGGCCGAATAGCAGGAT	274	179	6	CACGAC
STTR7	VIC	CGCGCAGCCGTTCTCACT TGTTCCAGCGCAAAGGTATCTA	477	265	39	GTAGCGCCGCAGCCGCAGTATCAGCAGCCGCA GCAACCG
SE-2	PET	CTT**AC**GATTATACCTGGATTG TGGACGGAGGCGATAG	201	168	7	GATGCCG
SENTR1	VIC	GCAACAGCAGCAGCAACAG CCGAGCTGAGATCGCCAAG	440	85	45	GAAGCGGCGAAAGCGGCGGCGGACGCGAAGA AGAAAGCGGAAGCC
SENTR3	NED	CTAAACAAGCCGCTCATCCG ACAACCTGCTGCTGTGCTG	493	80	93	CGACCCGAGTAAAGCTGCAGTGGAGGCAGCCA TCGCCCGCGCCAAAGCCCGTAAGCAGGAGCAG CAGGCCGGAAGCGAACCGGTCGAAGCGGT

[a]*the primers presenting underlined letters were designed in this study, the other primers refer to Kruy et al. (2011; review).*
[b]*sequence length in genome of S. Dublin strains CT_02021853 (accession No. NC_011205.1/CP001144.1) obtained from http://www.ncbi.nlm.nih.gov/genome/.*
[c]*length of 5′ and 3′ TR flanking region.*
[d]*Repeated Unit.*

marker (Applied Biosystems, France) and 10.5 μL of formamide for M2.

The samples were denatured 5 min to 95°C and cooled on ice before being subjected to capillary electrophoresis.

Capillary Electrophoresis and Data Analysis

The analyses were carried out on an AB3500 capillary electrophoresis system (Applied Biosystems, France) spectrally calibrated to run filter set G5. The instrument was prepared according to the procedures specified by Applied Biosystems. The standard fragment analysis protocol proposed by the manufacturer was used and positive and negative control isolates were included with each run to follow the drift in results due to the use of the instrument over long time periods. Data were automatically saved as .fsa files and imported into GeneMapper software (Applied Biosystems, France), where each fragment was identified according to color and size. The measured lengths attributed to each peak by GeneMapper were transferred to a .txt file to be normalized with the free access MLVA_Normalizer software[1], following the instructions of the author (Bachelerie et al., 2016). Then, the MLVA profiles were imported into BioNumerics software version 7.1 (Applied Maths, Belgium) as categorical data. A standard minimum-spanning tree (MST) was generated using the single and double locus variance priority rules, allowed to define the clonality and distance between strains.

The discriminatory power of the single VNTR and of the MLVA method compared to that of PFGE was calculated by Simpson's index of diversity (DI) according to the formula as described by Hunter and Gaston (1988).

Sequencing and Standardization Strains

For sequencing of the VNTR loci, genomic DNA was amplified in a simplex PCR with the same primer sequences used for VNTR detection but with unlabeled forward primers. Sequencing (Life Technologies, Germany) was performed in both directions using both the forward and the reverse primers for all loci to determine the sequence of the TRs and flanking regions. Twenty-four strains with different confirmed numbers of repeats at all loci were chosen as the reference strain panel (**Table 2**).

Reproducibility, Metrology, and Quality Safety Control

Distinct experimental trials were conducted including two different operators and different days of handling (from 2 to 5 days). All instruments were calibrated and metrologically controlled according to ISO NF 17025. The handling was performed under quality safety requirements.

RESULTS

Characterization, Diversity, and Allele Distribution of six VNTR Loci

Among the 21 loci included in this study, 8 displayed TRs in the genome of *S.* Dublin strains CT_02021853 by *in silico* analysis. Among these eight loci, two were discarded, STTR4 and SE-6. STTR4 was discarded because the length of the PCR amplicon exceeded the range of detection of the AB3500 capillary

[1]https://github.com/afelten-Anses/MLVA_normalizer

TABLE 2 | Reference strain panel of *Salmonella* Dublin (*n* = 24).

	Reference	MLVA fragment sizes[a] STTR5, STTR7, STTR3, SENTR1, SENTR3, SE-2	MLVA profile[b]
1	03EB8994SAL	229-594-409-440-494-243	8-8-10-8-4-11
2	10CEB141SAL	235-316-409-395-588-228	9-1-10-7-5-8
3	2014LSAL02934	313-594-409-395-588-187	22-8-10-7-5-3
4	2015LSAL00352	223-594-409-440-493-215	7-8-10-8-4-7
5	2015LSAL00380	325-594-409-395-588-194	24-8-10-7-5-4
6	02EB9210SAL	271-594-409-395-588-194	15-8-10-7-5-4
7	03EB3784SAL	239-594-343-440-588-194	10-8-8-8-5-4
8	09CEB6631SAL	289-594-409-395-588-194	18-8-10-7-5-4
9	10CEB3063SAL	223-316-409-395-588-222	7-1-10-7-5-8
10	10CEB472SAL	247-594-409-395-588-208	11-8-10-7-5-6
11	10CEB80SAL	277-594-409-395-399-194	16-8-10-7-3-4
12	10CEB8244SAL	277-594-409-395-588-194	16-8-10-7-5-4
13	10CEB8798SAL	295-594-409-395-588-187	19-8-10-7-5-3
14	11CEB386SAL	229-594-409-395-588-194	8-8-10-7-5-4
15	11CEB6136SAL	259-594-409-395-588-194	13-8-10-7-5-4
16	11CEB65SAL	217-594-409-440-494-243	6-8-10-8-4-11
17	11CEB6804SAL	301-594-409-395-587-201	20-8-10-7-5-5
18	11CEB6847SAL	289-594-409-395-588-194	18-8-10-7-5-4
19	11CEB6876SAL	295-594-409-395-588-187	19-8-10-7-5-3
20	12CEB3654SAL	265-594-409-395-588-194	14-8-10-7-5-4
21	10CEB2371SAL	229-316-409-395-588-208	8-1-10-7-5-6
22	04_4663	253-478-409-440-493-215	12-5-10-8-4-7
23	201501982	235-316-409-395-588-236	9-1-10-7-5-10
24	201500719	271-478-409-440-493-201	15-5-10-8-4-5

Isolate Nos. 1–21 were from the Salmonella Network (ANSES) and isolate Nos. 22–24 were from the National Reference Centre (Institut Pasteur).

[a]*The fragment sizes are the true size according to sequence results.*

[b]*The MLVA profile is based on the number of repeated units as described in Nadon et al. (2013). For the incomplete repeats, the copy number is rounded down to the nearest complete copy number.*

electrophoresis system (length of PCR amplicon >1200 bp). SE-6 corresponded to the locus STTR3 even though different authors have given different names to these loci (Kruy et al., 2011, review). For the development of the MLVA protocol, the STTR3 locus, initially described by Lindstedt et al. (2003) was chosen.

Finally, the six selected VNTRs (**Table 1**) enabled identification of 75 different MLVA profiles for the overall panel of 401 strains tested in this study. The most common MLVA profile (19-9-10-7-5-3) was observed for 29% of all strains. The STTR5 and SE-2 loci showed the highest Simpson's diversity index (DI), 0.805 and 0.625, respectively, with the highest number of different TR alleles (18 and 14 alleles). The lowest Simpson's diversity values were observed for the STTR3 locus (DI 0.040) with four alleles (**Table 3**). The fraction of strains that have the most frequent allele was calculated by the max(pi) value (range 0.0–1.0). For the STTR5 locus, 39% of the analyzed strains possessed the most common allele (**Table 3**). This result is an additional indicator of the diversity shown by the alleles' frequencies within the loci.

TABLE 3 | Variability of selected VNTRs in 401 strains of *Salmonella enterica* serovar Dublin.

Locus	Simpson's diversity index	No. of alleles*	max (pi)
STTR5	0.805	17	0.390
STTR7	0.110	5	0.943
STTR3	0.014	2	0.989
SENTR1	0.185	3	0.898
SENTR3	0.195	3	0.895
SE-2	0.625	10	0.435

**Number of different TRs present at the locus.*

Reference Strains for MLVA of *Salmonella enterica* Serovar Dublin

Twenty-four reference strains were selected from the 401 examined strains in order to represent most of the allele diversity observed for each locus. The amplicons from each locus were sequenced (**Table 2**). Data on the 24 reference strains are shown in **Supplementary Table S3**. This table, once compiled with the raw data, can be used as input file for using the MLVA workflow for normalizing MLVA results cited above (Bachelerie et al., 2016). Sequencing confirmed the number of TRs and alignments revealed that the six loci (STTR5, STTR7, STTR3, SENTR3, SENTR1, and SE-2) exhibit no variation in the sequence of the TR unit within a strain and between strains. Single nucleotide polymorphisms (SNPs) were identified in the TRs of the STTR7, STTR3, SENTR3, and SENTR1 loci for 12CEB3654SAL, 09CEB6631SAL and 03EB3784SAL reference strains compared

with the genome of *S.* Dublin strains CT_02021853 (**Figure 1**). The TR units at the six loci for the 24 reference strains were aligned. The alignment showed an incomplete repeat for a few strains (**Figure 1**). The number of alleles of such strains was rounded down to the nearest complete copy number, in accordance with the guidelines published by Nadon et al. (2013).

Discriminatory Power of PFGE and MLVA

Finally, the discriminatory power of the MLVA method developed was compared with that of the PFGE method for the 51 strains for which both methods were performed. PFGE and MLVA profiles are listed in **Supplementary Table S4**. PFGE analysis with *Xba*I sorted the strains into 13 different patterns displaying a discriminatory power with a value of 0.625. MLVA analysis provided 27 different MLVA profiles with a discriminatory value of 0.954. No linear correspondence between the two methods was observed, meaning that MLVA profiles can match, or do not match with a PFGE pattern. The most frequent PFGE pattern (SDUBXB0003) comprised strains characterized by 18 different MLVA profiles.

Minimum-Spanning Tree Analysis

A MST was set up from MLVA profiles of the 401 strains according to the human, animal and food sources, and the context of isolation (FBO; see **Supplementary Table S1** and **Figure 2**). The MST displayed a high degree of polymorphism of strains (**Figure 2**). A total of 71 MST groups were observed and 44 of them were represented by a single strain. The main MST group was characterized by the MLVA profile 19-8-10-7-5-3 (**Figure 2**, group A). This profile included 115 strains isolated from 2010 to 2015 from humans, food and animals. This group also includes strains related to an FBO that occurred in 2015. A type of raw milk cheese was suspected to be the source of this FBO, but in the end, no confirmation of the source was possible. Strains isolated during the FBO that occurred in 2012 (**Figure 2**, groups B and C) showed no epidemiological links with those observed in 2015. The six most represented MST groups, after the main one (**Figure 2**, groups B–G), were characterized by 22–38 strains. Human strains are dispersed among each MST group. Among the 27 groups including two or more strains, 54% comprised exclusively human strains. The 94 human, 109 animal, and 198 food strains were grouped into 44, 25, and 33 MLVA profiles, respectively.

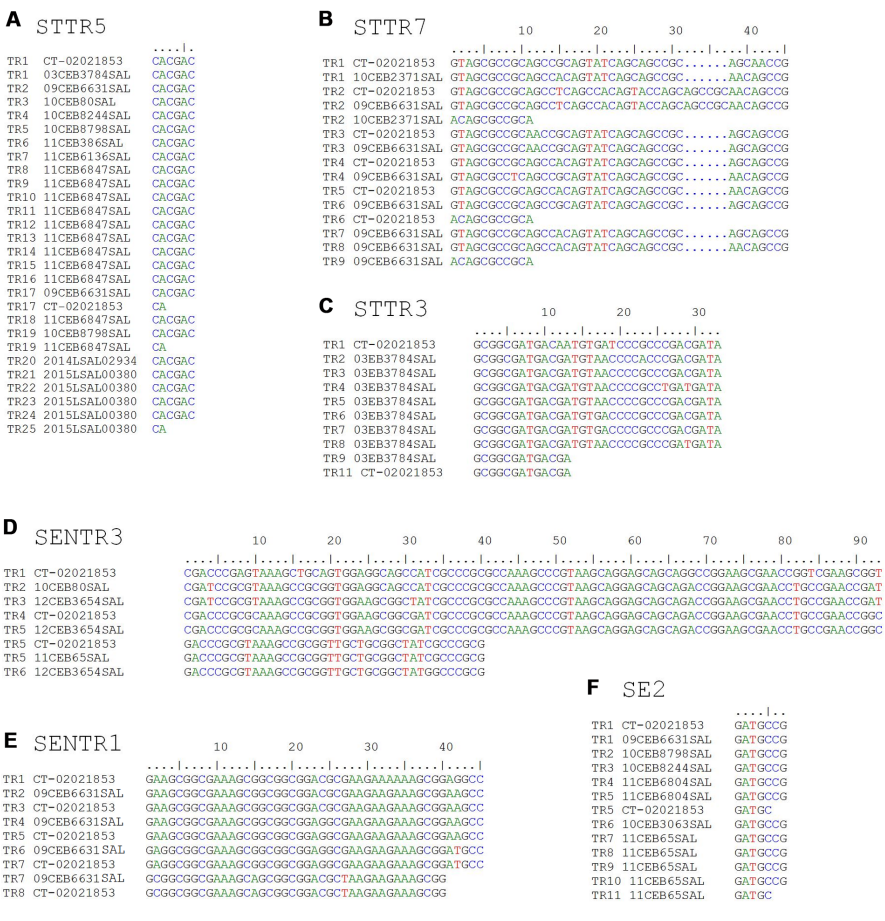


FIGURE 1 | Alignment of the TR units at the six loci for some reference stains compared with the genome of *S.* Dublin strains CT_02021853. (A) Locus STTR5; **(B)** locus STTR7; **(C)** locus STTR3; **(D)** locus SENTR3; **(E)** locus SENTR1; **(F)** locus SE-2.

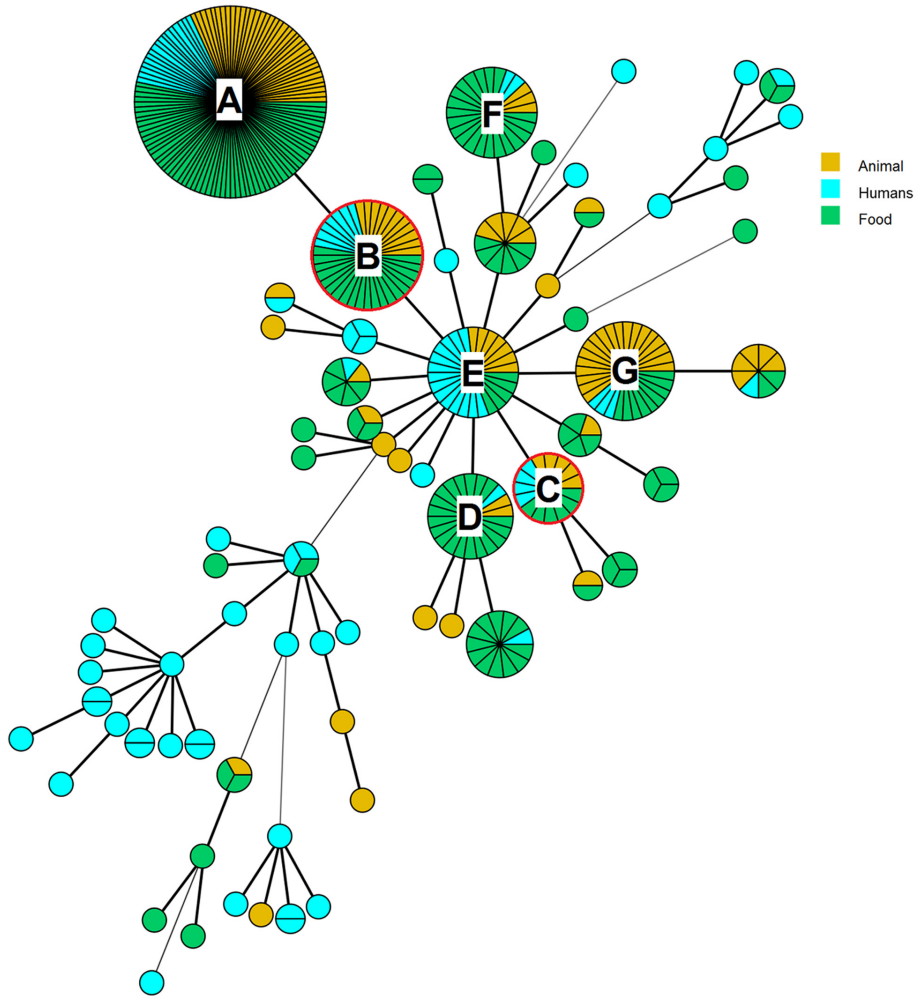


FIGURE 2 | Minimum-spanning tree on the basis of 6-loci MLVA profiles of 401 *S.* Dublin strains from 1929 to 2015. The main group (A) is characterized by the MLVA profile 19-8-10-7-5-3. This group includes human strains isolated in 2011, 2012, and 2015 but not related to the Saint-Nectaire outbreak described. The groups (B and C) surrounded in red include strains from the Saint-Nectaire outbreak that occurred in summer 2012 in France. The group (B) is characterized by the MLVA profile 19-8-10-7-5-4. This group includes also human strains isolated in 2015 during an outbreak which source confirmation was not possible. The group (C) is characterized by the MLVA profile 14-8-10-7-5-4. The group (D) is characterized by the MLVA profile 16-8-10-7-5-4, the group (E) by the profile 17-8-10-7-5-4, the group (F) by the profile 15-8-10-7-5-3 and the group (G) by the profile 18-8-10-7-5-4.

Saint-Nectaire Isolate Analysis from the FBO that Occurred in 2012 and *In vivo* Stability of the VNTRs Selected

The MLVA method was retrospectively applied on a set of food and human strains suspected of being related in the framework of a Saint-Nectaire outbreak in 2012. The *S.* Dublin strains isolated from two different cheese producers in France were differentiated in two distinct MLVA profiles (19-8-10-7-5-4 and 14-8-10-7-5-4; **Figure 2**, groups B and C, respectively). These profiles were those identified for the human strains, leading us to suspect that two different clones were implicated in this FBO (**Table 4**). The MLVA enabled us to discriminate epidemiologically related strains from sporadic case strains, while PFGE assigned only one PFGE profile (SDUBXB0003) for all the FBO and sporadic case strains, and was therefore not discriminant. Two strains from Saint-Nectaire cheese of producer 2 and sampled in the remainder of the meal consumed by patients 3 and 4 (each one from a different district) displayed the same MLVA profiles (14-8-10-7-5-4) as the strains from patients 3 and 4. Two strains recovered from a filter for milk and cheese directly from producer 1 and two strains sampled in the remainder of the meal of patients 1 and 2 (from two different districts) displayed the same MLVA profile 19-8-10-7-5-4. These two MLVA profiles (14-8-10-7-5-4 and 19-8-10-7-5-4) differed for five RUs of the TR STTR5.

DISCUSSION

Sensitive and specific molecular epidemiological tools are needed to identify the transmission route when FBO events are investigated. Moreover, given the multinational distribution of

TABLE 4 | Multiple-locus variable number tandem repeat analysis profiles of FBO strains assigned to the same PFGE profile (SDUBXB0003).

Origin	MLVA profile	Producer	Strain
Ripened Saint-Nectaire	19 – 8 – 10 – 7 – 5 – 4	1	12CEB3653SAL
Ripened Saint-Nectaire	19 – 8 – 10 – 7 – 5 – 4		12CEB3771SAL
Non-ripened Saint-Nectaire	19 – 8 – 10 – 7 – 5 – 4		12CEB3592SAL
Filtered milk	19 – 8 – 10 – 7 – 5 – 4		12CEB3537SAL
Patient 1	19 – 8 – 10 – 7 – 5 – 4		201206592
Patient 2	19 – 8 – 10 – 7 – 5 – 4		201206547
Ripened Saint-Nectaire	14 – 8 – 10 – 7 – 5 – 4	2	12CEB3654SAL
Ripened Saint-Nectaire	14 – 8 – 10 – 7 – 5 – 4		12CEB3657SAL
Patient 3	14 – 8 – 10 – 7 – 5 – 4		201207167
Patient 4	14 – 8 – 10 – 7 – 5 – 4		201207452

some food products, collaboration between countries can be crucial in identifying cases and in tracing the source of infection. In this study, we developed an MLVA scheme with 6-loci MLVA to subtype *Salmonella* Dublin strains. This scheme was developed following the guidelines published by Nadon et al. (2013). Twenty-four reference strains were characterized in depth and a scheme for normalization of results was proposed. The discriminatory power of this MLVA scheme was higher than that of the gold standard PFGE method. The 51 strains from the ANSES *Salmonella* Network Collection studied to determine the polymorphism of the 6 *loci* selected for the development of the Dublin MLVA analysis were clustered in 27 different MLVA profiles. PFGE was able to discriminate the same panel of strains in only 13 PFGE profiles displaying a low level of discriminatory power (MLVA DI 0.954 and PFGE DI 0.625, respectively). Previous studies have already shown the higher subtyping sensitivity of MLVA for some *Salmonella* serovars such as *S.* Typhimurium and *S*. Enteritidis, compared to historical methods (Wattiau et al., 2011 review). The MLVA method developed in this study, provided sufficient allelic variation to subdivide the 401 human, animal and food *S*. Dublin strains from France into 71 MLVA profiles. The STTR5 and SE-2 loci had the highest number of alleles and genetic diversity values in agreement with the results of Kjeldsen et al. (2014). These authors reported a four locus MLVA protocol in 2014 with three genomic loci SE-2, SE-5 (equal to the STTR5 locus) and SE-1, plus one locus, SD1, present in the plasmid pCT02021853_74. They also tested five others loci, among which SE-6, that were ultimately not selected because of their low discriminatory power within the panel of 272 strains analyzed. In contrast, we retained the SE-6 locus, called in our study STTR3, because it showed higher discriminatory power within the panel of French strains analyzed. A specificity of higher allelic mutation for the French strains compared to the Danish one for this locus cannot be excluded. Therefore, we did not select loci present on plasmids because of the high variability of the presence or absence of plasmids in *Salmonella* and the ability to acquire or lose such plasmids (Rychlik et al., 2006 review). Nevertheless, we looked for the plasmid-located SD1 locus described by Kjeldsen et al. (2014) and it was in fact not present in any of the studied strains. We also investigated the STTR10pl repeat located on the pSLT plasmid (Lindstedt et al., 2004) within the panel of 401 strains and it was found only in 19 strains (4%).

The MLVA scheme was then used to re-investigate an FBO that occurred in France in 2012 and it was shown to successfully cluster strains from an epidemiologically confirmed outbreak. The *in vivo* stability of the sequences of the RUs was investigated through the isolates analyzed for this FBO. The six *loci* exhibit *in vivo* stability, although the strains differ by sources, time period of sampling and geographical origin. The higher polymorphism identified for the human strains allowed us to distinguish the epidemiologically related strains from other strains isolated among sporadic cases.

Sequencing confirmed the number of TRs. Alignments of sequences revealed that the six loci do not exhibit variation in the flanking sequences and in the sequence of the TR unit within a strain and between strains, even though some SNPs were identified. The SNPs compared with the genome of *S.* Dublin strains CT_02021853 were in the TRs of the STTR3, STTR7, SENTR1, and SENTR3 loci. Sequencing also showed that no insertions and deletions were present in repeat units, except for the STTR7 VNTR for which an insertion of six bases was observed in the reference strain 09CEB6631SAL and in the genome of *S*. Dublin strains CT_02021853.

Given the importance of normalizing the raw results for the comparison of MLVA profiles between laboratories, in this study we defined a set of 24 reference strains and therefore recommend using them for better comparability of results (**Table 2**). This set of reference strains is available from the collection at the ANSES *Salmonella* Network and National Reference Centre of Institut Pasteur. For some serovars, such as Typhimurium (Larson et al., 2009) and Enteritidis (Hopkins et al., 2011), this reference strain set has already been published with the name of the reference strains, the correct MLVA profile, and the true length of each VNTR locus analyzed. The MLVA_normalizer workflow (Bachelerie et al., 2016) enables correction of the raw data obtained with the Dublin MLVA protocol proposed here. The conversion table described herein to ensure compatibility of *S.* Dublin MLVA data between laboratories is also available in **Supplementary Table S3**.

The 6-loci MLVA exhibited high discriminatory power for the 401 strains analyzed. Nine of the MST groups were represented both by human, animal and food strains. The rate of VNTR variation among human strains was higher than that among animal and food strains. We identified 44 different MST groups among the human strains ($n = 94$), 33 among the food strains ($n = 198$), and 25 among the animal strains ($n = 109$). The higher variability of MLVA profiles observed for the human strains could be explained by the longer period analyzed, from 1929 to 2015 for human strains, and from 1972 to 2015 for animal and food strains. The most frequently encountered MLVA profile

(19-8-10-7-5-3) included 116 strains that were recovered from cattle ($n = 37$), milk and cheese ($n = 53$) and clinical strains ($n = 17$). When analyzing the presence of strains in animals (mainly cattle) and in humans in our panel of strains from France, we observed substantial overlap of MLVA profiles between these strains. This could indicate that the same strains were responsible for animal and human cases in France. France's cheese manufacturing sector using cow's milk (including pressed cheeses and uncooked cheeses) produced 2,50,000 tons in 2012, and grew by 6% in 2013. This segment represents 11% of the total cheese production sector in the country and includes cheeses under protected designation of origin (PDO) or protected geographical indication (PGI). In France, cheese consumption *per capita* is high and relatively stable in the long term. In total, including purchases by households, industry and the consumer via catering, cheese consumption is estimated at more than 26 kg *per capita* per year (Ministere de l'Agriculture, de l'Agroalimentaire, et de la Forêt, 2014). Because of the importance of milk product consumption and production in France, and taking into account recent FBO events, it appears essential to have a typing method that discriminates *S*. Dublin strains for purposes of monitoring and surveillance, investigation of outbreaks, in the frame of in-house and official controls.

CONCLUSION

The ANSES Laboratory for Food Safety has been using this MLVA scheme to subtype *S*. Dublin since 2012. The results of this study are in complete agreement with the work presented by Kjeldsen et al. (2014), indicating that MLVA is a beneficial tool for investigating *Salmonella* Dublin in different epidemiological situations.

AVAILABILITY OF DATA AND MATERIAL

The reference strains for Dublin MLVA typing are from the *Salmonella* Network Collection of ANSES and from the National Reference Centre (Institut Pasteur). They are available by writing to: Réseau *Salmonella*, Laboratoire de Sécurité des Aliments, ANSES; 14 rue Pierre et Marie Curie; 94701 Maisons-Alfort or by emailing: sabrina.cadelsix@anses.fr.

AUTHOR CONTRIBUTIONS

SC-S designed, developed, and piloted the experiments for the MLVA scheme. ECha performed experiments during MLVA scheme development. M-LV performed experiments during the development of the MLVA scheme, and analyzed and interpreted the data for the work. MM performed PFGE analyses. VM and SLH provided strains. EChe performed the MST analysis. NJDS, SLH, RL, SC-S, and M-LV were involved in collecting FBO data, strains and analyses. SC-S and M-LV drafted the manuscript. NJDS, RL, SLH, and AB participated in the discussion and reviewed the report. All authors read, commented, and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by funding from the *Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt* and the *Association de Coordination Technique pour l'Industrie Agro-Alimentaire* (ACTIA-UMT ARMADA) and by the *Salmonella* Network, part of the Laboratory for Food Safety at ANSES (France).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00295/full#supplementary-material

TABLE S1 | List of strains analyzed in the present study.

TABLE S2 | Twenty-one VNTR markers tested. The 21 VNTR markers published from 2003 to 2009 and collected in the review of Kruy et al. (2011) selected for the study and blasted on the genomic sequence of *Salmonella* Dublin strains CT_02021853 (accession No. NC_011205.1/CP001144.1).

TABLE S3 | Multiple-locus variable number tandem repeat analysis and PFGE profiles for 51 strains used for calculating the discriminatory power of the two typing methods.

TABLE S4 | Input file with data on the 24 standardization strains of *Salmonella* Dublin. This table can be used within an MLVA_normalizer workflow for normalizing MLVA results (Bachelerie et al., 2016).

REFERENCES

Bachelerie, P., Felten, A., Vignaud, M. L., Glasset, B., Feurer, C., Lailler, R., et al. (2016). MLVA_normalizer: workflow for normalization of MLVA profiles and data exchange between laboratories. *J. Proteomics Bioinform.* 9, 25–27. doi: 10.4172/jpb.1000385

Beltran, P., Musser, J. M., Helmuth, R., Farmer, J. J., Frerichs, W. M., Wachsmuth, I. K., et al. (1988). Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes *S*. choleraesuis, *S*. derby, *S*. dublin, *S*. enteritidis, *S*. heidelberg, *S*. infantis, *S*. newport, and *S*. typhimurium. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7753–7757. doi: 10.1073/pnas.85.20.7753

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27, 573–580. doi: 10.1093/nar/27.2.573

Boqvist, S., and Vagsholm, I. (2005). Risk factors for hazard of release from *Salmonella*-control restriction on Swedish cattle farms from 1993 to 2002. *Prev. Vet. Med.* 71, 35–44. doi: 10.1016/j.prevetmed.2005.05.003

Centers for Disease Control [CDC] (1984). *Salmonella* dublin and raw milk consumption – California. *MMWR Morb. Mortal. Wkly. Rep.* 33, 196–198.

Chowdry, N., Threlfall, E. J., Rowe, B., and Stanley, J. (1993). Genotype analysis of faecal and blood isolates of *Salmonella* dublin from humans in England and Wales. *Epidemiol. Infect.* 110, 217–225. doi: 10.1017/S0950268800068138

Clegg, F. G., Wray, C., Duncan, A. L., and Appleyard, W. T. (1986). Salmonellosis in two dairy herds associated with a sewage farm and water reclamation plant. *J. Hyg.* 97, 237–246. doi: 10.1017/S0022172400065323

European Food Safety Authority [EFSA] (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA J.* 13:4329. doi: 10.2903/j.efsa.2015.4329

Findlay, C. R. (1972). The persistence of *Salmonella* dublin in slurry in tanks and on pasture. *Vet. Rec.* 91, 233–235. doi: 10.1136/vr.91.10.233

Giron, S., Le Hello, S., Salah, S., Mazur, M., Lailler, R., Barataud, D., et al. (2013). "*Salmonella* Dublin: What is its potential severity? Investigating a national foodborne outbreak of Salmonellosis associated with the consumption of raw milk cheese, France, August-September 2012," in *Proceedings of the International Symposium Salmonella and Salmonellosis (I3S) March 2013*, Saint Malo.

Grimont, P. A. D., and Weill, F. X. (2007). *Antigenic Formulae of the Salmonella Serovars*, 9th Edn. Paris: WHO Collaborating Center for Reference and Research on Salmonella, Institut Pasteur.

Hopkins, K. L., Peters, T. M., de Pinna, E., and Wain, J. (2011). Standardisation of multilocus variable-number tandem-repeat analysis (MLVA) for subtyping of *Salmonella enterica* serovar Enteritidis. *Euro. Surveill.* 16:19942.

Hunter, P. R., and Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466.

Inventaire du Réseau Salmonella (2010). *Sérotypage des Salmonella d'origine non humaine*. Available at: https://sites.anses.fr/fr/system/files/private/SML-Ra-INVRS2010.pdf.

Kerouanton, A., Brisabois, A., Grout, J., and Picard, B. (1996). Molecular epidemiological tools for *Salmonella* Dublin typing. *FEMS Immunol. Med. Microbiol.* 14, 25–29. doi: 10.1111/j.1574-695X.1996.tb00263.x

Kjeldsen, M. K., Torpdahl, M., Campos, J., Pedersen, K., and Nielsen, E. M. (2014). Multiple-locus variable-number tandem repeat analysis of *Salmonella enterica* subsp. *enterica* serovar Dublin. *J. Appl. Microbiol.* 116, 1044–1054. doi: 10.1111/jam.12441

Kruy, S. L., van Cuyck, H., and Koeck, J. L. (2011). Multilocus variable number tandem repeat analysis for *Salmonella enterica* subspecies. *Eur. J. Clin. Microbiol. Infect. Dis.* 30, 465–473. doi: 10.1007/s10096-010-1110-0

Lailler, R., Moury, F., Granier, S., and Brisabois, A. (2012). *Le Réseau Salmonella, un outil pour la surveillance des salmonelles de la; fourche à la fourchette, EuroReference, N° 8, ER08-12RX01*. Available at: https://sites.anses.fr/fr/system/files/private/ER08-Reseaux-Salmonella.pdf.

Larson, J. T., Torpdahl, M., Petersen, R. F., Sorensen, G., Lindstedt, B. A., and Nielsen, E. M. (2009). *Development of a New Nomenclature for Salmonella Typhimurium Multilocus Variable Number of Tandem Repeats Analysis (MLVA)*. Available at: http://www.eurosurveillance.org/images/dynamic/EE/V14N15/art19174.pdf

Liebana, E., Garcia-Migura, L., Clouting, C., Cassar, C. A., Clifton-Hadley, F. A., Lindsay, E. A., et al. (2002). Investigation of the genetic diversity among isolates of *Salmonella enterica* serovar Dublin from animals and humans from England, Wales and Ireland. *J. Appl. Microbiol.* 93, 732–744. doi: 10.1046/j.1365-2672.2002.01737.x

Lindstedt, B. A., Heir, E., Gjernes, E., and Kapperud, G. (2003). DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar typhimurium with emphasis on phage type DT104 based on variable number of tandem repeat loci. *J. Clin. Microbiol.* 41, 1469–1479. doi: 10.1128/JCM.41.4.1469-1479.2003

Lindstedt, B. A., Vardund, T., Aas, L., and Kapperud, G. (2004). Multiple-locus variable-number tandem-repeats analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. *J. Microbiol. Methods* 59, 163–172. doi: 10.1016/j.mimet.2004.06.014

Maguire, H., Cowden, J., Jacob, M., Rowe, B., Roberts, D., Bruce, J., et al. (1992). An outbreak of *Salmonella* dublin infection in England and Wales associated with a soft unpasteurized cows' milk cheese. *Epidemiol. Infect.* 109, 389–396. doi: 10.1017/S0950268800050378

McDonough, P. L., Fogelman, D., Shin, S. J., Brunner, M. A., and Lein, D. H. (1999). *Salmonella enterica* serotype Dublin infection: an emerging infectious disease for the northeastern United States. *J. Clin. Microbiol.* 37, 2418–2427. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC85243/pdf/jm002418.pdf

Ministere de l'Agriculture, de l'Agroalimentaire, et de la Forêt (2014). *Panorama des Iindustries Agroalimentaire, Fabrication de Fromages 2014*. Available at: http://agriculture.gouv.fr/sites/minagri/files/documents/IAA-Panorama-2014-web_cle4c47a6-2.pdf

Nadon, C. A., Trees, E., Ng, L. K., Nielsen, E. M., Reimer, A., Maxwell, N., et al. (2013). Development and application of MLVA methods as a tool for inter-laboratory surveillance. *Euro. Surveill.* 18:20565. doi: 10.2807/1560-7917.es2013.18.35.20565

Nielsen, L. R. (2009). *Overview of Pathogenesis, Epidemiology and Diagnostic Tools Necessary for Successful Surveillance and Eradication of Salmonella Dublin from the Danish cattle population: prize assignment "Professor Dr.med.h.c. C.O. Jensens Mindefond."* Available at: http://www.diku.dk/Ansatte/?pure=files%2F32981191%2FC.O._Jensen_fonds_prisopgave_om_Salmonella_Dublin.pdf

Nielsen, T. D., Kudahl, A. B., Østergaard, S., and Nielsen, L. R. (2013). Gross margin losses due to *Salmonella* Dublin infection in Danish dairy cattle herds estimated by simulation modelling. *Prev. Vet. Med.* 111, 51–62. doi: 10.1016/j.prevetmed.2013.03.011

O'Leary, C. (2014). *Salmonella* dublin in Irish cattle. *Vet. Irel. J.* 4, 642–643.

Olsen, J. E., and Skov, M. (1994). Genomic lineage of *Salmonella enterica* serovar Dublin. *Vet. Microbiol.* 40, 271–282. doi: 10.1016/0378-1135(94)90116-3

Plym-Forshell, L., and Ekesbo, I. (1996). Survival of salmonellas in urine and dry faeces from cattle–an experimental study. *Acta Vet. Scand.* 37, 127–131.

PulseNet (2013). *Standard Operating Procedure for PulseNet PFGE of Escherichia coli O157:H7, Escherichia coli non-O157 (STEC), Salmonella Serotypes, Shigella sonnei and Shigella flexneri*. Available at: http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL05_Ec-Sal-ShigPFGEprotocol.pdf

Ramisse, V., Houssu, P., Hernandez, E., Denoeud, F., Hilaire, V., Lisanti, O., et al. (2004). Variable number of tandem repeats in *Salmonella enterica* subsp. *enterica* for typing purposes. *J. Clin. Microbiol.* 42, 5722–5730. doi: 10.1128/JCM.42.12.5722-5730.2004

Rychlik, I., Gregorova, D., and Hradecka, H. (2006). Distribution and function of plasmids in *Salmonella enterica*. *Vet. Microbiol.* 112, 1–10. doi: 10.1016/j.vetmic.2005.10.030

Wattiau, P., Boland, C., and Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Appl. Environ. Microbiol.* 77, 7877–7885. doi: 10.1128/AEM.05527-11

Weill, F., and Le Hello, S. (2013). *Bilan des Activités 2013 du Centre National de Référence des Esherichia coli, Shigella et Salmonella*. Available at: https://www.pasteur.fr/fr/sante-publique/CNR/les-cnr/escherichia-coli-shigella-salmonella/rapports-d-activite

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Vignaud, Cherchame, Marault, Chaing, Le Hello, Michel, Jourdan-Da Silva, Lailler, Brisabois and Cadel-Six. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 March 2017
doi: 10.3389/fmicb.2017.00552

Antibacterial Action of Jineol Isolated from *Scolopendra subspinipes mutilans* against Selected Foodborne Pathogens

Vivek K. Bajpai[1†], Shruti Shukla[2†], Woon K. Paek[3], Jeongheui Lim[3], Pradeep Kumar[4]* and MinKyun Na[5]**

[1] Microbiome Laboratory, Department of Applied Microbiology and Biotechnology, Yeungnam University, Gyeongsan, South Korea, [2] Department of Energy and Materials Engineering, Dongguk University, Seoul, South Korea, [3] National Science Museum, Ministry of Science, ICT and Future Planning, Daejeon, South Korea, [4] Department of Forestry, North Eastern Regional Institute of Science and Technology (Deemed University), Nirjuli, India, [5] College of Pharmacy, Chungnam National University, Daejeon, South Korea

This study was undertaken to assess the antibacterial potential of 3,8-dihydroxyquinoline (jineol) isolated from *Scolopendra subspinipes mutilans* against selected foodborne pathogens *Escherichia coli* O157:H7 and *Staphylococcus aureus* KCTC-1621. Jineol at the tested concentration (50 μL; corresponding to 250 μg/disk) exhibited significant antibacterial effects as a diameter of inhibition zones (11.6–13.6 mm), along with minimum inhibitory concentration (MIC) and minimum bactericidal concentration values found in the range of (62.5–125 μg/mL) and (125–250 μg/mL), respectively. Jineol also exhibited significant antibacterial effects as confirmed by the reduction in bacterial cell viabilities, increasing release of potassium (K^+) ions (650 and 700 mmole/L) and 260 nm materials (optical density: 2.98–3.12) against both the tested pathogens, *E. coli* O157:H7 and *S. aureus* KCTC-1621, respectively. Moreover, changes in the cell wall morphology of *E. coli* O157:H7 and *S. aureus* KCTC-1621 cells treated with jineol at MIC further confirmed its inhibitory potential against the tested pathogens, suggesting its role as an effective antimicrobial to control foodborne pathogens.

Keywords: antimicrobial effect, jineol, foodbrone pathogens, scanning electron microscopy

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Alejandro Castillo, Texas A&M University, USA
Soner Soylu, Mustafa Kemal University, Turkey

***Correspondence:**
Pradeep Kumar
pkbiotech@gmail.com
Jeongheui Lim
jhlim1226@naver.com
MinKyun Na
mkna@cnu.ac.kr

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 January 2017*
Accepted: *16 March 2017*
Published: *28 March 2017*

Citation:
Bajpai VK, Shukla S, Paek WK, Lim J, Kumar P and Na M (2017) Antibacterial Action of Jineol Isolated from Scolopendra subspinipes mutilans against Selected Foodborne Pathogens. Front. Microbiol. 8:552. doi: 10.3389/fmicb.2017.00552

INTRODUCTION

Foodborne illnesses caused by foodborne pathogenic bacteria affect a huge number of population world-wide. Development of natural alternative means as safe antimicrobials is essential in combating serious foodborne pathogens which pose significant threat to humans (Alwash et al., 2013). Among several studied foodborne pathogens, to some extent, *Escherichia coli* O157:H7, and *Staphylococcus aureus* are known to be causative agents of foodborne diseases. The development of resistance of foodborne pathogens in the commercial antibiotics and the emergence of new strains are widespread concerns (Alwash et al., 2013).

Staphylococcus aureus possess the ability to produce enterotoxins as well as contributes in hospital-acquired diseases and food-poisoning, thus, considered a serious foodborne pathogen among others (Pereira et al., 2009). The occurrence of *S. aureus* toxicity depends on the capability of the strain to survive, multiply under a variety of conditions and produce extracellular toxic

compounds. Contaminated food, especially undercooked ground beef, raw milk, soft cheese, raw fruits and vegetables are major sources of *E. coli* O157:H7 associated illness. *E. coli* O157:H7 has the ability to produce Shiga toxin, thereby causes bloody diarrhea and sometimes kidney failure (Du et al., 2008).

Consumers, the food industry and the regulatory authorities are concerned about the safety of food from the contamination by foodborne pathogens (Al-Zorekya and Al-Taher, 2015). In the USA, foodborne pathogens have been reported to be the cause of 75% of the foodborne disease outbreaks, involving 68% of all reported cases of foodborne illness (Scot, 2003). Additionally, in Canada, a worth of approximately 500 million dollars is imposed on the treatment of diseases caused by foodborne pathogens surviving on meat or meat-products (Oussalah et al., 2007). In addition, failures in preservation technologies to control foodborne and food spoilage pathogens have reinforced the suggestion for exploring other effective classes of antimicrobials (Awaisheh and Ibrahim, 2009; Negi, 2012). Furthermore, prevalence of synthetic and chemical additives in food and food products has urged an urgent need of application of natural preservatives to meet the consumer acceptability (Shakiba et al., 2011; Shen et al., 2014).

Previous findings support the fact that the use of chemical or synthetic food preservatives imposes a higher rate of health complications, therefore, food processors are often looking for safe and effective antimicrobials of natural origin for food protection and food preservation purposes (Taguri et al., 2004; Santas et al., 2010; Soylu et al., 2010). Also, development of pathogen resistance to commercially available antimicrobials evidences employment of innovative research strategies to explore safe, and effective antimicrobial treatments (Militello et al., 2011). Hence, the present study was designed to isolate a biologically effective 3,8-dihydroxyquinoline (jineol) from a centipede *Scolopendra subspinipes mutilans*, and to determine its antimicrobial potential against selected foodborne pathogenic bacteria.

MATERIALS AND METHODS

Chemicals and Reagents

The nutrient broth (NB) medium was purchased from Difco Ltd., USA. Highly pure quality reagents and chemicals were employed for the test assays. Test samples of jineol were prepared in 1% dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Germany). For absorbance reading, an enzyme-linked immunosorbent assay (ELISA) (Tecan, Infinite M200, Männedorf, Switzerland) was used.

Test Foodborne Pathogens

Staphylococcus aureus KCTC1621 (Gram+), and *E. coli* O157:H7 (Gram−) foodborne pathogens procured from the Korean Collection for Type Cultures (KCTC, Korea) were used in this study. For the growth and culture of strains, NB was used and cultures were incubated at 37°C, followed by maintenance of strains on nutrient agar (NA) slants at 4°C. To reactivate the cultures, cultures were taken out and a loop-full of colonies were inoculated in the fresh NB medium, and incubated for 24 h and 37^{o}C. Further, sub-culturing was maintained in NB medium.

Insect Material

Dried *Scolopendra subspinipes mutilans* specimens were purchased from the herbal market at Geumsan, South Korea, and identified by in-charge of the department. A voucher specimen (CNU-INS 1408) was deposited at the Pharmacognosy Laboratory of the College of Pharmacy, Chungnam National University (Daejeon, South Korea).

Extraction, Isolation, and Characterization of Jineol

Jineol was isolated from specimens using a chromatographic approach (Lee et al., 2016). Briefly, the dried ethanol (EtOH) extract (110.0 g) was suspended in water and fractionated successively with ethyl acetate (EtOAc) and then *n*-butanol (BuOH) to yield EtOAc-soluble (60.0 g) and *n*-BuOH-soluble (8.0 g) fractions, and residue (40.0 g). The EtOAc-soluble fraction was subjected to vacuum-liquid chromatography (VLC) using hexane-EtOAc 40:1, 20:1, 10:1, and 4:1; hexane-EtOAc-MeOH 2:1:0.2; $CHCl_3$-MeOH 6:1; $CHCl_3$-MeOH-H_2O 3:1:0.1 and then washed with MeOH to yield eight fractions (E1–E8). Fraction E4 (8.0 g), which was obtained by eluting with hexane-EtOAc 4:1 was partitioned with hexane and MeOH to afford jineol (60.0 mg).

Jineol: yellowish amorphous powder; ^{1}H NMR (300 MHz, CD_3OD) δ_H 8.46 (1H, d, $J = 2.4$ Hz, H-2), 7.42 (1H, d, $J = 2.4$ Hz, H-4), 7.29 (1H, t, $J = 8.0$ Hz, H-6), 7.14 (1H, d, $J = 8.0$ Hz, H-5), 6.87 (1H, d, $J = 8.0$ Hz, H-7), ^{13}C NMR (75 MHz, CD_3OD) δc 154.2 (C-8), 153.0 (C-3), 142.3 (C-2), 134.8 (C-8a), 131.9 (C-4a), 129.0 (C-6), 117.9 (C-5), 117.2 (C-4), 109.0 (C-7) (Moon et al., 1996).

Determination of Antibacterial Activity of Jineol

A method of agar diffusion was employed to determine the antibacterial activity of jineol using Luria-Bertani (LB) agar plates (Bajpai and Kang, 2010). To make the desired concentrations (0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 cells/mL) of the tested strains, pathogens were cultured in NB medium following incubation at 37°C for 24 h and serially diluted. Further, a method of the microbial plate-count was employed to determine the viable cell numbers. A 100 μL inoculum containing 10^7 CFU/mL was poured on dried agar plates and spread uniformly using a bacterial plate spreader followed by drying at room temperature. The compound was dissolved in 5% DMSO, and finally 50 μL jineol solution, corresponding to 250 μg/disk was impregnated on a sterilized filter paper (Whatman No. 1) disk. The same solvent used for dissolving the sample was also tested as a negative control. Further, plates followed the incubation of 37°C for 24 h, and zones of inhibition around the disks were measured to confirm the antibacterial activity of test compound in triplicate measurements.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Jineol

A method of twofold serial dilution was employed to determine the MIC of jineol (Bajpai et al., 2013). At first, jineol was dissolved in the DMSO (5%), followed by incorporation into the NB medium to make a 500 μg/mL solution of jineol. Further, serial dilutions of jineol solution were made in NB to obtain 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/mL concentrations of jineol. A 10 μL standardized suspension (~10^7 CFU/mL) of each tested organism was inoculated into each tube. Controls were devoid of sample and contained only bacterial inoculum. No bacterial growth in the lowest concentration (μg/mL) of jineol following macroscopic analysis confirmed the MIC of jineol. The cultures (50 μL each) in which jineol concentrations did not show any visual bacterial growth were spread on NA plates in triplicates following incubation of 37°C for 24 h. Finally, the lowest concentration which completely inhibited the formation of CFU on NA plate was referred as MBC of jineol.

Determination of the Effect of Jineol on Bacterial Viabilities

Freshly grown bacterial colonies of the selected pathogenic bacteria were inoculated in NB and incubated at 37°C for 24 h, and then bacterial cultures were serially diluted to 10^7 CFU/mL (Shin et al., 2007). To determine the effect of jineol on cell viabilities, each of the tubes containing the bacterial suspension (10 μL; ~10^7 CFU/mL) of *S. aureus* KCTC1621 and *E. coli* O157:H7 was inoculated with 100 μL of jineol at its MIC in 890 μL NB broth at 37°C. A time interval of 0, 40, 80, 120, 160, and 200 min was followed to take the sample for counting cell viabilities on NA plates (Bajpai et al., 2013). Counting of CFU was performed after 24 h of incubation at 37°C. Controls were prepared in a similar manner except the treatment of jineol in triplicate.

Determination of the Effect of Jineol on the Release of Potassium (K^+) Ions

A previously developed method was adopted for the determination of the effect of jineol on K^+ ion efflux from the cells of tested bacteria (Bajpai et al., 2013). The concentration of free K^+ ions from the cell suspensions of *S. aureus* KCTC1621 and *E. coli* O157:H7 was determined for 0, 30, 60, 90, and 120 min following the jineol exposure to bacterial cells at MIC employing sterilized peptone-water. Photometeric measurement of extracellular K^+ ion was performed at each above-mentioned time interval, using a commercial kit for Calcium/Potassium detection. Controls were also tested in a similar way without the addition of jineol. Data were presented as the release of extracellular K^+ ion concentration (mmol/L) in a triplicate set.

Determination of the Effect of Jineol on the Release of 260-nm Absorbing Cellular Materials

The measurements of the release of 260-nm-absorbing components (DNA/RNA) from *S. aureus* KCTC1621 and *E. coli* O157:H7 cells were performed in a 2 mL aliquot of the bacterial inoculum in a sterilized peptone-water. The loss of 260 nm cellular materials is considered a good indication of the antimicrobial efficacy of any test compound. The reaction solution was added of MIC of jineol following incubation at 37°C. Subsequently, cultures treated at 0, 30, and 60 min were collected separately following centrifugation (3,500 × *g*, 10 min) to obtain the cell free supernatants and read for absorbance at 260-nm using an ELISA (Bajpai et al., 2013). Controls tested devoid of jineol. Data were collected at each time point and presented as optical densities (ODs) of the samples.

Determination of the Effect of Jineol on the Cell Wall Morphology of Foodborne Pathogens

Scanning electron microscopic (SEM) study was executed according to Kim et al. (2007) to examine the effects of jineol on the morphological changes in the cell wall of the selected pathogens, *S. aureus* KCTC1621 and *E. coli* O157:H7 at MIC. Control samples were prepared without jineol. Microscopic examination was performed using a S-4300 SEM Analyzer (Hitachi, Japan).

Statistical Analysis

Experiments were carried out in a set of triplicate, and the data obtained were presented as mean ± SD following one-way ANOVA statistical analysis coupled with Duncan's multiple test.

RESULTS

Identification and Characterization of Jineol

^{1}H NMR data showed signals for five aromatic protons at δ_H 8.46 (1H, d, J = 2.4 Hz, H-2), 7.42 (1H, d, J = 2.4 Hz, H-4), 7.29 (1H, t, J = 8.0 Hz, H-6), 7.14 (1H, d, J = 8.0 Hz, H-5), and 6.87 (1H, d, J = 8.0 Hz, H-7). Typical proton signals for 3-hydroxy quinoline alkaloid were observed at 8.46 (1H, d, J = 2.4 Hz, H-2) and 7.42 (1H, d, J = 2.4 Hz, H-4). Inspection of the ^{13}C NMR spectra revealed nine aromatic carbon signals. Based on its NMR spectroscopic data analyses, the compound was identified as 3,8-dihydroxyquinoline (jineol) (Moon et al., 1996) (**Figure 1**).

Antibacterial Activity

This study showed antibacterial effects of the test compound jineol as confirmed by the presence of inhibitory zones in agar plates against the tested foodborne pathogenic bacteria, *S. aureus* KCTC1621 and *E. coli* O157:H7. In this assay, jineol exhibited a significant inhibitory effect against both the employed foodborne pathogenic bacteria. The inhibitory effect of jineol in agar plates

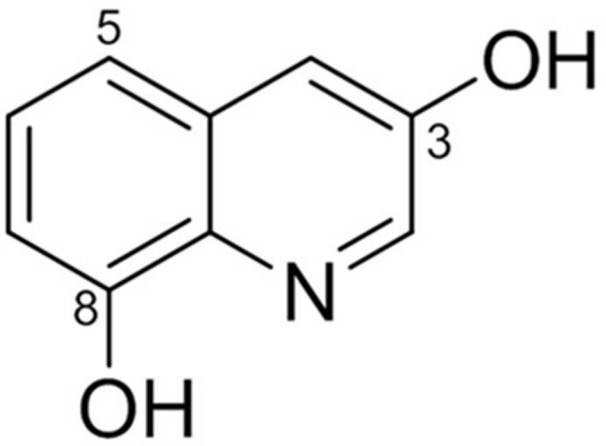


FIGURE 1 | Chemical structure of jineol isolated from *Scolopendra subspinipes mutilans*.

TABLE 1 | Antibacterial activity of jineol against foodborne pathogens *Staphylococcus aureus* KCTC1621 and *Escherichia coli* O157:H7.

Pathogens	Jineol		
	Zones of inhibition[x]	Susceptibility	
		MIC[y]	[z]MBC
Staphylococcus aureus KCTC1621	13.6 ± 0.2[a]	62.5[a]	125[a]
Escherichia coli O157:H7	11.6 ± 0.3[b]	125[b]	250[b]

[x]*Diameters of inhibition zones in millimeter;* [y]*Minimum inhibitory concentration (values in μg/mL);* [z]*Minimum bactericidal concentration (values in μg/mL). Values in the same column with different superscripts are significantly different according to Duncan's Multiple Range Test ($P < 0.05$). All values were expressed as mean ± SD of three parallel measurements ($n = 3$). Zones of inhibition around the disk were measured in millimeter (mm) using a Vernier's caliper.*

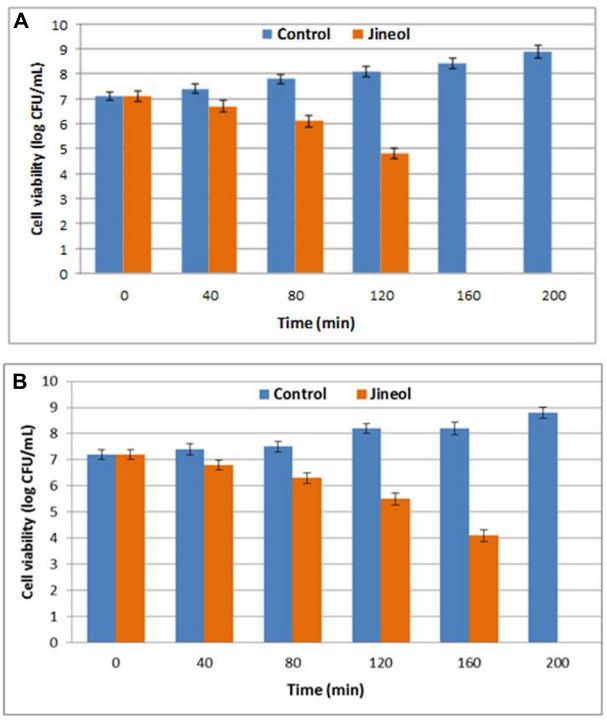


FIGURE 2 | Effect of jineol on the viability of the tested pathogenic bacteria of *S. aureus* KCTC1621 (A) and *E. coli* O157:H7 **(B)**. Control without treatment. Data are expressed as mean ± SD ($n = 3$). Values in the same column with different superscripts are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

was confirmed through the diameters of zones of inhibition, which were found to be 11.6–13.6 mm (**Table 1**). It was observed that jineol exhibited antibacterial effects against both the tested bacterial isolates.

MIC and MBC

This assay revealed different susceptibilities of test compound jineol against the tested foodborne pathogens as confirmed by the low and variable MIC and MBC values. As a result, the MIC and MBC values of jineol against the tested pathogens were ranged 62.5–125, and 125–250 μg/mL, respectively (**Table 1**). In this assay, it was observed that jineol had inhibitory effects against both Gram-positive and Gram-negative bacteria.

Effect on Bacterial Cell Viability

This assay confirmed the antibacterial potential of quinoline alkaloid compound jineol, as confirmed by the reduction in the cell viabilities of the tested pathogens, *S. aureus* KCTC-1621 and *E. coli* O157:H7 when inoculated at MIC (**Figure 2**). Bacterial pathogens when exposed to test compound jineol for 80 min showed no remarkable decrease in the cell viabilities. However, exposure of jineol to pathogen for 160 and 200 min completely inhibited the growth of both the tested pathogens, *S. aureus* KCTC-1621 and *E. coli* O157:H7, respectively (**Figure 2**).

K^+ Ion Efflux

Loss of extracellular K^+ ions from the bacterial cells upon the treatment of specific antimicrobial indicates loss of cell integrity, thus establishing its antimicrobial effect againt the tested bacteria. It was found in this study that jineol at the used concentration exhibited significant antibacterial effect as confirmed by the significant release of K+ ions from the treated bacterial cells of *S. aureus* KCTC-1621 (**Figure 3A**) and *E. coli* O157:H7 (**Figure 3B**). Jineol exhibited time dependent inhibitory effect in this assay. However, no significant release of K+ ions from the control sets were observed.

Release of 260 nm Absorbing Cellular Materials

Since loss of 260 nm cellular materials is considered a good indication of the antimicrobial efficacy of any test compound, this assay confirmed that bacterial cells of tested foodborne pathogens, *S. aureus* KCTC-1621 and *E. coli* O157:H7 upon treatment with jineol at MIC had a severe inhibitory effect in terms of release of 260 nm absorbing materials (DNA and RNA) from them (**Figure 4**). As a result, it was observed that bacterial

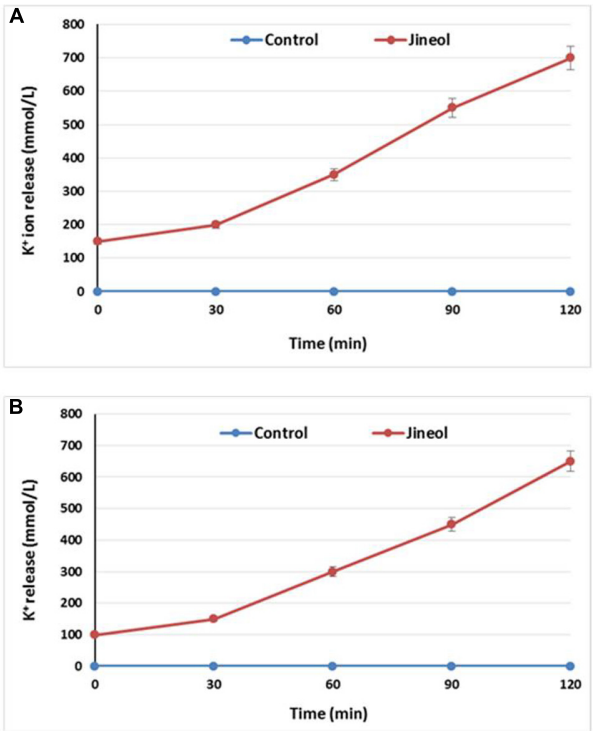


FIGURE 3 | Effect of jineol on the release rate of extracellular K^+ ions from *S. aureus* KCTC1621 (A) and *E. coli* O157:H7 **(B)**. Data are expressed as mean ± SD (n = 3). Values in the same column with different superscripts are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

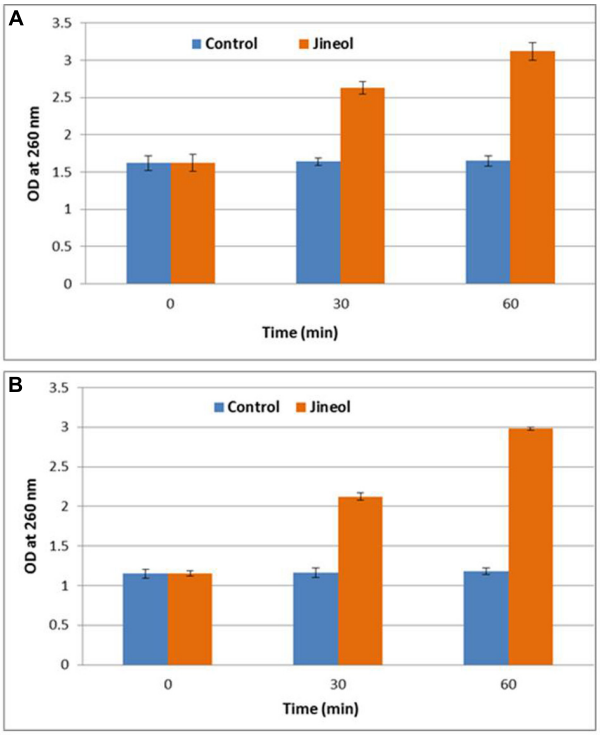


FIGURE 4 | Effect of jineol on the release rate of 260-nm absorbing material from *S. aureus* KCTC1621 (A) and *E. coli* O157:H7 **(B)**. Data are expressed as mean ± SD (n = 3). Values in the same column with different superscripts are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

cells of tested foodborne pathogens, *S. aureus* KCTC-1621 (1.62-3.12) and *E. coli* O157:H7 (1.15-2.98) showed significant differences in their respective ODs as compared to control groups (1.62–1.65) and (1.15–1.18) measured at 260 nm. No significant differences in ODs of control groups were observed in this assay.

Observation of Morphological Changes in Bacterial Cell Wall

It is a vital phenomenon that exposure of the antimicrobial agent to bacterial cells results in the disruption of cell wall, therefore, we performed SEM analysis to further confirm the deteriorating effects of jineol on the cell wall physiologies and morphologies of *S. aureus* KCTC-1621 and *E. coli* O157:H7 cells (**Figure 5**). As a result, jineol significantly altered the cell wall morphology of both the tested pathogens *S. aureus* KCTC-1621 and *E. coli* O157:H7 used at MIC with clear visualization of cell wall damage and cell lysis (**Figures 5B,D**). However, as expected, cells without treatment as a control had intact shape and no morphological changes were observed (**Figures 5A,C**).

DISCUSSION

The jineol showed significant antibacterial effects against both the tested bacterial isolates. To support the findings our study, recently, a number of quinolines and their derivatives have shown significant ability to inhibit the growth of various pathogenic microbes including foodborne pathogenic bacteria (Cherdtrakulkiat et al., 2016). A number of phytochemicals exhibit higher amount of inhibitory effect against a wide range of pathogenic microbes especially Gram-positive bacteria. Similarly, in the present study, jineol exhibited higher inhibitory effects against *S. aureus* KCTC1621, a Gram-positive bacterium than that of a Gram-negative bacterium, *E. coli* O157:H7. This can be encountered by the phenomenon that the hydrophilic thick cell wall of Gram-negative bacteria made by lipopolysaccharide has ability to block and avoid the accumulation of jinol in the target cell membrane than the single membrane, cell wall structure of Gram-positive bacteria, which might be more permeable to the jineol (Bezic et al., 2003).

The jineol shows the variable MIC and MBC value against the both the foodborne pathogens and the similar reports have confirmed antibacterial efficacy of quinoline alkaloid derivatives in MIC assay, which found to elicit significant antibacterial effects against various foodborne pathogenic bacteria with varied MIC and MBC values (Sibi et al., 2014). Also, isoquinoline alkaloids isolated from the rhizome *Coptis chinensis* were found to exhibit a remarkable inhibitory effect in MIC assay against various pathogens, including *S. aureus* and *E. coli* (Kim et al., 2004).

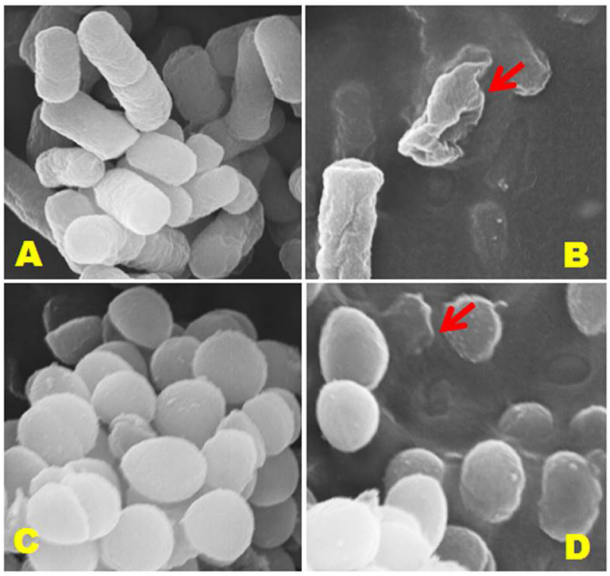


FIGURE 5 | Scanning electron microscopic (SEM) analysis of *E. coli* 0157:H7 and *S. aureus* KCTC-1621 cells treated with jineol at minimum inhibitory concentration (MIC). Controls **(A,C)** showing a regular and smooth surface; whereas treated cells **(B,D)** arrows showing disruption and cell lysis, respectively.

The exposure of jineol to pathogen for 160 and 200 min completely inhibited the growth of both the tested pathogens. Previous reports have confirmed the inhibitory effects of various phytochemicals isolated from different sources on the cell viabilities of foodborne pathogenic bacteria (Rastogi et al., 2008; Bajpai and Kang, 2010).

The significant release of K+ ions from the jineol treated bacterial cells and no release of K^+ ions from the control sets were observed. Similar findings were observed when Patra et al. (2015) tested the efficacy of bio-oil on the release of extracellular K^+ ions from *Bacillus cereus* and *Listeria monocytogenes* cells. Permeability of essential ions such as K^+ ions is regulated by the bacterial plasma membrane where membrane chemo-structural composition plays a significant role on the release of such ions, and extensive release of these ions from the target bacterial cells could be considered as a disruptive effect of treatment agent on the plasma membrane, hence, validating the antimicrobial role of jineol, as also reported previously (Cox et al., 2001).

This study validated that the increasing release of 260-nm absorbing cellular materials from the target bacterial cells mediated by jineol had a huge influence on the release of cellular metabolites indicating structural damage to the plasma membrane, thereby causing cell death. Such findings on specific antimicrobials have also been confirmed previously (Farag et al., 1989). The results clearly indicate that regulation of nucleic acid or cellular materials from the tested pathogens could be considered a significant indication of membrane structural damage. Similar to our findings, Souza et al. (2013) tested the antibacterial efficacy of essential oil components, carvacrol and thymol against foodborne pathogens, and it was noted that both the compounds were able to cause membrane damaging effects, and eventually caused efflux of 260-nm nuclear components.

Using SEM analysis, it was confirmed that upon treatment with jineol, morphology of treated bacterial cells was dramatically changed or hampered. Similar reports on morphological deteriorating effects of several phytochemicals have been observed previously against various foodborne pathogens (Soylu et al., 2007; Alwash et al., 2013). Such morphological and physiological changes could be caused by the changes in the integrity and fluidity of plasma membrane as well as changes in the composition of the lipid profile of plasma membrane directly hampered by the antimicrobial action of treating agent (Sikkema et al., 1994).

CONCLUSION

This study reports isolation of a quinoline compound jineol from *Scolopendra subspinipes mutilans* which was enough able to exhibit significant inhibitory effects against two selected foodborne pathogens. The findings of this study suggest that the antimicrobial action of jineol could be mediated through its efficacy to alter cell membrane permeability parameters of the tested pathogens as demonstrated by the excessive release of K^+ ions and 260-nm absorbing materials, thus causing membrane disruption as also confirmed during SEM analysis. We conclude that jineol, exhibiting significant antibacterial activity against selected foodborne pathogens could be potential efficacy for using in food and pharma industries as an alternative means of antimicrobial.

AUTHOR CONTRIBUTIONS

Author VB and SS designed performed the experiments and write the manuscript. WP assist during experimental work. PK helps in manuscript preparation and editing. JL and MN help in editing and finalizing the manuscript.

ACKNOWLEDGMENT

This work was supported by National Research Foundation of Korea (2013M3A9A5047052, 2008-2004707 and 2012-0006701).

REFERENCES

Alwash, M. S., Ibrahim, N., and Ahmad, W. Y. (2013). Identification and mode of action of antibacterial components from *Melastoma malabathricum* Linn leaves. *Am. J. Infect. Dis.* 9, 46–58. doi: 10.3844/ajidsp.2013.46.58

Al-Zorekya, N. S., and Al-Taher, A. Y. (2015). Antibacterial activity of spathe from *Phoenix dactylifera* L. against some food-borne pathogens. *Ind. Crops Prod.* 65, 241–246. doi: 10.1016/j.indcrop.2014.12.014

Awaisheh, S. S., and Ibrahim, S. A. (2009). Screening of antibacterial activity of lactic acid bacteria against different pathogens found in vacuum-packaged meat products. *Foodborne Pathog. Dis.* 6, 1125–1132. doi: 10.1089/fpd.2009.0272

Bajpai, V. K., and Kang, S. C. (2010). Antibacterial abietane-type diterpenoid, taxodone from *Metasequoia glyptostroboides* Miki ex Hu. *J. Biosci.* 35, 533–538. doi: 10.1007/s12038-010-0061-z

Bajpai, V. K., Sharma, A., and Baek, K. H. (2013). Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. *Food Control* 32, 582–590. doi: 10.1016/j.foodcont.2013.01.032

Bezic, N., Skocibusic, M., Dinkic, V., and Radonic, A. (2003). Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. *Phytother. Res.* 17, 1037–1040. doi: 10.1002/ptr.1290

Cherdtrakulkiat, R., Boonpangrak, S., Sinthupoom, N., Prachayasittikul, S., Ruchirawat, S., and Prachayasittikul, V. (2016). Derivatives (halgen, nitro and amino) of 8-hydroxyquinoline with highly potent antimicrobial and antioxidant activities. *Biochem. Biophys. Rep.* 6, 135–141.

Cox, S. D., Mann, C. M., Markhan, J. L., Gustafson, J. E., Warmington, J. R., and Wyllie, S. G. (2001). Determining the antimicrobial action of tea tree oil. *Molecules* 6, 87–91. doi: 10.3390/60100087

Du, W. X., Olsen, C. W., Avena-Bustillos, R. J., Mchugh, T. H., Levin, C. E., and Friedman, M. (2008). Antibacterial activity against *E. coli* O157:H7, physical properties, and storage stability of novel carvacrol-containing edible tomato films. *J. Food Sci.* 73, 378–383. doi: 10.1111/j.1750-3841.2008.00892.x

Farag, R. S., Daw, Z. Y., Hewedi, F. M., and El-Baroty, G. S. A. (1989). Antimicrobial activity of some Egyptian spice essential oil. *J. Food Prot*. 52, 665–667. doi: 10.1021/JF101620C

Kim, J. E., Choi, N. H., and Kang, S. C. (2007). Anti-listerial properties of garlic shoot juice at growth and morphology of *Listeria monocytogenes*. *Food Control* 18, 1198–1203. doi: 10.1016/j.foodcont.2006.07.017

Kim, S. H., Shin, D. S., Oh, M. N., Chung, S. C., Lee, J. S., and Oh, K. B. (2004). Inhibition of the bacterial surface protein anchoring transpeptidase sortase by isoquinoline alkaloids. *Biosci. Biotechnol. Biochem.* 68, 421–424. doi: 10.1271/bbb.68.421

Lee, W., Lee, J., Kulkarni, R., Kim, M. A., Hwang, J. S., Na, M., et al. (2016). Antithrombotic and antiplatelet activities of small-molecule alkaloids from *Scolopendra subspinipes* mutilans. *Sci. Rep.* 6:e21956. doi: 10.1038/srep21956

Militello, M., Settanni, L., Aleo, A., Mammina, C., Moschetti, G., Giammanco, G. M., et al. (2011). Chemical composition and antibacterial potential of *Artemisia arborescens* L. essential oil. *Curr. Microbiol.* 62, 1274–1281. doi: 10.1007/s00284-010-9855-3

Moon, S. S., Cho, N., Shin, J., Seo, Y., Lee, C. O., and Choi, S. U. (1996). Jineol, a cytotoxic alkaloid from the centipede Scolopendra subspinipes. *J. Nat. Prod.* 59, 777–779. doi: 10.1021/np960188t

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *Int. J. Food Microbiol.* 156, 7–17. doi: 10.1016/j.ijfoodmicro.2012.03.006

Oussalah, M., Cailet, S., Soucier, I., and Iacroix, M. (2007). Inhibitory effect of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control* 18, 414–420. doi: 10.1016/j.foodcont.2005.11.009

Patra, J. K., Hwang, H., Choi, J. W., and Baek, K. H. (2015). Bactericidal mechanism of bio-oil obtained from fast pyrolysis of *Pinus densiflora* against two foodborne pathogens, *Bacillus cereus* and *Listeria monocytogenes*. *Foodborne Pathog. Dis.* 12, 529–535. doi: 10.1089/fpd.2014.1914

Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., and Teixeira, P. (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolated from various foods in Portugal. *Food Microbiol.* 26, 278–282. doi: 10.1016/j.fm.2008.12.008

Rastogi, N., Domadia, P., Shetty, S., and Dasgupta, D. (2008). Screening of natural phenolic compounds for potential to inhibit bacterial cell division protein FtsZ. *Ind. J. Exp. Biol.* 46, 783–787.

Santas, J., Almajano, M., and Carbó, R. (2010). Antimicrobial and antioxidant activity of crude onion (*Allium cepa* L.) extracts. *Int. J. Food Sci. Technol.* 45, 403–409. doi: 10.1111/j.1365-2621.2009.02169.x

Scot, E. (2003). Food safety and foodborne disease in 21st century homes. *Can. J. Microbiol.* 14, 277–280.

Shakiba, M., Kariminik, A., and Parsia, P. (2011). Antimicrobial activity of different parts of *Phoenix dactylifera*. *Int. J. Mol. Clin. Microbiol.* 1, 107–111.

Shen, X., Sun, X., Xie, Q., Liu, H., Zhao, Y., Pan Hwang, Y., et al. (2014). Antimicrobial effect of blueberry (*Vaccinium corymbosum* L.) extracts against the growth of *Listeria monocytogenes* and *Salmonella enteritidis*. *Food Control* 35, 159–165. doi: 10.1016/j.foodcont.2013.06.040

Shin, S. Y., Bajpai, V. K., Kim, H. R., and Kang, S. C. (2007). Antibacterial activity of bioconverted eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) against foodborne pathogenic bacteria. *Int. J. Food Microbiol.* 113, 233–236. doi: 10.1016/j.ijfoodmicro.2006.05.020

Sibi, G., Venkategowda, A., and Gowda, L. (2014). Isolation and characterization of antimicrobial alkaloids from *Plumeria alba* flowers against foodborne pathogens. *Am. J. Life Sci.* 2, 1–6.

Sikkema, J., De Bont, J. A. M., and Poolman, B. (1994). Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* 269, 8022–8028.

Souza, E. L., Oliveira, C. E. V., Stamford, T. L. M., Conceicao, M. L., and Gomes Neto, N. J. (2013). Influence of carvacrol and thymol on the physiological attributes, enterotoxin production and surface characteristics of *Staphylococcus aureus* strains isolated from foods. *Braz. J. Microbiol.* 44, 29–35. doi: 10.1590/S1517-83822013005000001

Soylu, E. M., Kurt, Ş., and Soylu, S. (2010). *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. *Int. J. Food Microbiol.* 143, 183–189. doi: 10.1016/j.ijfoodmicro.2010.08.015

Soylu, S., Yigitbas, H., Soylu, E. M., and Kurt, S. (2007). Antifungal effects of essential oils from oregano and fennel on *Sclerotinia sclerotiorum*. *J. Appl. Microbiol.* 103, 1021–1030. doi: 10.1111/j.1365-2672.2007.03310.x

Taguri, T., Tanaka, T., and Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol. Pharm. Bull.* 27, 1965–1969. doi: 10.1248/bpb.27.1965

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Bajpai, Shukla, Paek, Lim, Kumar and Na. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 April 2017
doi: 10.3389/fmicb.2017.00579

Turn Up the Heat—Food and Clinical *Escherichia coli* Isolates Feature Two Transferrable Loci of Heat Resistance

Erik J. Boll[1†], *Roger Marti*[2†], *Henrik Hasman*[1], *Søren Overballe-Petersen*[1], *Marc Stegger*[1], *Kim Ng*[1], *Susanne Knøchel*[3], *Karen A. Krogfelt*[1], *Joerg Hummerjohann*[2] *and Carsten Struve*[1*]

[1] *Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark,* [2] *Agroscope, Division of Food Microbial Systems, Microbiological Safety of Foods of Animal Origin Group, Bern, Switzerland,* [3] *Department of Food Science, University of Copenhagen, Copenhagen, Denmark*

Heat treatment is a widely used process to reduce bacterial loads in the food industry or to decontaminate surfaces, e.g., in hospital settings. However, there are situations where lower temperatures must be employed, for instance in case of food production such as raw milk cheese or for decontamination of medical devices such as thermo-labile flexible endoscopes. A recently identified locus of heat resistance (LHR) has been shown to be present in and confer heat resistance to a variety of *Enterobacteriaceae*, including *Escherichia coli* isolates from food production settings and clinical ESBL-producing *E. coli* isolates. Here, we describe the presence of two distinct LHR variants within a particularly heat resistant *E. coli* raw milk cheese isolate. We demonstrate for the first time in this species the presence of one of these LHRs on a plasmid, designated pFAM21805, also encoding type 3 fimbriae and three bacteriocins and corresponding self-immunity proteins. The plasmid was highly transferable to other *E. coli* strains, including Shiga-toxin-producing strains, and conferred LHR-dependent heat resistance as well as type 3 fimbriae-dependent biofilm formation capabilities. Selection for and acquisition of this "survival" plasmid by pathogenic organisms, e.g., in food production environments, may pose great concern and emphasizes the need to screen for the presence of LHR genes in isolates.

Keywords: heat resistance, *E. coli*, transfer of heat resistance, food production, biofilms, clpK

OPEN ACCESS

Edited by:
Giovanna Suzzi,
University of Teramo, Italy

Reviewed by:
Ute Römling,
Karolinska Institutet, Sweden
Catherine Maeve Burgess,
Teagasc—The Irish Agriculture and Food Development Authority, Ireland

***Correspondence:**
Carsten Struve
cas@ssi.dk

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 December 2016*
Accepted: *21 March 2017*
Published: *07 April 2017*

Citation:
Boll EJ, Marti R, Hasman H, Overballe-Petersen S, Stegger M, Ng K, Knøchel S, Krogfelt KA, Hummerjohann J and Struve C (2017) Turn Up the Heat—Food and Clinical Escherichia coli Isolates Feature Two Transferrable Loci of Heat Resistance. Front. Microbiol. 8:579. doi: 10.3389/fmicb.2017.00579

INTRODUCTION

Heat-treatment is an efficient and widely used measure to reduce bacterial contamination. If the material to be decontaminated is heat-stable, autoclaving can be used for complete sterilization. However, there are often situations where this is not feasible, both in food production and in the medical field, and lower temperatures must be employed. This is the case for heat treatment of flexible endoscopes (where thermochemical treatment below 60°C is being used; Jørgensen et al., 2016) or in case of thermization of raw milk for the production of specific cheeses (57–68°C for 15 s or more; Peng et al., 2013a), where protection of certain enzymes is aimed for. These treatments are generally sufficient to reduce the vegetative bacterial load to safe levels, unless the contaminating bacteria are especially heat-resistant.

Exposure of bacteria to severe heat stress results in massive misfolding and aggregation of proteins, and the potential toxic effects of these aggregates, coupled with net loss of active proteins, may cause cell death. Bacteria have evolved elaborate strategies to counteract these effects. Cytosolic chaperone systems with protein folding capacities such as DnaKJE and GroESL, as well as ATP-dependent proteolytic systems such as the Clp ATPases are ubiquitous in bacterial species (Hecker et al., 1996; Frees et al., 2014; Li and Gänzle, 2016). However, we recently identified a novel Clp ATPase, ClpK, uniquely found in Gram-negative bacteria, which also confers heat resistance (Bojer et al., 2010).

The *clpK* gene is located within a cluster termed the locus of heat resistance (LHR) comprising up to 16 open reading frames (ORFs) (Mercer et al., 2015). Located immediately upstream of *clpK* is a gene encoding a small heat shock protein, sHsp20c, which—like ClpK—has been shown to contribute to heat resistance (Lee et al., 2015). The remaining ORFs remain largely uncharacterized, but some are predicted to possess functional properties such as proteases or sodium/hydrogen transporters, implying that overall, the LHR may play a more generalized role in response to external stress (Mercer et al., 2015).

The LHR was originally described in *Klebsiella pneumoniae* and was found in roughly 1/3 of nosocomial *K. pneumoniae* isolates. The high prevalence of LHR in *K. pneumoniae* is likely due to its plasmid-located nature in this organism (Bojer et al., 2010). Notably, LHR-encoding plasmids often also carry multidrug-resistance genes and are transferable by conjugation to other *K. pneumoniae* isolates (Bojer et al., 2010, 2012). The LHR was since discovered in a *Cronobacter sakazakii* isolate, a pathogen associated with serious infections in neonates, which are thought to be linked to contaminated dried infant milk formula (Gajdosova et al., 2011).

Quite recently, a comparative genomic analysis of 29 *E. coli* strains identified a putatively chromosomally located ∼14 kb region with >99% identity to the LHR clusters in *K. pneumoniae* and *C. sakazakii*. In contrast to the *K. pneumoniae* population examined, the LHR only occurred at a frequency of ∼2% among the *E. coli* whole genomes and genome shotgun sequences published at that time (Mercer et al., 2015). We observed a similar frequency of LHR among extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates collected from Danish patients in 2008–2009 (Boll et al., 2016). This could suggest that the LHR does not provide significant general benefits to *E. coli*, or that transfer of LHR only rarely occurs in this organism. On the other hand, previous studies have investigated *E. coli* strains isolated from raw milk cheeses in Switzerland and shown that many of them exhibit increased heat resistance in milk at subpasteurization temperatures and during cheese ripening (Peng et al., 2012, 2013a,b). We recently screened a total of 256 of these *E. coli* raw milk cheese isolates for heat resistance markers *clpK* and an additional LHR marker gene, demonstrating that 93 (36.3%) of them were positive for both (while 24 and 9 isolates, respectively, were positive for one marker). We hypothesize that these increased numbers reflect a thermal selection pressure in this environment (Marti et al., 2016), due to the mild heat treatment employed during processing.

In this study, we focus on a raw milk cheese isolate, FAM21805, exhibiting a significantly increased level of heat resistance. We show that FAM21805 harbors two LHR variants, both of which confer heat resistance and both of which are transferrable by means of horizontal gene transfer. Moreover, one of the LHRs is located on a conjugative plasmid belonging to the IncFII group (pFAM21805) also harboring *mrkABCDF*, a locus encoding type 3 fimbriae (Burmølle et al., 2008). When transferred to *E. coli* K-12 MG1655, the plasmid increases both the heat resistance and biofilm formation properties of that strain. Finally, the plasmid is also transferable to strains of pathogenic *E. coli*. To the best of the authors' knowledge, this is the first description of a plasmid-borne LHR in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in **Table 1**. Bacteria were routinely cultured at 37°C on Luria-Bertani (LB) agar and in LB broth.

Screening PCRs Used in This Study

A total of 90 *E. coli* dairy isolates were screened for LHR marker genes *clpK1* and *clpK2*, and the *mrkABCDF* locus encoding type 3 fimbriae using the primers and annealing temperatures indicated in **Table 2**.

Phenotypic Heat Resistance Screen

E. coli dairy isolates were screened for phenotypic heat resistance by incubation of overnight cultures LB Lennox broth (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride, pH 7.0) at 55°C for 30 min. Strains were diluted 1:10 into pre-heated LB broth and sampled at 0, 15, and 30 min. (duplicate plating). Strains showing a reduction in colony forming units (CFU) of less than one $\log_{10}$ after 30 min. were considered phenotypically heat resistant. When comparing wildtype strains with their LHR mutants or when testing LHR transconjugant strains, a fourth time point at 45 min incubation was added and the assay was performed at least in biological triplicate. Relative survival of a strain at a given time point was calculated by dividing the CFU/ml of that time point by the initial CFU/ml (time point 0).

Genome Sequencing

FAM21805 and FAM21843 genomic DNA was extracted with the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Buchs, Switzerland). Illumina sequencing was done in a 101-bp paired-end run (University of Bern), and *de novo* assembly was performed using CLCbio Genomic Workbench (v9.0.1). For all other strains, genomic DNA was extracted from isolates using a DNeasy Blood and Tissue Kit (QIAGEN, Copenhagen, Denmark). MiSeq libraries were made using the NexteraTMKit (Illumina) and sequencing was performed as 250-bp paired-end runs. Reads were assembled *de novo* using CLCbio Genomic Workbench (v9.5.2). The annotated sequence of $LHR1_{FAM21805}$ has been deposited at GenBank under the accession KY646173. The Whole Genome Shotgun projects of FAM21805 and FAM21843 have been deposited at

TABLE 1 | *E. coli* strains used in this study.

Strain	Misc.[a]	Origin	References
FAM21805	Heat resistant; harbors LHR1 and LHR2	Swiss raw milk cheese	Peng et al., 2012, 2013a; Marti et al., 2016
FAM21805 ΔLHR1	Knock-out mutant, LHR1 (*orf2-orf16*) replaced with *kan*^{r}		This study
FAM21805 ΔLHR2	Knock-out mutant, LHR2 (*orf2-orfF*) replaced with *kan*^{r}		This study
FAM21805 ΔLHR1&2	Knock-out mutant, LHR1&2 replaced with *kan*^{r}		This study
FAM21805 Δ*mrk*	Knock-out mutant, *mrkABCDF* replaced with *tet*^{r}		This study
FAM21805 Δ*orfE*	Knock-out mutant, *orfE* replaced with *tet*^{r}		This study
FAM21805 LHR1 *kan*^{r}	LHR1 tagged with *kan*^{r} without knock-out of known ORFs; donor of LHR1 in HGT experiments		This study
FAM21805 LHR2 *tet*^{r}	LHR2 tagged with *tet*^{r} without knock-out of known ORFs; donor of LHR2 in HGT experiments		This study
C604-10	Heat resistant, ESBL-producing	Human clinical isolate	Olesen et al., 2013; Boll et al., 2016
C604-10 LHR1 *kan*^{r}	LHR1 tagged with *kan*^{r} without knock-out of known ORFs; donor of LHR1 in HGT experiments		Boll et al., 2016
C604-10 LHR2 *tet*^{r}	LHR2 tagged with *tet*^{r} without knock-out of known ORFs; donor of LHR2 in HGT experiments		Boll et al., 2016
MG1655 *nal*^{r} *rif*^{r}	Recipient in HGT experiments, laboratory strain	Commensal K-12 strain	Møller et al., 2003
MG1655 LHR2 (21805)	MG1655 transconjugant harboring LHR2 plasmid of FAM21805 tagged with *tet*^{r} without knock-out of known ORFs		This study
MG1655 LHR1&2 (21805)	MG1655 transconjugant harboring LHR1&2 of FAM21805; LHR1 tagged with *kan*^{r} without knock-out of known ORFs		This study
MG1655 LHR2 Δ*orfE* (21805)	MG1655 transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacing a putative di-guanyl cyclase (*orfE*) with *tet*^{r}		This study
MG1655 LHR2 Δ*mrk* (21805)	MG1655 transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacing *mrkABCDF* with *tet*^{r}		This study
MG1655 LHR1 (C604-10)	MG1655 transconjugant harboring LHR1 of C604-10; tagged with *kan*^{r} without knock-out of known ORFs		This study
MG1655 LHR2 (C604-10)	MG1655 transconjugant harboring LHR2 of C604-10 tagged with *tet*^{r} without knock-out of known ORFs		This study
FAM21843	Heat resistant; harbors LHR1	Swiss raw milk cheese	Peng et al., 2012, 2013a; Marti et al., 2016
FAM22873	*eaeA* (intimin) +, *stx1*+, serotype O26+	Swiss raw milk	This study
FAM22873 LHR2 (21805)	FAM22873 transconjugant harboring LHR2 plasmid of FAM21805 tagged with *tet*^{r} without knock-out of known ORFs		This study
FAM22873 ΔLHR2 (21805)	FAM22873 transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacement of LHR with *kan*^{r}		This study
FAM23288	*stx2*+	Swiss raw milk	This study
FAM23288 LHR2 (21805)	FAM23288 transconjugant harboring LHR2 plasmid of FAM21805 tagged with *tet*^{r} without knock-out of known ORFs		This study
FAM23288 ΔLHR2 (21805)	FAM23288 transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacement of LHR with *kan*^{r}		This study
55989	Enteroaggregative *E. coli* (EAEC) strain	Human clinical isolate	Bernier et al., 2002
55989 LHR2 (21805)	55989 transconjugant harboring LHR2 plasmid of FAM21805 tagged with *tet*^{r} without knock-out of known ORFs		This study
55989 ΔLHR2 (21805)	55989 transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacement of LHR with *kan*^{r}		This study
C227-11 φcu	Shiga toxin-producing EAEC strain cured of the Stx2a-bearing phage	Human clinical isolate	Zangari et al., 2013
C227-11 φcu LHR2 (21805)	C227-11 φcu transconjugant harboring LHR2 plasmid of FAM21805 tagged with *tet*^{r} without knock-out of known ORFs		This study
C227-11 φcu ΔLHR2 (21805)	C227-11 φcu transconjugant harboring LHR2 plasmid of FAM21805 tagged by replacement of LHR with *kan*^{r}		This study.

[a] *kan*^{r}, *kanamycin resistance cassette; tet*^{r}, *tetracycline resistance cassette; nal*^{r}, *nalidixic acid resistant; rif*^{r}, *rifampicin resistant.*

TABLE 2 | Primers and annealing temperatures used in this study.

Name	Sequence (5′-3′)	Target	Use	$T_{ann.}$ (°C)	References
K12-R K12IS-L	ATCCTGCGCACCAATCAACAA CGCGATGGAAGATGCTCTGTA	orf264 and the IS element inserted only in K-12 strains e.g., MG1655	Confirmation of transconjugant identity	54	Kuhnert et al., 1995
clpK1_F clpK1_R	TGCTGTTGTGCGACGACCATTACC TTGCCGACCACCTTGCTGACCTGT	*clpK1* gene specific PCR	Screening of strains for presence of *clpK1*	64	Boll et al., 2016
clpK2_F clpK2_R	ACGATCACTATCGCCAACTG AGTATTTATCCAGCTCGGGCGTG	*clpK2* gene specific PCR	Screening of strains for presence of *clpK2*	64	Boll et al., 2016
orfE_F orfE_R	CGGTCGTTCTGGCAAAGGTG CGTCCTGACGAAATCGCTCC	putative di-guanyl-cyclase (dgc) of FAM21805 LHR2	PCR specific for *orfE*, a putative dgc, part of LHR2 plasmid	64	This study
mrkD_F mrkF_R	TCGAAGGGTCGCGCTTTACG CATGGTAGCGGTAGTGCTGGTGG	*mrkD* of the *mrkABCDF* locus	Screening of strains for presence *mrk* locus encoding type 3 fimbriae	60	This study

DDBJ/ENA/GenBank under the accessions MVEA00000000 and MVIN00000000, respectively.

Plasmid Sequencing

Plasmid DNA was sequenced both on a MiSeq instrument (Illumina) and on a MinION flow cell (Oxford Nanopore Technologies). The MiSeq library was made using the Nextera XT kit (Illumina) and sequencing was performed as a paired-end 250 bp run yielding 372,720 reads with an average length of 237 bp. MinION library with native barcode (NB01 from EXP-NDB002) was prepared using the R9 Genomic Sequencing kit (SQK-NSK007) and was sequenced on a FLO-MIN105 SpotON Mk1 flow cell according to the manufacturer's instructions. Fast5 read files were subjected to base calling via a two-direction (2D) workflow using ONT's Metrichor software yielding 12,327 passed read files. Mixed assembly was performed by combining MiSeq and MinION reads using the SPAdes (v3.9.0) assembler. Finally, CLCbio Genomic Workbench (v9.5.2) was used for end trimming of the assembled plasmid and for final error correction by mapping trimmed MiSeq reads against the plasmid contig obtained after the mixed SPAdes assembly. ORFs were predicted by RAST annotation (Aziz et al., 2008) and then manually curated. The sequence has been deposited at GenBank under the accession KY416992.

Phylogenetic Analysis

For phylogenetic analysis of the LHR, genomes containing homologous sequences with >80% coverage of LHR2 of raw milk cheese *E. coli* isolate FAM21805 were retrieved from NCBI. In addition, sequences from clinical ESBL-producing *E. coli* isolates C598-10 and C604-10 (Boll et al., 2016), beef *E. coli* isolates AW1.3, AW1.7, and GM16.6-6 (Mercer et al., 2015), raw milk cheese *E. coli* isolate FAM21843 (Peng et al., 2012, 2013a) as well as five clinical isolates from Statens Serum Institut were included. Single nucleotide polymorphisms (SNPs) were identified after alignment of all sequences to the $LHR1_{FAM21805}$ reference using the NUCmer component (Kurtz et al., 2004) as implemented in the Northern Arizona SNP Pipeline (NASP) v1.0 (http://biorxiv.org/content/early/2016/01/25/037267). A total of 1,270 SNPs were identified from 45% of the 15 kb $LHR1_{FAM21805}$ excluding any repetitive regions. The relatedness of the elements was inferred using the maximum-likelihood algorithm implemented in PhyML (http://www.atgc-montpellier.fr/phyml-sms/) with Smart Model Selection using the Bayesian Information Criterion with 100 bootstrap replicates using random starting trees.

Modification of Bacterial Strains

LHR1 in FAM21805 was deleted by allelic exchange with a kanamycin resistance (*kan*^{r}) encoding cassette, flanked by regions homologous to sequences at the beginning and end of the loci, as previously described (Bojer et al., 2010). The *kan*^{r} cassette was then removed from FAM21805ΔLHR1 using pCP20. The LHR2 locus was deleted in FAM21805 and FAM21805ΔLHR1 by allelic exchange with *kan*^{r} flanked by regions homologous to sequences at the beginning and end of LHR2. Using the same technique, the *mrkABCDF* cluster and *orfE* (encoding a putative digyanylate cyclase within LHR2) in FAM21805 were deleted by allelic exchange with a tetracycline resistance (*tet*^{r})-encoding cassette. Finally, *kan*^{r} or *tet*^{r} were introduced immediately downstream of the last ORFs of LHR1 and LHR2 without disrupting the flanking mobile elements (**Table 1**).

Horizontal Gene Transfer Experiments

Horizontal gene transfer of loci of heat resistance was assessed in plate matings as previously described (Marti et al., 2016). In short, 500 μl overnight culture of donor and recipient strains were mixed and centrifuged (12,000 × g, 2 min), the supernatant completely removed and resuspended in 50 μl NA (8 g/L NaCl, 1 g/L peptone) solution and spotted onto LB Agar plates. The rifampicin (RIF) and nalidixic acid (NAL) resistant *E. coli* K-12 MG1655 rif^r nal^r (Møller et al., 2003) was used as recipient. The donors were various heat resistant strains with LHRs tagged with either *tet*^{r} or *kan*^{r} cassettes (**Table 1**). After incubation at 37°C for 24 h (if not stated otherwise), the cells were scraped off, suspended in 3 ml NA and plated onto LB plates selective for donors (LB_{TET} or LB_{KAN}), recipients ($LB_{NAL/RIF}$), and transconjugants ($LB_{NAL/RIF/TET}$ or $LB_{KAN/NAL/RIF}$). After overnight incubation

at 37°C, CFU were counted and transconjugant frequencies per recipient calculated. The antibiotics were used in the following concentrations: Kanamycin: 50 μg/ml, nalidixic acid: 30 μg/ml, tetracycline: 15 μg/ml, and rifampicin: 100 μg/ml (Sigma-Aldrich, Buchs, Switzerland). The assay was carried out in biological triplicate except where frequencies were too low for quantitative evaluation. Three presumptive K-12 MG1655 transconjugants per replicate were confirmed by screening with MG1655-specific primers and various combinations of specific PCRs for *clpK1*, *clpK2*, *orfE,* or *mrkD* (**Table 2**).

Biolog Phenotype MicroArrays

Stress responses of *E. coli* FAM21805 wild-type and its LHR mutants, as well as *E. coli* K-12 transconjugants, were assessed using the Phenotype MicroArrays (PM) 9 and 10 of the Biolog system screening for growth depending on osmolytes and pH (BIOLOG, Inc., Hayward, CA, USA). Strains were prepared and plates inoculated as follows: Strains from glycerol stocks were re-activated by two overnight incubations at 37°C on blood agar plates (Columbia agar + 5% sheep blood, bioMérieux, Geneva, Switzerland). The second plate was less than 24 h old when used for inoculation of the PM plates. Inoculating fluids (IF) were prepared by addition of 25 ml sterile water to 125 ml IF-0a and addition of 1.5 ml dye mix A and 23.5 ml water to 125 ml IF-10b. Cells were inoculated into IF-0 to a transmittance of 42 ± 2% using a sterile cotton swab and diluted (1:5) into fresh IF-0 resulting in a final transmittance of 85 ± 2%. This suspension was diluted 1:200 into IF-10b + dye mix A and used to inoculate PM plates (100 μl per well). Metabolic activity was assessed at 37°C for 72 h under standard atmosphere. PM plates, IF-0 and IF-10b were obtained from Endotell AG, Allschwil, Switzerland.

Hydrogen Peroxide Growth Challenge

In a first step, overnight cultures were diluted 1:100 into fresh tryptic soy broth (TSB, Oxoid, Pratteln, Switzerland). This was followed by a second 1:10 dilution into pre-warmed TSB containing hydrogen peroxide (H_2O_2) resulting in 1 ml aliquots with final concentrations of 0, 0.5, 1, 2, 5, 7.5, 10, 12.5, and 15 mM H_2O_2. For each concentration, a quadruplicate of 200 μl per well was added to a 96-well plate and incubated at 37°C for 24 h in a microplate reader (model: ELx808, BioTek, Luzern, Switzerland). Optical density at $\lambda = 600$ nm (OD_{600}) was measured in intervals of 30 min. The plates were only shaken for 5 s prior to each measurement on the fast setting. The experiment was carried out in biological duplicate.

Hydrogen Peroxide Inactivation Assay

Overnight cultures were diluted 1:10 into 0.9% NaCl solution pre-warmed to 37°C with a final H_2O_2 concentration of 50 mM and statically incubated at 37°C. Following 0, 15, and 30 min of incubation, 50 μl samples were taken (mixed by pipetting), and immediately diluted 1:10 into 450 μl 0.9% NaCl solution. This immediately reduced the H_2O_2 concentration and stopped further reduction of CFU. Once all samples were taken, further dilutions were spotted in triplicate on TSA (Oxoid, Pratteln, Switzerland) and incubated overnight at 37°C.

Crystal Violet (CV) Biofilm Formation Assay

Overnight cultures of strains grown in LB broth were diluted 1:100 in minimal media with 0.5% casamino acids as carbon source (ABTCAA, Reisner et al., 2006) and 150 μl were added per well (eight wells per strain and biological replicate) in 96-well plates (untreated PS surface, CytoOne, StarLab, Hamburg, Germany). Plates were wrapped in plastic bags and partially closed to reduce evaporation of media and incubated at 12, 28, and 37°C for 48 h. After incubation, the plates were emptied by throwing out and removing residual liquid by touching the inverted plate on paper tissue. Plates were then washed three times with 200 μl NA per well and the biofilms subsequently stained with 200 μl 0.1% crystal violet (CV) solution (Sigma-Aldrich, Buchs, Switzerland) per well for 20 min. Staining was followed by three washes with ddH_2O and biofilms were dissolved in 200 μl 96% ethanol per well. Biofilm formation was assessed by measurement of OD_{600} values, which are reported as average and standard deviation of three biological replicates of OD_{600} of strains minus OD_{600} of the media control.

Plasmid Profiling

For plasmid size determination, plasmid preparation was carried out using a modified version of the protocol by Kado and Liu (1981) as previously described (Schjørring et al., 2008). As a plasmid marker, *E. coli* strain 39R861 was used, containing four plasmids of 147, 63, 36, and 7 kb (Threlfall et al., 1986).

Statistical Analysis

Statistical analysis of data (*t*-tests, Mann-Whitney rank sum tests and 1-way ANOVA) was performed using SigmaPlot 13.0 (Systat Software, San Jose, CA) as indicated in the main text and legends ($\alpha = 0.05$).

RESULTS

Two Distinct Heat Resistance Clusters Are Present in *E. coli* Raw Milk Cheese Isolates

We have recently described the presence of two loci of heat resistance (LHR1 and LHR2) in a clinical ESBL-producing *E. coli* isolate (C604-10), both of which contributed to a highly heat resistant phenotype (Boll et al., 2016). In a separate study, we reported a remarkably high frequency of *clpK*-positive *E. coli* raw milk or raw milk cheese isolates (93 out of 256) (Marti et al., 2016). Here, we examined a subset of these isolates (both *clpK*-positive and—negative) for the potential presence of both LHRs with PCRs specific for *clpK1* and *clpK2*, the marker genes of LHR1 and LHR2, respectively. Of the 90 tested isolates, 23 contained both, 26 were positive for *clpK1* only and one only for *clpK2*. Next, we correlated PCR results and phenotypical heat resistance to determine the predictive value of the PCRs. All of the 49 *clpK1* positive strains were heat resistant and only one out of 50 heat resistant strains was *clpK1* negative. Thus, there was a strong correlation of heat resistance with *clpK1*. Every strain positive for *clpK2* (Stahlhut et al., 2013) tested phenotypically heat resistant, but only 24 of 50 heat resistant strains were *clpK2* positive. It is important to note that the *clpK1* negative, yet heat resistant strain was *clpK2* positive. Thus, the combination of these two PCRs resulted in perfect prediction of phenotypic heat

resistance in this set of strains (double PCR negative strains all tested heat sensitive).

Strains testing positive for both *clpK1* and *clpK2* by PCR showed significantly increased survival in our phenotypic heat resistance screening assay compared to the *clpK1* single positive strains, which in turn were significantly more resistant than double negative strains. The average relative survival after 30 min at 55°C was $4.64 \pm 6.61 \times 10^{-3}$ for double negatives ($n = 40$), $3.27 \pm 1.75 \times 10^{-1}$ for *clpK1* single positives ($n = 26$), and $5.39 \pm 1.96 \times 10^{-1}$ for *clpK1* and *2* double positive strains ($n = 23$). The differences between groups are statistically significant (p-values < 0.001 for all pairwise comparisons, Mann-Whitney Rank Sum Test). For further characterization of the two different LHR clusters in our collection of raw milk and raw milk cheese isolates, we focused our attention on isolate FAM21805, which harbors both *clpK* gene variants. This strain has previously been shown to exhibit an increased degree of heat resistance compared to strains harboring a single LHR in milk at sub-pasteurization temperatures (Peng et al., 2013a).

Characterization of a Plasmid-Encoded LHR in Raw Milk Cheese Isolate FAM21805

Whole genome sequencing (Illumina) analysis revealed that raw milk cheese isolate FAM21805 harbored an LHR (here designated LHR1) ~15 kb in size with a G+C content of 62% and highly similar (98–99%) to the one previously described in four heat resistant *E. coli* isolated from beef (Mercer et al., 2015). It contained fourteen putative ORFs and was flanked by mobile elements (**Figure 1**). We moreover detected additional homologs to several of these ORFs, strongly implying the presence of a second LHR in this isolate. However, *de novo* assembly failed to demonstrate the location of these homologs within a single genetic locus.

Since in *K. pneumoniae*, the LHR is thought to predominantly be located on plasmids, we sought to determine whether this was also the case for this putative additional LHR in *E. coli* isolate FAM21805. Gel electrophoresis of purified plasmids revealed that FAM21805 harbored two large plasmids of slightly different sizes around 110–120 kb (Supplementary Figure 1). We therefore introduced a kanamycin resistance encoding gene (*kan*^{r}) within the *clpK2* gene, purified the plasmids from the resulting FAM21805 Δ*clpK2* strain and transformed the plasmids into laboratory *E. coli* strain NEB-10β. Plating on kanamycin-containing plates yielded several colonies, and plasmid profiling from one of these demonstrated the presence of a single plasmid in NEB-10β corresponding in size to the lower-size plasmid in isolate FAM21805 (Supplementary Figure 1), strongly suggesting that the *kan*^{r}–disrupted *clpK2* gene was in fact located on that plasmid.

Using MinIon Nanopore R9 technology, we sequenced the *kan*^{r}-tagged plasmid from the transformed NEB-10β strain. We then manually replaced the *kan*^{r}–disrupted *clpK2* gene with the intact *clpK2* from Illumina sequencing within the complete closed sequence, thus reconstructing the original plasmid sequence. The resulting plasmid, titled pFAM21805, is 114,916 bp in length and has an average G+C content of 53.6% (**Figure 2**). RAST annotation predicted a total of 136 ORFs, 99 of which were functionally assigned. A single RepII replicon was identified with the F plasmid type FAB formula F96:A-:B-.

The plasmid sequence confirmed the presence of a single LHR locus ($LHR2_{FAM21805}$) ~19.0 kb in length and containing 15 putative functional ORFs (**Figure 1** and Supplementary Table 1). It had a G+C content similar to LHR1 (61%) and was flanked by various mobile elements. Homologs to key elements of LHR1 were also present in $LHR2_{FAM21805}$, including: (1) the Clp ATPase ClpK (*orf3*); (2) the two small heat shock proteins, sHsp20c (*orf2*) and sHsp20 (*orf7*); (3) a putative sodium/hydrogen exchanger with a Kef-type membrane component (*orf13*); (4) a putative zinc-dependent protease (*orf15*); and (5) a putative 2-alkenal reductase with a trypsin-like protease domain (*orf16*). Overall, 75% of $LHR2_{FAM21805}$ was present in $LHR1_{FAM21805}$ with a corresponding average identity of 88% (**Figure 1**).

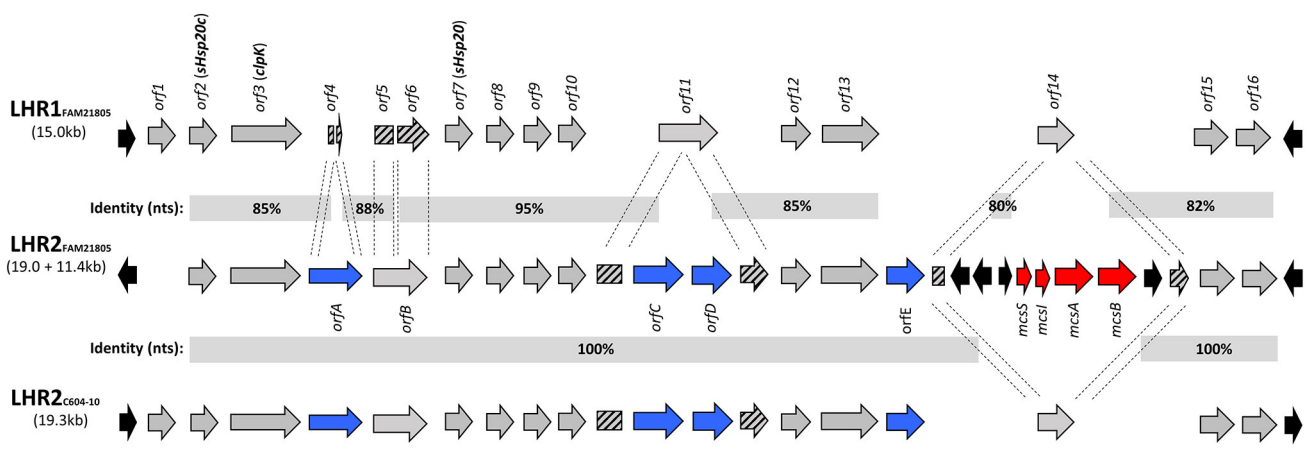


FIGURE 1 | Comparison of loci of heat resistance in FAM21805 (LHR1 and -2) and C604-10 (LHR2). ORFs present in all three LHRs are marked in gray (except for *orf1* which is absent in $LHR2_{FAM21805}$), ORFs unique to LHR2 are marked in blue, partially disrupted ORFs are marked with stripes, and mobile elements are marked in black. Regional nucleotide identities (%) are depicted. A 11.4 kb region containing the *mcsSIAB* cluster (Microcin S system, marked in red) has been inserted into $LHR2_{FAM21805}$. For additional information on the ORFs, see Supplementary Table 1.

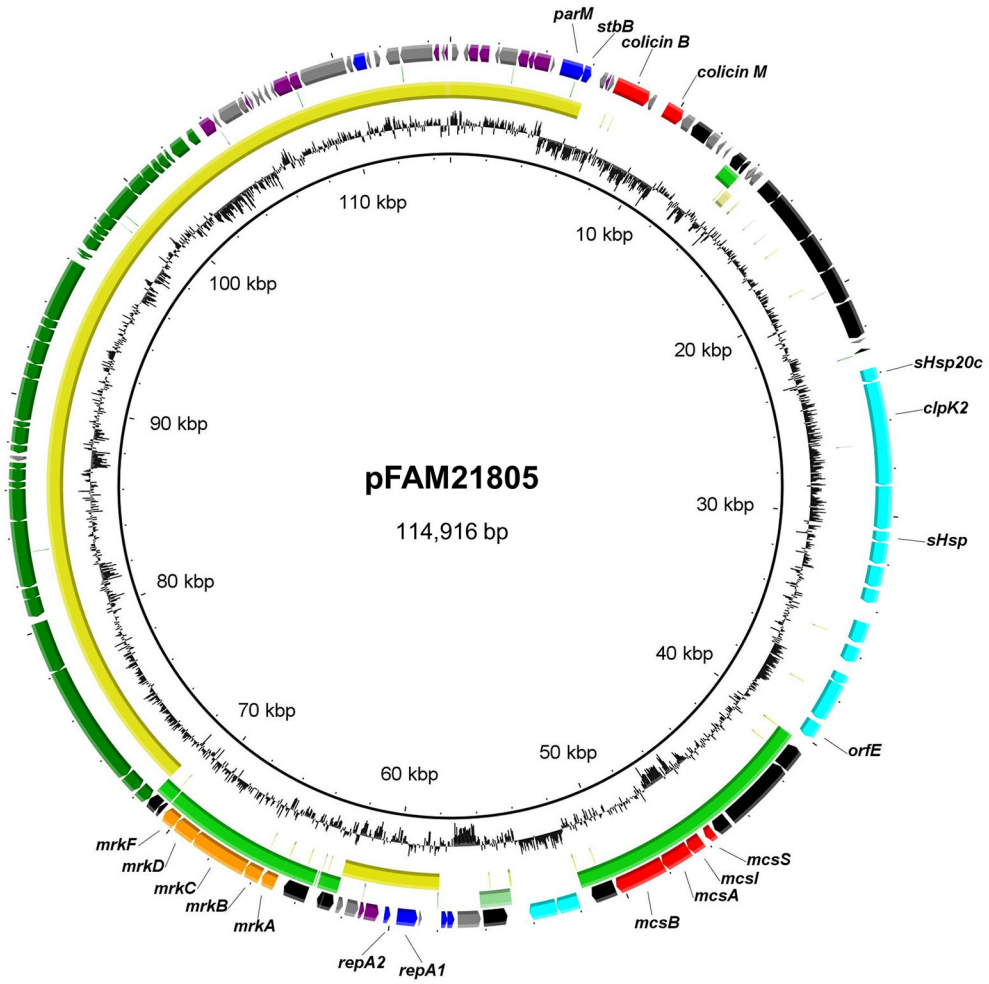


FIGURE 2 | Circular representation of pFAM21805 and BLASTn comparison of plasmids with shared regions. The outer ring shows predicted ORFs. Colors represent different putative functions: Gray, hypothetical proteins; blue, plasmid replication and maintenance; red, bacteriocins and accessory genes; light blue, LHR2 genes; orange, adhesins; dark blue, regulatory genes; purple, miscellaneous; and black, mobile elements. Within circles two (pCPO13026, yellow) and three (pSYM1, green), the darkest color indicates nucleotide identity exceeding 90%, whereas the lightest color represents identity exceeding 80%. Innermost circle, G+C content. The circular map was generated using BRIG.

The previously found LHR2 in ESBL-producing *E. coli* isolate C604-10 was highly identical to $LHR2_{FAM21805}$ (Boll et al., 2016). However, whereas $LHR2_{C604-10}$ (and $LHR1_{FAM21805}$) was initiated by an ORF encoding a protein with a putative helix-turn-helix (HTH) motif capable of binding DNA (*orf1*), this ORF was absent in $LHR2_{FAM21805}$. Moreover, a region spanning 11.4 kb with a G+C content of 50% had been inserted into *orf14* of $LHR2_{FAM21805}$ (**Figures 1**, **2**). This region comprised the *mcsSIAB* gene cluster encoding an antibacterial peptide Microcin S (MccS) along with a self-immunity protein and transport apparatus recently identified on plasmid pSYM1 in probiotic *E. coli* strain G3/10 (Zschüttig et al., 2012).

LHR2 of FAM21805 and C604-10 both contained four ORFs not found in LHR1: (1) a putative cardiolipin synthase (*orfA*); (2) a putative mechanosensitive ion channel (*orfC*) followed by a hypothetical protein of unknown function (*orfD*); and (3) an ORF containing a GGDEF domain characteristic of a putative di-guanyl cyclase (*orfE*) (**Figure 1** and Supplementary Table 1). Interestingly, remnants of the N- and C-terminal parts of *orfA* were present in LHR1, indicating evolutionary partial loss of this ORF in LHR1. In addition, *orfC* and *orfD* were inserted in the middle of *orf11* encoding a hypothetical protein in LHR1, thereby disrupting this ORF. Finally, *orfE* appeared to have been inserted between *orf13* and *orf14* present in both LHRs (**Figure 1**).

In addition to LHR2 itself, pFAM21805 also contained the *mrkABCDF* cluster, encoding type 3 fimbriae, which mediate bacterial biofilm formation on abiotic surfaces such as plastic, and on biotic surfaces such as human epithelial cells. The *mrk* operon is present in nearly all *K. pneumoniae* isolates but is only rarely found in *E. coli* (Stahlhut et al., 2013). Notably, plasmid co-localization of the *mrk* operon and the *mcs* gene cluster was also observed in the probiotic G3/10 strain (Zschüttig et al., 2012). pFAM21805 also harbored a 32 kb *tra* region (24 *tra* genes, 8 *trb* genes, and *finO*), implying that the plasmid

may be transferable by conjugation. Also present on the plasmid were genes associated with plasmid stability including *parM* and *stbB* as well as the genes encoding the antimicrobial compounds Colicin B and Colicin M (**Figure 2**). Thus, the plasmid likely confers both enhanced heat resistance, the ability to kill other *E. coli* and enhanced adhesive properties to FAM21805.

A large portion of pFAM21805, including the *tra* region, *repA* replicon and plasmid maintenance genes also comprised the backbone of plasmid pCPO13026 of Shiga Toxin-producing *E. coli* (STEC) strain 2009C-3133 isolated from a patient in New York in 2009 (Lindsey et al., 2015) (**Figure 2**).

Presence of Loci of Heat Resistance in Other Pathogenic Species

Previous studies have described the presence of LHR in several distinct pathogenic species, including *E. coli*, *K. pneumoniae*, *Enterobacter* species, and *Pseudomonas aeruginosa* (Bojer et al., 2010; Gajdosova et al., 2011; Lee et al., 2015; Mercer et al., 2015). Based on a BLAST search using the entire LHR, Mercer et al. recently observed two distinct phylogenetic groups harboring LHRs—one predominantly comprising *Enterobacteriaceae* and one primarily comprising *P. aeruginosa* (Mercer et al., 2015). We performed the same analysis using LHR2 from FAM21805 as input, which retrieved 27 sequences with more than 80% coverage. SNPs within the LHRs were identified and used to calculate a maximum-likelihood phylogenetic tree. As expected, $LHR1_{FAM21805}$ and $LHR1_{C604-10}$ both clustered tightly with the other *E. coli* LHR1s within the *Enterobacteriaceae* group (**Figure 3**). Not surprisingly, $LHR2_{FAM21805}$ and $LHR2_{C604-10}$ both clustered tightly together. Remarkably, however, they were located within the *Pseudomonas* group of the phylogenetic tree. These findings demonstrate the ability of *E. coli* to acquire LHRs from both phylogenetic groups.

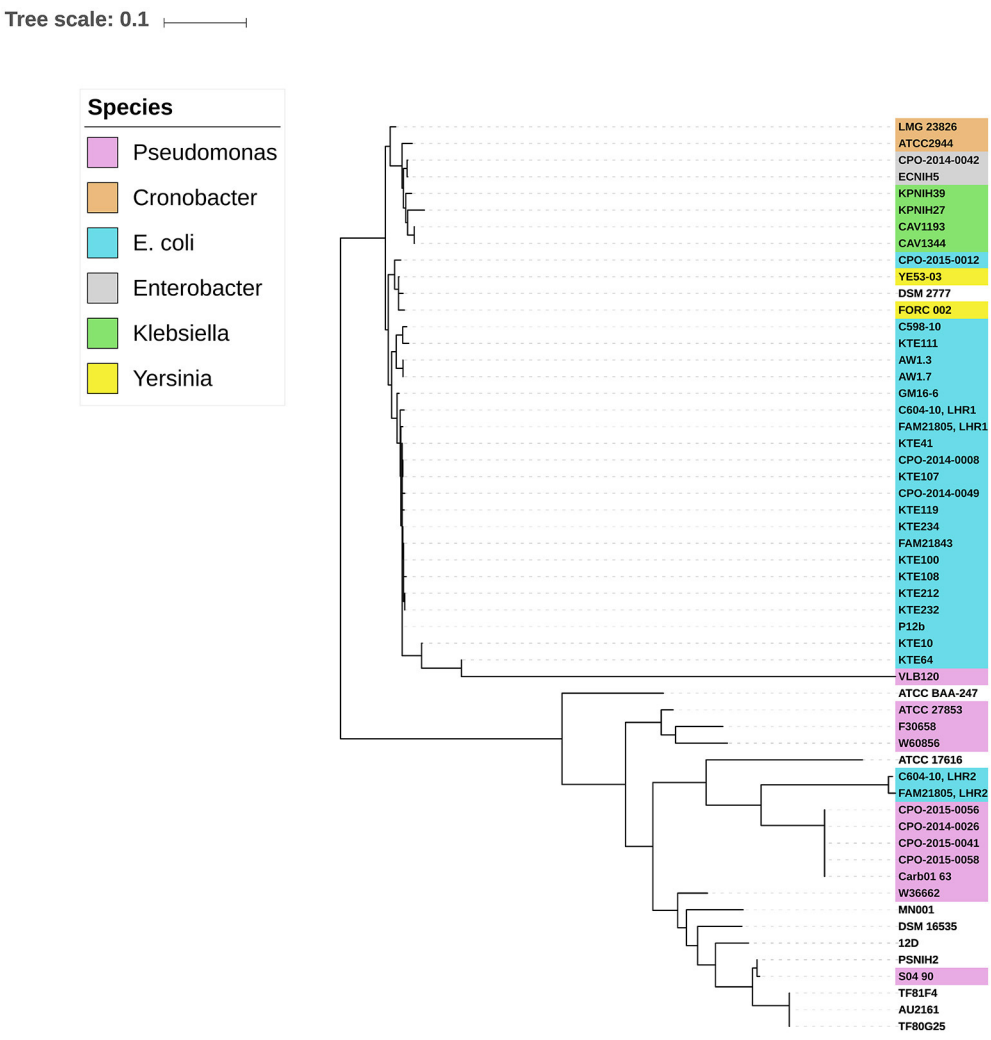


FIGURE 3 | Midpoint-rooted maximum-likelihood phylogenetic tree of aligned LHR sequences (>80% coverage of $LHR1_{FAM21805}$) from different bacterial species. Two distinct clusters representing predominantly *Enterobacteriaceae* and strains of *Pseudomonas*, respectively, are observed. LHR2 of FAM21805 and C604-10 cluster within the *Pseudomonas* group.

Both the LHR1 and LHR2 Confer Heat Resistance and Are Transferable by Conjugation

We have previously demonstrated that both LHRs in ESBL-producing *E. coli* isolate C604-10 confer heat resistance to this strain (Boll et al., 2016). To determine whether this was also the case with raw milk cheese *E. coli* isolate FAM21805, we generated a panel of LHR mutant strains with allelic replacements of designated regions with the *kan*^{r} gene (**Table 1**). For FAM21805 ΔLHR1, we deleted the region spanning from *orf2* (*sHsp20c)* to *orf16*. With regard to $LHR2_{FAM21805}$, we deleted the region spanning from orf2 to the disrupted *orf14*, thereby excluding potential influence of removal of the inserted Microcin S system. As shown in **Figure 4A**, removal of either one of the LHRs did not affect survival of FAM21805 upon heat exposure. In contrast, survival of the FAM21805 double LHR mutant was severely reduced, thereby demonstrating that both LHRs of FAM21805 are functionally active and confer heat resistance.

We next sought to determine whether the LHRs of FAM21805 and C604-10 were amenable to horizontal gene transfer. We tagged LHR1 in both FAM21805 and C604-10 as well as $LHR2_{C604-10}$ (all presumably chromosomally located) with genes encoding *kan*^{r} or *tet*^{r} by introducing them in the region immediately downstream of *orf16* (**Table 1**). Likewise, we inserted *kan*^{r} in a non-coding region on pFAM21805. We then carried out conjugative transfer assays using either one of the four LHR-tagged strains as donors and commensal *E. coli* strain K-12 MG1655 as recipient. Plate mating assays resulted in very high transfer frequencies for the tagged donor $LHR2_{FAM21805}$ ($7.56 \pm 2.00 \times 10^{-1}$ transconjugants per recipient). The donor with a tagged $LHR2_{C604-10}$ produced transconjugants at very low levels. Standard plate matings with tagged LHR1 of both FAM21805 and C604-10 yielded no transconjugants at all. We proceeded to increase incubation time, spotting separate plate matings for each time point and incubating up to 12 days. This way, LHR1 transconjugants of both strains were generated and confirmed by PCR, albeit once again at very low levels. Notably, all of the $LHR1_{FAM21805}$ transconjugants also appeared to have acquired pFAM21805. In contrast, we did not observe co-transfer of LHRs not selected for in any other case.

Confirming retained post-transfer functionality of the LHRs, all four types of K-12 MG1655 transconjugants exhibited significantly elevated survival upon heat exposure compared to the native strain (**Figures 4B,C**). The highest level of survival was observed in MG1655 carrying both $LHR1_{FAM21805}$ and $LHR2_{FAM21805}$ (**Figure 4B**), illustrating that both loci contribute to heat resistance in this bacterial background. In contrast, $LHR2_{C604-10}$ appeared to more modestly confer heat protection. Finally, given the relative ease by which pFAM21805 was transferable to MG1655, we examined whether the plasmid could also be transferred to isolates belonging to diarrheagenic *E. coli* (DEC) and Shiga-toxin (Stx)-encoding *E. coli* (STEC) pathotypes. Through plate mating assays, we successfully transferred pFAM21805 tagged with antibiotic resistance cassettes to two STEC strains, FAM22873, and FAM23288, as well as two enteroaggregative *E. coli* (EAEC) strains, 55989 and the Stx-phage-cured German outbreak strain C227-11 φcu. As shown in **Table 3**, pFAM21805 conferred significantly elevated heat resistance to all four pathogenic *E. coli* strains. Strains conjugated with pFAM21805 ΔLHR2 exhibited the same levels of heat killing as did the non-conjugated strains, confirming that this effect was attributable to LHR2 (**Table 3**).

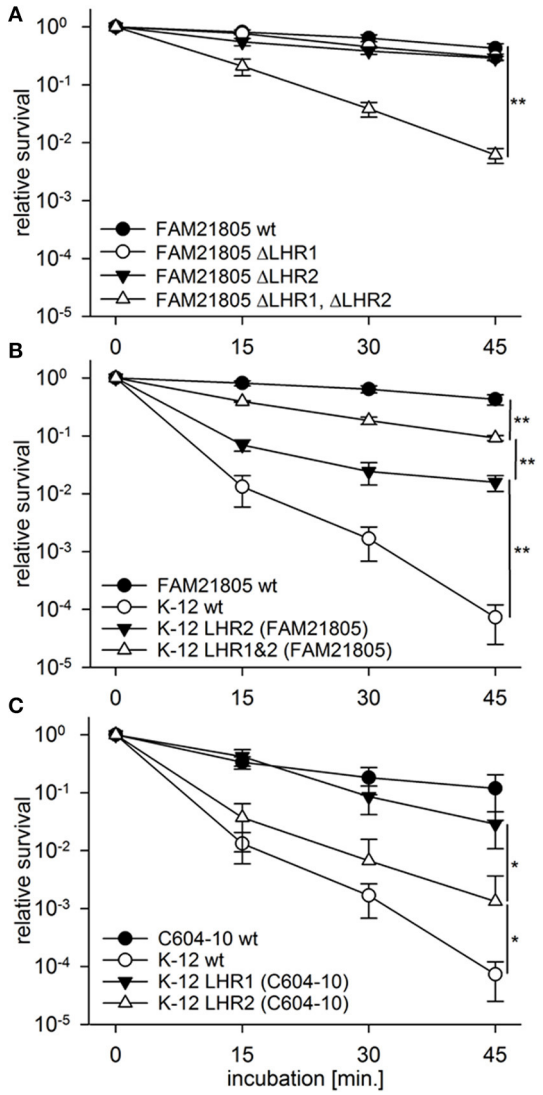


FIGURE 4 | Heat resistance assays of *E. coli* wild-type strains, FAM21805 mutants, and K-12 MG1655 transconjugants. Relative survival compared to time point 0 of strains incubated at 55°C for 15, 30, and 45 min. **(A)** FAM21805 wild-type, -ΔLHR1, -ΔLHR2, and ΔLHR1ΔLHR2. **(B)** FAM21805 wild-type, K-12 MG1655 wild-type, and K-12 MG1655 transconjugants harboring LHR2 or both LHRs of FAM21805. **(C)** C604-10, K-12 MG1655 wild-type, and K-12 MG1655 transconjugants featuring LHR1 or LHR2 of C604-10. Error bars indicate standard deviations. Significances are indicated for the 45 min. time points based on one-tailed *t*-tests in **(A,B)**, and Mann-Whitney rank sum tests in **(C)**; $^*p < 0.05$, $^{**}p < 0.01$.

TABLE 3 | Heat resistance assays with pathogenic *E. coli* (EAEC and STEC) wild-type strains and the strains conjugated with pFAM21805 or with pFAM21805 ΔLHR2.

Strain	Relative survival[a]		*p*-value[c]
	Average	*SD*[b]	
FAM22873 wild-type	5.64E-04	1.64E-04	–
FAM22873 ΔLHR2 (21805)	9.49E-04	1.08E-03	0.322
FAM22873 LHR2 (21805)	4.28E-02	2.24E-02	0.028
FAM23288 wild-type	9.03E-04	2.04E-04	–
FAM23288 ΔLHR2 (21805)	4.79E-04	5.38E-04	0.124
FAM23288 LHR2 (21805)	1.02E-02	8.12E-03	0.029
55989 wild-type	3.60E-04	1.54E-04	–
55989 ΔLHR2 (21805)	5.05E-04	1.98E-04	–
55989 LHR2 (21805)	3.91E-01	1.23E-01	0.007
C227-11 φcu	3.23E-04	4.89E-04	–
C227-11 φcuΔ LHR2 (21805)	1.20E-04	1.08E-04	–
C227-11 φcu LHR2 (21805)	4.27E-01	1.39E-01	0.001

[a] *Relative survival at time point 45 min. compared to 0 min. incubation at 55°C.*
[b] *SD, standard deviation.*
[c] *p-value of direct comparison (one-tailed t-test or Mann-Whitney rank sum) against corresponding wild-type strain.*

Screening for Other Locus of Heat Resistance-Related Stress Response Phenotypes

We next sought to determine whether the LHRs confer other stress-related advantages to their bacterial host in addition to enhanced thermotolerance. Since homologs of most of the ORFs are present in both of the LHR, we focused on the combined effect of removing both loci in FAM21805.

We first looked at the ability of FAM21805 wild-type and the ΔLHR1ΔLHR2 mutant to survive oxidative stress. However, neither killing nor growth challenge assays with hydrogen peroxide revealed any significant differences between the wild-type and mutant strain (data not shown). Thus, there seems to be no clear protective effect of either LHR against the oxidative action of H_2O_2.

To examine the potential effect of osmolarity and pH, we next screened the wild-type and ΔLHR1ΔLHR2 mutant strain using phenotypic microarrays (PM) 9 and 10 from the Biolog system, which measures activity of the bacterial metabolism via respiratory action (reduction of a redox dye). A biological triplicate with both PM 9 and 10 revealed only one consistent phenotypical difference between the two strains. PM9 contains four wells challenging bacteria with sodium benzoate at concentrations of 20, 50, 100, and 200 mM (each at pH 5.2). At a concentration of 100 mM, the wild-type strain consistently started respiring after ∼36 h of incubation, while the LHR double mutant was unable to do so. Both strains were able to respire at 20 and 50 mM sodium benzoate while both were unable to do so at 200 mM over the entire 72 h incubation. FAM21805 ΔLHR1&2 complemented with either LHR2 or LHR1&2 were able to respire at 100 mM sodium benzoate (pH 5.2) after ∼36 h, like the wild-type (single replicate). Notably, K-12 MG1655 transconjugants with LHR2 or LHR1&2 of FAM21805 did not show this sodium benzoate related phenotype (biological duplicate).

The pFAM21805 Plasmid Increases Biofilm Formation of *E. coli* MG1655

Biofilm formation is an important contributor to persistence of bacteria in both food processing and clinical settings (Abdallah et al., 2014). A combination of biofilm formation with heat resistance would increase a strain's persistence even further (Bojer et al., 2011). As described above, pFAM21805 contains the *mrk* gene cluster encoding type 3 fimbriae, which are strongly associated with bacterial biofilm formation (Burmølle et al., 2008; Hufnagel et al., 2015). Moreover, *orfE* of $LHR2_{FAM21805}$ encodes a putative di-guanyl cyclase, and c-di-GMP signaling has been shown to affect biofilm formation (Burmølle et al., 2008; Schroll et al., 2010; Hufnagel et al., 2015). Thus, both factors could potentially contribute to increased biofilm formation in *E. coli*. To investigate if this was the case, we replaced either *mrkABCDF* or *orfE* in FAM21805 with tet^r (**Table 1**), and transferred pFAM21805 from the two corresponding mutant strains to K-12 MG1655. We then performed crystal violet (CV) assays with K-12 MG1655 nal^r rif^r and its transconjugants in ABTCAA for 48 h at 12, 28, and 37°C. As shown in **Figure 5**, the presence of intact pFAM21805 significantly increased biofilm production of MG1655. Moreover, our results show that the *mrk* locus—but not the putative LHR2-encoded di-guanyl cyclase—was required to increase biofilm formation in K-12 MG1655 transconjugants. Thus, pFAM21805 has the potential to confer both enhanced survival during heat stress and adhesive properties at a wide range of temperatures to *E. coli* recipient strains.

DISCUSSION

Heat treatment is a commonly used process in the food industry and the main technology to reduce bacterial load. It is therefore crucially important to understand the mechanisms mediating increased heat resistance in potentially pathogenic bacteria as well as its origin, potential for distribution, and possible cross-protective effects associated with this phenotype.

We and others have recently described the LHR, which is present in and confers heat resistance to a variety of *Enterobacteriaceae* as well as *Pseudomonas* spp. (Bojer et al., 2010; Lee et al., 2015; Mercer et al., 2015; Boll et al., 2016). In contrast to *K. pneumoniae*, where the LHR appears to predominantly be located on plasmids, thus far, only chromosomally located LHRs have been reported in *E. coli*, including the fully sequenced strain P12b (Bojer et al., 2010; Liu et al., 2012; Mercer et al., 2015). This likely explains the much lower overall predicted prevalence of LHR in *E. coli* (∼2%) compared to *K. pneumoniae* (1/3 of clinical isolates) (Bojer et al., 2010; Mercer et al., 2015). However, when screening for LHR marker genes in a collection of *E. coli* isolates from dairy production, we observed a much higher fraction of LHR-positive isolates (∼36%). This most likely reflects a selection process in which LHR-positive isolates survive the thermal processing steps to a much greater extent than LHR-negative isolates (Marti et al., 2016). Further studies are

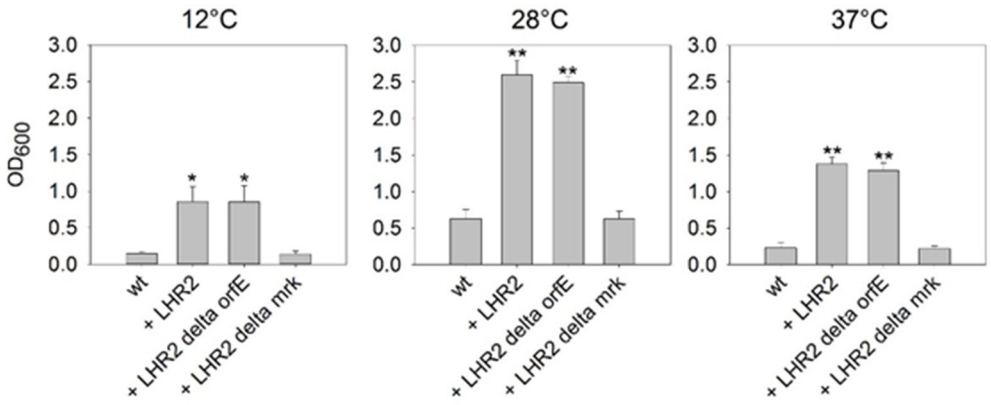


FIGURE 5 | Impact of pFAM21805 on biofilm formation of K-12 MG1655. Biofilm formation of *E. coli* K-12 MG1655 wild-type and K-12 MG1655 transconjugants harboring pFAM21805, pFAM21805 Δ*orfE* (deletion of LHR2-encoded putative di-guanyl cyclase), or pFAM21805 Δ*mrk* (deletion of type 3 fimbriae genes). Bacteria were grown in ABTCAA media for 48 h at 12, 28, and 37°C, after which biofilm formation was quantified by crystal violet (CV) staining. Bars represent averages and standard deviations (*$p < 0.05$, **$p < 0.001$, tested by 1-way ANOVA vs. K-12 wt control, $\alpha = 0.05$, Holm-Sidak).

needed to evaluate the occurrence of similar selection processes under sub-pasteurization heat treatments in clinical usage and food production.

In this study, we were able to more closely characterize a subset (90 isolates) of the collection of *E. coli* dairy isolates with regard to the presence of LHR. While distinct variants of LHRs exist in different species, the *clpK* gene (as well as the upstream located *sHsp20c*) are always present. We therefore used PCRs specifically targeting *clpK1* and *clpK2* as markers for *E. coli* LHR1 (Mercer et al., 2015) and the newly discovered variant LHR2, found in the ESBL-encoding *E. coli* isolate C604-10 (Boll et al., 2016), respectively. We observed a strong correlation between the presence of LHR and a heat resistant phenotype, with all of the heat resistant isolates harboring LHR1, with the exception of FAM22891, which tested *clpK2* single positive. Nearly half of the LHR1-positive isolates additionally also harbored LHR2, and these isolates exhibited the highest levels of heat resistance overall. These findings stress the need for screening for both LHR1 and LHR2 to detect highly heat resistant isolates and validate the use of primers targeting the *clpK* genes as markers for the LHRs.

We also examined whether the LHRs would be amenable to horizontal gene transfer. Focusing on C604-10 and *E. coli* raw milk cheese isolate FAM21805, both of which harbor both LHR1 and LHR2, we indeed found that all four LHRs were transferrable to *E. coli* K-12; however, with very different rates of transfer. Interestingly, compared to the other three LHRs, $LHR2_{FAM21805}$ was transferred at a much higher rate. MinIon-based sequencing revealed that, unlike the other LHRs, $LHR2_{FAM21805}$ was encoded on a large IncFII-type plasmid, pFAM21805. This plasmid also contained a *tra* operon (Lawley et al., 2003), thus explaining why this plasmid was so readily transferred by conjugation. Importantly, pFAM21805 was also readily conjugated into STEC and EAEC, both of which are pathotypes associated with foodborne outbreaks (Rasko et al., 2011; Farrokh et al., 2013; Robertson et al., 2016). While thus far, no studies have described the natural presence of LHR in these diarrheal pathotypes, this finding highlights the potential for such cases to occur.

The fact that LHR1 was transferred at a much lower rate than the plasmid-encoded LHR2 from FAM21805 suggests that LHR1 is located either on the chromosome or on a non-conjugative plasmid in this strain. Gel electrophoresis of the LHR1 K-12 MG1655 transconjugant (also harboring pFAM21805) demonstrated the presence of a single plasmid identical in size to that of the pFAM21805 K-12 MG1655 transconjugant (data not shown). This rules out the location of LHR1 on the second large plasmid present in FAM21805. We instead aligned Illumina sequences from the LHR1 K-12 MG1655 transconjugant with the genome sequence of MG1655. Based on this, we identified 1,708 SNPs within a region spanning ∼700 kb of the MG1655 genome. The same high SNP frequency occurred across the entire MG1655 genome following alignment with Illumina sequences from FAM21805. Taking together, these findings suggest that $LHR1_{FAM21805}$ was transferred horizontally to MG1655 as part of a 700 kb region of FAM21805. While the underlying mechanism for this phenomenon remains to be determined, the LHR-containing DNA region could have been transferred to FAM21805 by conjugal mating with a high frequency recombinant (Hfr) donor strain with a conjugative plasmid integrated into its genome (O'Gorman et al., 1996). A similar scenario describing a chimeric *K. pneumoniae* strain having taken up a large portion of genomic DNA from another strain has previously been described (Struve et al., 2015). In any case, such a transfer mechanism would expectedly occur at a very low frequency, co-inciding with very low transfer frequency of $LHR1_{FAM21805}$ in our study.

In addition to LHR2, pFAM21805 also contained the *mrkABCDF* locus encoding type 3 fimbriae, which are considered a major virulence factor of *K. pneumoniae* allowing the organism to produce extensive biofilm (Schroll et al., 2010; Andrade et al., 2014). In K-12 MG1655, conjugation with pFAM21805 lead to significantly increased biofilm production at 12, 28, and 37°C,

which may in turn increase frequency of horizontal gene transfer (Burmølle et al., 2014; Rossi et al., 2014). The fact that the entire range of temperatures tested saw an increase in biofilm formation suggests potential beneficial effects for a host of this plasmid both in the environment and *in vivo*.

Moreover, the plasmid also contained three bacteriocins (along with their respective self-immunity genes): Colicin B, Colicin M, and Microcin S. Although the functionality of the bacteriocins remains to be verified, having the ability to produce multiple bacteriocins most likely provides the bacterial host of the plasmid with an expanded killing range, and thus a competitive advantage in multispecies communities (Gordon and O'Brien, 2006).

The pFAM21805 plasmid did not encode any antimicrobial resistance genes. This is in contrast to *K. pneumoniae*, where the LHR is often present on plasmids harboring resistances to tetracycline, neomycin, trimethoprim, sulfamethoxazole as well as encoding ESBL genes such as CTX-M-15, which confers resistance to third-generation cephalosporins (Bojer et al., 2012). Importantly, FAM21805 has been shown to be able to harbor ESBL-encoding conjugative plasmids and is able to act as donor of these (Marti et al., 2016), and the *tra* operon on pFAM21805 could likely enable conjugation of mobilizable (resistance) plasmids in other hosts of this LHR2 plasmid.

Interestingly, we observed a strong correlation between the presence of the *mrk* gene cluster and LHR in our collection of dairy *E. coli* isolates (all 13 *mrk* positive isolates also harbored *clpK1* and some also *clpK2*). In light of this, we performed whole-genome sequencing on two *mrk*- and *clpK2*-positive isolates but found that neither isolate harbored pFAM21805 or a highly similar plasmid. Thus, in spite of the fact that the plasmid was readily transferred and provided clear benefits to its host, it did not appear to be widespread among these isolates. However, this does not rule out the possibility that other LHR-encoding plasmids exist, some of which may potentially harbor LHR1-like heat resistance clusters and *mrk*, similar to pFAM21805. Notably, thus far in *E. coli*, the *mrk* gene cluster has only been reported as located on conjugative plasmids (Burmølle et al., 2008), which is in agreement with our finding of *mrk* on pFAM21805. Further studies are needed to clarify the role of the *mrk* gene cluster and biofilm production capacity in *E. coli* isolates being persistent in hospitals or food-production.

The exact function of many of the ORFs of the LHRs remains to be unraveled. However, the fact that the majority of them are highly conserved strongly suggests that they all play a beneficial role for their host. Notably, some of them are predicted to act as ion-exchangers or proteases/peptidases suggesting that they may be involved in handling osmotic- or heat stress (Mercer et al., 2015). The ClpK chaperone itself shares many structural properties with ClpB, which is known to play a critical role in survival following various types of stress (Squires et al., 1991; Ekaza et al., 2001; Lourdault et al., 2011). We therefore endeavored to identify other stress response phenotypes associated with LHRs in FAM21805. However, both H_2O_2 growth challenge and killing assays revealed no significant differences between the FAM21805 wildtype and LHR double mutant strain in response to this stressor. Assays with the phenotypic microarrays 9 and 10 of the Biolog system did reveal one phenotype: The FAM21805 wildtype was able to respire in 100 mM sodium benzoate (pH 5.2) after 36 h, while its LHR1&2 double mutant could not. This phenotype could be complemented in FAM21805 ΔLHR1&2 with either LHR2 or LHR1&2, but not transferred to K-12 MG1655 by conjugation. The phenotype appears to be dependent on the genomic background around LHR2. Benzoate is being used as a preservative in a range of foods. Although a difference was only observed in one scenario here, it indicates that there could be non-thermal stresses where LHR would confer an advantage to the host isolate.

In conclusion, we have characterized in detail the presence of a recently discovered variant of LHR (LHR2) and demonstrated its presence on a plasmid in the highly heat resistant dairy *E. coli* isolate FAM21805. This plasmid was transferable at much higher rates than the presumably chromosomal LHRs tested, and conferred LHR2-dependent heat resistance as well as *mrk*-dependent biofilm formation capabilities to recipient *E. coli*, including pathogenic strains. In addition, the plasmid also harbored three bacteriocins and corresponding self-immunity proteins. Selection for and acquisition of this "survival" plasmid by pathogenic organisms, e.g., in food production environments, may pose great concern and emphasizes the need to screen for the presence of LHR genes in isolates.

AUTHOR CONTRIBUTIONS

EB and RM designed the work, collected, analyzed and interpreted the data and drafted the article. HH, SO, and MS analyzed and interpreted the data and critically revised the article. KN analyzed and interpreted the data. SK critically revised the article. KK and JH interpreted the data and critically revised the article. CS designed the work, interpreted the data and critically revised the article.

ACKNOWLEDGMENTS

We thank Michala T. Sørensen (Statens Serum Institut) for carrying out plasmid profiling, Javorka Naskova (Agroscope) for technical assistance, and Daniel Wüthrich (University of Bern) for performing some of the Illumina sequencing. Ulrich Zürcher is acknowledged for the management of the Agroscope research program REDYMO. This work was financially supported by the Danish Council for Research grant DFF-1331-00161 to CS, BacFoodNet, the Agroscope research program REDYMO and in part by COST action FA1202.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00579/full#supplementary-material

REFERENCES

Abdallah, M., Benoliel, C., Drider, D., Dhulster, P., and Chihib, N. E. (2014). Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. *Arch. Microbiol.* 196, 453–472. doi: 10.1007/s00203-014-0983-1

Andrade, L. N., Vitali, L., Gaspar, G. G., Bellissimo-Rodrigues, F., Martinez, R., and Darini, A. L. (2014). Expansion and evolution of a virulent, extensively drug-resistant (polymyxin B-resistant), QnrS1-, CTX-M-2-, and KPC-2-producing *Klebsiella pneumoniae* ST11 international high-risk clone. *J. Clin. Microbiol.* 52, 2530–2535. doi: 10.1128/JCM.00088-14

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. doi: 10.1186/1471-2164-9-75

Bernier, C., Gounon, P., and Le Bouguénec, C. (2002). Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as a sensitive probe for detecting the AAF-encoding operon family. *Infect. Immun.* 70, 4302–4311. doi: 10.1128/IAI.70.8.4302-4311.2002

Bojer, M. S., Hammerum, A. M., Jørgensen, S. L., Hansen, F., Olsen, S. S., Krogfelt, K. A., et al. (2012). Concurrent emergence of multidrug resistance and heat resistance by CTX-M-15-encoding conjugative plasmids in *Klebsiella pneumoniae*. *APMIS* 120, 699–705. doi: 10.1111/j.1600-0463.2012.02885.x

Bojer, M. S., Krogfelt, K. A., and Struve, C. (2011). The newly discovered ClpK protein strongly promotes survival of *Klebsiella pneumoniae* biofilm subjected to heat shock. *J. Med. Microbiol.* 60, 1559–1561. doi: 10.1099/jmm.0.032698-0

Bojer, M. S., Struve, C., Ingmer, H., Hansen, D. S., and Krogfelt, K. A. (2010). Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. *PLoS ONE* 5:e15467. doi: 10.1371/journal.pone.0015467

Boll, E. J., Frimodt-Møller, J., Olesen, B., Krogfelt, K. A., and Struve, C. (2016). Heat resistance in extended-spectrum beta-lactamase-producing *Escherichia coli* may favor environmental survival in a hospital setting. *Res. Microbiol.* 167, 345–349. doi: 10.1016/j.resmic.2016.02.002

Burmølle, M., Bahl, M. I., Jensen, L. B., Sørensen, S. J., and Hansen, L. H. (2008). Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in Enterobacteriaceae strains. *Microbiology* 154, 187–195. doi: 10.1099/mic.0.2007/010454-0

Burmølle, M., Ren, D., Bjarnsholt, T., and Sørensen, S. J. (2014). Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22, 84–91. doi: 10.1016/j.tim.2013.12.004

Ekaza, E., Teyssier, J., Ouahrani-Bettache, S., Liautard, J. P., and Köhler, S. (2001). Characterization of Brucella suis clpB and clpAB mutants and participation of the genes in stress responses. *J. Bacteriol.* 183, 2677–2681. doi: 10.1128/JB.183.8.2677-2681.2001

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., et al. (2013). Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. *Int. J. Food Microbiol.* 162, 190–212. doi: 10.1016/j.ijfoodmicro.2012.08.008

Frees, D., Gerth, U., and Ingmer, H. (2014). Clp chaperones and proteases are central in stress survival, virulence and antibiotic resistance of *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 304, 142–149. doi: 10.1016/j.ijmm.2013.11.009

Gajdosova, J., Benedikovicova, K., Kamodyova, N., Tothova, L., Kaclikova, E., Stuchlik, S., et al. (2011). Analysis of the DNA region mediating increased thermotolerance at 58°C in Cronobacter sp. and other enterobacterial strains. *Antonie Van Leeuwenhoek* 100, 279–289. doi: 10.1007/s10482-011-9585-y

Gordon, D. M., and O'Brien, C. L. (2006). Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. *Microbiology* 152, 3239–3244. doi: 10.1099/mic.0.28690-0

Hecker, M., Schumann, W., and Volker, U. (1996). Heat-shock and general stress response in *Bacillus subtilis*. *Mol. Microbiol.* 19, 417–428. doi: 10.1046/j.1365-2958.1996.396932.x

Hufnagel, D. A., Depas, W. H., and Chapman, M. R. (2015). The biology of the *Escherichia coli* extracellular matrix. *Microbiol. Spectr.* 3:MB-0014-2014. doi: 10.1128/microbiolspec.mb-0014-2014

Jørgensen, S. B., Bojer, M. S., Boll, E. J., Martin, Y., Helmersen, K., Skogstad, M., et al. (2016). Heat-resistant, extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in endoscope-mediated outbreak. *J. Hosp. Infect.* 93, 57–62. doi: 10.1016/j.jhin.2016.01.014

Kado, C. I., and Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145, 1365–1373.

Kuhnert, P., Nicolet, J., and Frey, J. (1995). Rapid and accurate identification of *Escherichia coli* K-12 strains. *Appl. Environ. Microbiol.* 61, 4135–4139.

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for comparing large genomes. *Genome Biol.* 5:R12. doi: 10.1186/gb-2004-5-2-r12

Lawley, T. D., Klimke, W. A., Gubbins, M. J., and Frost, L. S. (2003). F factor conjugation is a true type IV secretion system. *FEMS Microbiol. Lett.* 224, 1–15. doi: 10.1016/S0378-1097(03)00430-0

Lee, C., Wigren, E., Trcek, J., Peters, V., Kim, J., Hasni, M. S., et al. (2015). A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ. Microbiol.* 17, 4511–4526. doi: 10.1111/1462-2920.12915

Li, H., and Gänzle, M. (2016). Some like it hot: heat resistance of *Escherichia coli* in food. *Front. Microbiol.* 7:1763. doi: 10.3389/fmicb.2016.01763

Lindsey, R. L., Knipe, K., Rowe, L., Garcia-Toledo, L., Loparev, V., Juieng, P., et al. (2015). Complete genome sequences of two shiga toxin-producing *Escherichia coli* strains from serotypes O119:H4 and O165:H25. *Genome Announc.* 3:e01496-15. doi: 10.1128/genomeA.01496-15

Liu, B., Hu, B., Zhou, Z., Guo, D., Guo, X., Ding, P., et al. (2012). A novel non-homologous recombination-mediated mechanism for *Escherichia coli* unilateral flagellar phase variation. *Nucleic Acids Res.* 40, 4530–4538. doi: 10.1093/nar/gks040

Lourdault, K., Cerqueira, G. M., Wunder, E. A. Jr., and Picardeau, M. (2011). Inactivation of clpB in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. *Infect. Immun.* 79, 3711–3717. doi: 10.1128/IAI.05168-11

Marti, R., Muniesa, M., Schmid, M., Ahrens, C. H., Naskova, J., and Hummerjohann, J. (2016). Short communication: heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum beta-lactamases and Shiga toxin-encoding phages in dairy. *J. Dairy Sci.* 99, 8622–8632. doi: 10.3168/jds.2016-11076

Mercer, R. G., Zheng, J., Garcia-Hernandez, R., Ruan, L., Ganzle, M. G., and McMullen, L. M. (2015). Genetic determinants of heat resistance in *Escherichia coli*. *Front. Microbiol.* 6:932. doi: 10.3389/fmicb.2015.00932

Møller, A. K., Leatham, M. P., Conway, T., Nuijten, P. J., de Haan, L. A., Krogfelt, K. A., et al. (2003). An *Escherichia coli* MG1655 lipopolysaccharide deep-rough core mutant grows and survives in mouse cecal mucus but fails to colonize the mouse large intestine. *Infect. Immun.* 71, 2142–2152. doi: 10.1128/IAI.71.4.2142-2152.2003

O'Gorman, L. E., Krejany, E. O., Bennett-Wood, V. R., and Robins-Browne, R. M. (1996). Transfer of attaching and effacing from a strain of enteropathogenic *Escherichia coli* to *E. coli* K-12. *Microbiol. Res.* 151, 379–385. doi: 10.1016/S0944-5013(96)80007-3

Olesen, B., Hansen, D. S., Nilsson, F., Frimodt-Moller, J., Leihof, R. F., Struve, C., et al. (2013). Prevalence and characteristics of the epidemic multiresistant *Escherichia coli* ST131 clonal group among extended-spectrum beta-lactamase-producing *E. coli* isolates in Copenhagen, Denmark. *J. Clin. Microbiol.* 51, 1779–1785. doi: 10.1128/JCM.00346-13

Peng, S., Hoffmann, W., Bockelmann, W., Hummerjohann, J., Stephan, R., and Hammer, P. (2013b). Fate of Shiga toxin-producing and generic *Escherichia coli* during production and ripening of semihard raw milk cheese. *J. Dairy Sci.* 96, 815–823. doi: 10.3168/jds.2012-5865

Peng, S., Hummerjohann, J., Stephan, R., and Hammer, P. (2013a). Short communication: heat resistance of *Escherichia coli* strains in raw milk at different subpasteurization conditions. *J. Dairy Sci.* 96, 3543–3546. doi: 10.3168/jds.2012-6174

Peng, S., Stephan, R., Hummerjohann, J., Blanco, J., and Zweifel, C. (2012). *In vitro* characterization of Shiga toxin-producing and generic *Escherichia coli* in respect of cheese production-relevant stresses. *J. Food Safety Food Qual.* 63, 136–141. doi: 10.2376/0003-925X-63-136

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., et al. (2011). Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. *N. Engl. J. Med.* 365, 709–717. doi: 10.1056/NEJMoa1106920

Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L., and Molin, S. (2006). *In vitro* biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. *J. Bacteriol.* 188, 3572–3581. doi: 10.1128/JB.188.10.3572-3581.2006

Robertson, K., Green, A., Allen, L., Ihry, T., White, P., Chen, W. S., et al. (2016). Foodborne outbreaks reported to the U.S. food safety, and inspection service, fiscal years 2007 through 2012. *J. Food Prot.* 79, 442–447. doi: 10.4315/0362-028X.JFP-15-376

Rossi, F., Rizzotti, L., Felis, G. E., and Torriani, S. (2014). Horizontal gene transfer among microorganisms in food: current knowledge and future perspectives. *Food Microbiol.* 42C, 232–243. doi: 10.1016/j.fm.2014.04.004

Schjørring, S., Struve, C., and Krogfelt, K. A. (2008). Transfer of antimicrobial resistance plasmids from *Klebsiella pneumoniae* to *Escherichia coli* in the mouse intestine. *J. Antimicrob. Chemother.* 62, 1086–1093. doi: 10.1093/jac/dkn323

Schroll, C., Barken, K. B., Krogfelt, K. A., and Struve, C. (2010). Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. *BMC Microbiol.* 10:179. doi: 10.1186/1471-2180-10-179

Squires, C. L., Pedersen, S., Ross, B. M., and Squires, C. (1991). ClpB is the *Escherichia coli* heat shock protein F84.1. *J. Bacteriol.* 173, 4254–4262. doi: 10.1128/jb.173.14.4254-4262.1991

Stahlhut, S. G., Chattopadhyay, S., Kisiela, D. I., Hvidtfeldt, K., Clegg, S., Struve, C., et al. (2013). Structural and population characterization of MrkD, the adhesive subunit of type 3 fimbriae. *J. Bacteriol.* 195, 5602–5613. doi: 10.1128/JB.00753-13

Struve, C., Roe, C. C., Stegger, M., Stahlhut, S. G., Hansen, D. S., Engelthaler, D. M., et al. (2015). Mapping the evolution of hypervirulent *Klebsiella pneumoniae*. *MBio* 6:e00630. doi: 10.1128/mBio.00630-15

Threlfall, E. J., Rowe, B., Ferguson, J. L., and Ward, L. R. (1986). Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain. *J. Hyg.* 97, 419–426. doi: 10.1017/S0022172400063609

Zangari, T., Melton-Celsa, A. R., Panda, A., Boisen, N., Smith, M. A., Tatarov, I., et al. (2013). Virulence of the Shiga toxin type 2-expressing *Escherichia coli* O104:H4 German outbreak isolate in two animal models. *Infect. Immun.* 81, 1562–1574. doi: 10.1128/IAI.01310-12

Zschüttig, A., Zimmermann, K., Blom, J., Goesmann, A., Pohlmann, C., and Gunzer, F. (2012). Identification and characterization of microcin S, a new antibacterial peptide produced by probiotic *Escherichia coli* G3/10. *PLoS ONE* 7:e33351. doi: 10.1371/journal.pone.0033351

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Boll, Marti, Hasman, Overballe-Petersen, Stegger, Ng, Knøchel, Krogfelt, Hummerjohann and Struve. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

MINI REVIEW
published: 10 April 2017
doi: 10.3389/fmicb.2017.00604

Helicobacter pullorum: An Emerging Zoonotic Pathogen

*Sundus Javed *, Farzana Gul, Kashaf Javed and Habib Bokhari*

Department of BioSciences, COMSATS Institute of Information Technology, Islamabad, Pakistan

Helicobacter pullorum (*H.*pullorum) commonly colonizes the gastrointestinal tract of poultry causing gastroenteritis. The bacterium may be transmitted to humans through contaminated meat where it has been associated with colitis and hepatitis. Despite the high prevalence of *H. pullorum* observed in poultry, little is known about the mechanisms by which this bacterium establishes infection in host and its virulence determinants. In this article we aim to provide an overview of this emerging zoonotic pathogen; its general characteristics, hosts, prevalence, and transmission as well as its pathogenic potential. We also discuss possible control strategies and risk of disease emergence.

Keywords: *Helicobacter*, enteric *Helicobacter* species, *H. pullorum*, zoonotic pathogen, foodborne pathogen

OPEN ACCESS

Edited by:
Rosanna Tofalo,
University of Teramo, Italy

Reviewed by:
Alessandra De Cesare,
University of Bologna, Italy
Heriberto Fernandez,
Austral University of Chile, Chile

****Correspondence:***
Sundus Javed
sundus.javed@comsats.edu.pk

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *26 January 2017*
Accepted: *23 March 2017*
Published: *10 April 2017*

Citation:
Javed S, Gul F, Javed K and Bokhari H (2017) Helicobacter pullorum: An Emerging Zoonotic Pathogen. Front. Microbiol. 8:604. doi: 10.3389/fmicb.2017.00604

INTRODUCTION

Helicobacter pullorum (*H. pullorum*) was first discovered by Stanley in 1994. He reported *Campylobacter*-like organisms in the liver, duodenum and caecum of chickens, as well as humans suffering from gastroenteritis. Due to its unique DNA homology and total protein electrophoretic patterns, it was classified as a novel species belonging to the *Helicobacter genus* (Stanley et al., 1994). The bacterium is an important member of the enterohepatic *Helicobacter* species (EHS) which predominantly colonize the intestine and the hepatobiliary system of the host (Hameed and Sender, 2011). In the following review we aim to provide a comprehensive overview of *H. pullorum* prevalence, its associated pathology as well as reported virulence and antibiotic resistance mechanisms.

GENERAL CHARACTERISTICS

H. pullorum is a gram-negative bacterium, slightly curved rod in shape, with a single polar flagellum which is non-sheathed. It is a motile, non-spore forming, microaerophilic bacterium, which best grows at 37–42°C (Hassan et al., 2014a). *H. pullorum* produces catalase, reduces nitrates, but lacks urease, indoxyl acetate esterase, or alkaline phosphatase activity.

Genome Sequence information from 5 *H. pullorum* strains including one human strain (MIT 98-5489) isolated from a patient suffering from gastroenteritis and four poultry isolates (229334/12, 229336/12, 229254/12, 229313/12) are available at the NCBI database. The database also includes plasmid sequences from 2 strains. The genomic DNA has 33% GC content with a 1,919 kb circular chromosome coding for 2,044 genes of which 2008 are protein coding (Shen et al., 2014).

LIPOPOLYSACCHARIDES (LPS)

Structural characterization of *Helicobacter pullorum* purified lipopolysaccharides (LPS) using electrophoretic, serological, and chemical methods reveals O-polysaccharide chain bearing lipopolysaccharides. 3-hydroxytetradecanoic acid and 3-hydroxyhexadecanoic acid are important components of *H. pullorum* LPS with low variability between chicken and human isolates.

The bacterium exhibits high hydrophilicity, therefore water based extraction instead of acid glycine is considered to be more effective. *H. pullorum* LPS has the highest relative *Limulus* amoebocyte lysate activity of all *Helicobacter* species lipopolysaccharides, indicating high endotoxin activity (Hynes et al., 2004). Polysaccharides of *H. pullorum* may play an important role in bacterial adhesion since competitive binding of sulphated groups of heparin results in marked reduction in host cell adhesion (Lutay et al., 2011). Ability of *H. pullorum* LPS to induce nuclear factor-Kappa B activation in host cells may play an important role in inflammation leading to the gastroenteritis observed in *H. pullorum* infection (Hynes et al., 2004).

N-LINKED GLYCOSYLATION SYSTEM

Bacterial N-linked glycosylation system was discovered in *Campylobacter jejuni*. Oligosaccharyltransferase PglB is the key enzyme of this system involved in the coupling of glycan to asparagine residues of the glycoprotein. Until now, all characterized *Helicobacter* species lacked *pgl* genes except *H. pullorum, H. canadensis,* and *H. winghamensis.*

H. pullorum possesses two unrelated pglB genes (pglB1 and pglB2), neither of which is located within a larger locus like *C. jejuni*. PglB1 protein of *H. pullorum* displays oligosaccharyltransferase activity in complementation experiments. On the other hand pglB2 lacks oligosaccharyltransferase activity *in vitro*. Moreover, insertional knockout mutagenesis of pglB2 gene proved lethal for the bacterium suggesting that it is essential for its survival (Jervis et al., 2010). N-linked glycosylation is common in eukaryotes but rarely seen in bacteria. The description of N-linked glycosylation in another bacterial system presents an interesting opportunity for protein glycoengineering and possibilities for future therapeutic applications.

SURVIVAL AND TRANSMISSION

Catalase enzyme plays a crucial role in protection of *H. pullorum* against oxidative stress of host and environment (Sirianni et al., 2013). The bacterium is able to tolerate high bile stress and variation in expression of certain bile stress response proteins has been suggested (Hynes et al., 2003). In a report by Bauer and colleagues, the *H. pullorum* two-component system (TCS) was shown to be involved in the control of nitrogen metabolism by regulating the expression of glutamate dehydrogenase. *H. pullorum* TCS is composed of an AmtB ammonium transporter and a PII protein consisting of the HPMG439 and its cognate histidine kinase (HK) HPMG440 (Bauer et al., 2013). In this respect the bacterium resembles *C. jejuni* than *H. pylori*. Moreover, the ability of the bacterium to tolerate oxidative stress and live under high bile stress enables it to occupy various niches in the enteric system of the host including the gall bladder, as mentioned in subsequent sections.

PREVALENCE

H. pullorum naturally infects many poultry birds, some rodent species as well as humans. Gastroeneteritis in farm raised birds, including chicken, turkey, and guinea fowl has been associated with *H. pullorum* infection. The infection has been linked to vibrionic hepatitis lesions in chickens (Burnens et al., 1994) and diarrhea in humans (Ceelen et al., 2005a). Meanwhile, natural infection of *H. pullorum* strains in rats and rabbits has also been reported (Van den Bulck et al., 2006; Cacioppo et al., 2012). *H. pullorum* prevalence reports from various regions have been summarized in **Table 1**.

Poultry

H. pullorum has been isolated from various poultry tissues. 76.4% of Turkeys were found to be infected with the bacterium in Finland whereas no bacterial growth in turkey, cloacal, cecal, and liver samples was observed in a report from Egypt (Zanoni et al., 2011; Hassan et al., 2014b). Meanwhile, in chickens variable prevalence rates have been reported from various regions. A Polish study depicted 23.5% fresh chicken meat samples from different producers to be positive for *H. pullorum* (Borges et al., 2015). Whereas, 57.1% free-range farm birds and 100% broiler, layer, and organic farm chickens were infected with *H. pullorum* in Italy (Zanoni et al., 2007; Manfreda et al., 2011). Bacterial isolates obtained from the gastrointestinal tract and liver of 110 broiler chickens in Belgium were tested through PCR where 33.6% (cecum), 31.8% (colon), 10.9% (jejunum), and 4.6% (liver) isolates tested positive for the bacterium (Ceelen et al., 2006a).

39.33% prevalence rate was observed in Egypt using a *H. pullorum* species-specific 16S rRNA PCR on isolates from 900 cloacal, cecal, and liver isolates of broiler chickens, while there was no bacterial growth from duck samples (Hassan et al., 2014b). A study spanning 32 villages in Selangor and Malaysia testing broiler chickens for culture and PCR based identification of *H. pullorum,* reported 24.72% prevalence rate, where 12.36% chickens were co-infected with *Campylobacter spp* (Wai et al., 2012). On the other hand a higher *H. pullorum* prevalence rate of 55.21% was reported from Turkey where 12 broiler chicken flocks were tested (Beren and Seyyal, 2013).

Meanwhile, in the province of Ardabil, Iran 120 samples of chickens with gastroenteritis were tested using biochemical tests for the identification of *H. pullorum***.** Results obtained through this study showed 7.5, 5, and 2.5% *H. pullorum* prevalence rates in cecum, liver and thigh meat samples, respectively (Shahram et al., 2015). However, another study from Iran evaluated 100 cecal samples from the gastrointestinal tract of broiler chickens using PCR and observed 61% prevalence of *H. pullorum* (Jamshidi et al., 2014). Cecum seems to be the preferred niche of the bacterium with fewer prevalence rates in the liver.

As discussed earlier *H. pullorum* is associated with vibrionic hepatitis in chickens, although the evidence seems singular. Later studies do not find any particular link between colonization of the bacterium and macroscopic liver lesions (Burnens et al., 1996; Ceelen et al., 2005b). This can be explained by the fact that *H.*

TABLE 1 | Summary of published *H. pullorum* prevalence data in poultry.

Region	Source	Sample size	Identification	Prevalence	References
Egypt	Poultry (Chickens, Turkey, Ducks)	1,800 (cloacal swabs, cecal swabs and liver)	16S rRNA gene PCR assay	60% (Chickens) 0% (Turkeys) 0% (Ducks)	Hassan et al., 2014b
Marmara Turkey	Broiler chickens	96 (cecum and colon)	16S rRNA gene PCR assay	55.21% 32.29% (cecum) 10.15% (colon) 15.63% (both)	Beren and Seyyal, 2013
Ardabil	Broiler chickens	120 (chicken with gastroenteritis)	Biochemical tests (oxidase, catalase, nitrate reduction, urease)	7.5% (cecum) 5% (liver)	Shahram et al., 2015
Iran	Human fecal samples	100 (gastroenteritis)		2.5% (Thigh) 6%	
	Broiler chickens	100 (cecum)	16SrRNA gene PCR assay	41%	Jamshidi et al., 2014
Belgium	Broiler chickens	110 samples from 11 flocks (gastrointestinal tract and liver)	16S rRNA gene PCR assay	33.6% (cecum) 31.8% (colon) 10.9% (jejunum) 4.6% (liver)	Ceelen et al., 2006a
Belgium	Human fecal samples	531 (Gastroenteritis) 100 (healthy individuals)	16S rRNA gene PCR assay	4.3% (gastroenteritis) 4.0% (healthy)	Ceelen et al., 2005b
Selangor Malaysia	Broiler and village chickens	89 samples, 32 (village). 57 (Broiler chickens)	16S rRNA gene PCR assay	24.72%	Wai et al., 2012
Libson	Chicken meat	17 samples	16S rRNA gene PCR assay	23.52%	Borges et al., 2015
Italy	Farms (broiler chicken)	169 ceca (30 conventional farms)	Genotyping analysis(AFLP, PFGE)	100% (conventional farms)	Manfreda et al., 2011
		39 ceca (8 organic farms)		100% (organic farms)	
		40 ceca (7 free-range farms)		57.1% (free-range farms)	
	Farms (Turkeys)	55 (cecum)	16SrRNA gene PCR assay	76.4%	Zanoni et al., 2011
	Broiler chickens and laying hens	60 (cecum)	16SrRNA gene PCR assay	100%	Zanoni et al., 2007

pullorum prevalence reported in the study was low and may be reflective of colonization with hypervirulent strains.

H. pullorum has the ability to contaminate the carcasses of the poultry and is considered a food borne pathogen (Mohamed et al., 2010). Overall it may be expected that the prevalence of *H. pullorum* is generally underestimated as noted by Wainø et al. (2003). Generally gross underestimation of prevalence rates may be expected when relying on phenotypic tests commonly employed for identification. This is may be expected since screening for *H. pullorum* is not undertaken and *H. pullorum* strains fail to thrive on the mCCDA medium employed in the laboratory to select for campylobacters. This underestimation is supported by PCR identification of *H. pullorum* from broiler chicken isolates that were originally denoted unspeciated cultures or falsely identified as *Campylobacter lari* (Wedderkopp et al., 2000; Wainø et al., 2003).

Presence of the bacterium in the gut of chickens may have a wider impact on the birds' gastrointestinal physiology than having a pathological outcome. As a recent study suggests that *H. pullorum* impacts the gastrointestinal microbiota of commercial broiler chickens, influencing the *Lactobacillus, Streptococcus, Ruminococcaceae* abundance as well as prevalence of *Corynebacterium* species in the chicken gut (Kaakoush et al., 2014). However, the overall impact on the birds' physiology and health (e.g., net weight gain) as well as susceptibility to infection remains to be investigated.

Humans

H. pullorum is a zoonotic bacterium that has also been associated with certain enteric infections in humans. *H. pullorum* has been associated with recurrent diarrheal illness in patients after treatment suggesting the possibility of chronic infection (Steinbrueckner et al., 1997). Case of a 35 year old male suffering from *H. pullorum* induced bacteraemia, presented with abdominal pain along with profuse diarrhea has also been reported (Tee et al., 2001). In Iran, human diarrheal samples were evaluated for presence of *H. pullorum* with a 6% prevalence rate (Shahram et al., 2015). On the other hand a Belgian study showed 4.3% *H. pullorum* prevalence in fecal samples from patients with gastroenteritis compared to clinically healthy individuals (Ceelen et al., 2005a). In another study 158 fecal samples were collected from under-five children with diarrhea and 35 bacterial

pathogens were isolated. The bacterial isolates comprised of *Campylobacter* species, 20 (12.7%), *Shigella* species, 11 (7.0%), and *Salmonella* species, 4 (2.5%) indicating that diarrheagenic pathogens other than *H. pullorum* are the main etiologic agents of diarrhea in children (Mulatu et al., 2014). Therefore, the evidence of the bacterium's association with diarrheal disease is weak; however it seems likely that Crohn's disease and cholelitiasis have more significant associations with *H. pullorum* infection. This association is not surprising since the bacterium along with *Helicobacter bilis* is able to tolerate high bile stress and is supported by several reports from Germany, Sweden, China, and Japan suggesting *H. pullorum* prevalence of 2–27% in gall bladder malignancies (Fukuda et al., 2002; Murata et al., 2004; Bohr et al., 2007; Chen et al., 2007; Karagin et al., 2010). Meanwhile a study in Chile and another from Ukraine report much higher prevalence rates (Fox et al., 1998; Apostolov et al., 2005). *H. pullorum* has also been found to be the predominant *Helicobacter* species in patients with Crohn's disease (Young et al., 2000; Bohr et al., 2004).

EXPERIMENTAL INFECTION MODEL

EHS, including *H. pullorum* and *H. pullorum*-like living organisms, have been found to bring about bacteraemia and systemic ailment in both immunocompromised and immunocompetent patients. As described earlier, *H. pullorum* identified by PCR tests has been associated with enteric and hepatobiliary illnesses in humans. In comparison to different EHS, *H. pullorum* is viewed as an emerging, zoonotic human pathogen justifying the need to create animal models in order to understand the underlying pathogenic mechanisms. The bacterium possesses broad host specificity with the ability to infect birds, rodents, and humans.

Routine observation testing at a business rat creation office distinguished *Helicobacter* infected animal groups by PCR in BN/MolTac rats as well as C57BL/6NTac, C3H/HeNTac, and DBA/2NTac mice. Of the 10 C57BL/6NTac mice, 8 of 10 caecal and seven of 10 colon samples were PCR positive for *Helicobacter sp*. Only the caecum from one of three C3H/HeNTac mice was positive for *H. pullorum* (Turk et al., 2012). *H. pullorum* was also reported to be the causative agent of an outbreak in C57BL/6NTac and C3H/HeNTac mice housed within one isolated barrier unit. The isolates were phylogenetically similar to a human isolate, depicting a shift in host specificity (Boutin et al., 2010). The importance of *H. pullorum* in clinical ailment requires further studies to establish causative link. C57BL/6NTac mice can be persistently infected with *H. pullorum* in experimental settings providing the opportunity to utilize a mouse model to study *H. pullorum* pathogenesis (Turk et al., 2012).

PATHOGENESIS

H. pullorum shows similarity with other *Helicobacter* species and *Campylobacter* species with regards to presence of several virulence factors. It has been isolated from patients suffering from cholecystitis, liver problems and cirrhosis (Ponzetto et al., 2000; Ananieva et al., 2002). Therefore, involvement of *Helicobacter* spp. including *H. pullorum* in pathogenesis and progression of cirrhosis, particularly in HCV-infected individuals seems plausible (Ponzetto et al., 2000). It has been also found that *H. pullorum* like organisms are present in individuals suffering from bacteremia, especially those who were immunocompetent. Later, association of *H. pullorum* infection with inflammatory bowel disease (IBD) has been speculated (Jamshidi et al., 2014).

Many virulence factors aid in the pathogenicity of the host cell by the bacterium including the bacterial flagellar apparatus, T3SS secreted toxin Cdt and the recently described T6SS. The cytopathogenic alterations induced by several human and avian *H. pullorum* strains were investigated on human intestinal epithelial cell lines. Human hepatocytes, gall bladder epithelial cells, and colon epithelial cells infected with *H. pullorum*, showed increased expression of MMP-2 and MMP-9 compared to uninfected controls in a bacterial dose dependent manner. These matrix metalloproteinases (MMPs) aid in degradation of extracellular matrix, allowing bacteria to interact with host cells (Yanagisawa et al., 2005).

H. pullorum is able to interact with host intestinal microvilli via its flagellum. This flagellum-microvilli interaction stimulates IL-8 production and intestinal cell colonization. This bacterial invasion process cause host damage via cellular edema and cell debris release (Sirianni et al., 2013). Recently it has been reported that the secretion of IL-8 leading to inflammatory reponses in gastric epithelial cells is dependent upon bacterial attachment to the epithelial lining (Sirianni et al., 2013). This inflammation is enhanced by cytolethal distending toxin (CDT) and lipopolysaccharide (LPS) via activation of the NF-kB pathway (Ceelen et al., 2005a).

More recently it has been observed that the bacterium is able to induce nitric oxide production in murine macrophages after internalization. The interaction of *H. pullorum* with host macrophages also stimulates secretion of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and MIP-2 (Parente et al., 2016).

VIRULENCE DETERMINANTS

H. pullorum causes gastroenteritis in poultry as well as humans; however few mechanisms of bacterial pathogenesis and its molecular determinants have so far been characterized. Following we list a few bacterial virulence factors described in *H. pullorum* (Summarized in **Figure 1**).

Adhesins

Bacterial co-culture experiments with the mammalian intestinal epithelial cell line, Caco-2 showed that *H. pullorum* is capable of host cell adhesion, albeit at a much lesser extent in comparison to *Salmonella typhimurium* and comparable to the adhesion rates observed for *C. jejuni* (Varon et al., 2009; Sirianni et al., 2013). Factors responsible for cytopathogenic effects of *H. pullorum* on epithelial cells have not been formally identified. However, cell-binding factor 2, flagellin, secreted protein Hcp, valine-glycine repeat protein G (VgrG), a type VI secretion protein and a protease were identified as important virulence

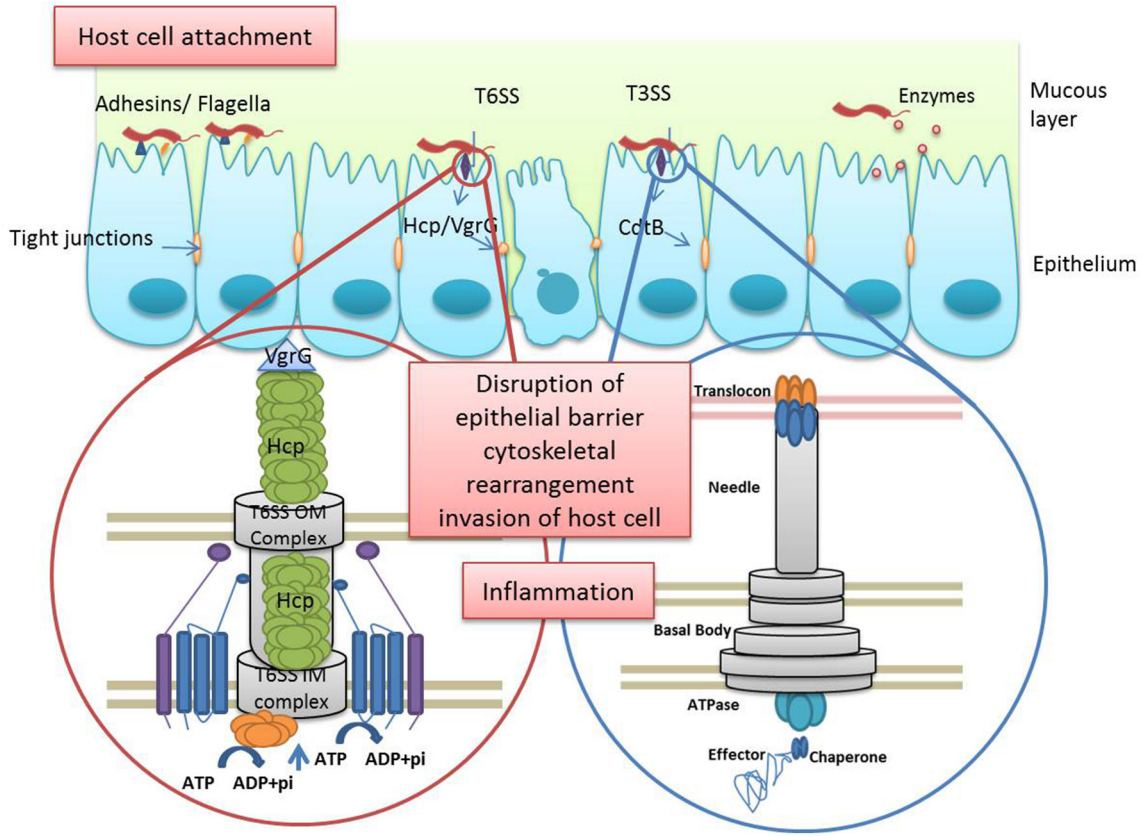


FIGURE 1 | Schematic representing major *H. pullorum* virulence factors. *H. pullorum* virulence factors aid in the pathogenecity and colonization of the host cell by the bacterium including the cell-binding factor 2, flagellin, as well as type 6 secretion proteins Hcp and VgrG. The polar flagellum mediates initial contact with host cells via a flagellum–microvillus interaction. This contact leads to injection of CdtB toxin in the host cell which along with the newly described T6SS is involved in cell invasion and cytoskeletal rearrangement of host cell leading to epithelial barrier disruption. The inflammation elicited by the bacterium is enhanced by cdt and LPS.

and colonization factors in *H. pullorum* (Sirianni et al., 2013). Furthermore, scanning electron microscopy suggests that the polar flagellum of *H. pullorum* mediates initial contact with host cells via a flagellum–microvillus interaction and that host cell contact is important for inflammation elicited by the bacterium (Sirianni et al., 2013; Varon et al., 2009).

Cdt (Cytolethal distending toxin)

Cytolethal distending toxin (Cdt) was first reported by Jhonson and Lior in *E. coli* in the year 1987 and was described to cause cellular anomalies and cell death in Chinese Hamster ovary (Ceelen et al., 2006b). Cdt causes edema, cytoskeleton anomalies and G2/M cycle arrest in host cell. Cdt has been identified in *Campylobacter* species as well as in different *Helicobacter* species like *H. bilis, H. canis, H. hepaticus,* and *H. pullorum* (Mohamed et al., 2010). *Two* soluble factors involved in cytotoxic activity were reported in *H. pullorum*: the Cdt toxin and a soluble toxic factor, still unidentified, causing a mitotic catastrophe resulting in primary necrosis of hepatic cells. CdtB induced a cellular and nuclear enlargement, accompanied by profound remodeling of the actin cytoskeleton with the formation of cortical actin-rich large lamellipodia and membrane ruffle structures. In addition, disturbance of focal adhesion and the microtubule network were also observed. These effects may have profound consequences on bacterial adherence and intestinal barrier integrity (Varon et al., 2014). Therefore, *H. pullorum* Cdt is responsible for major cytopathogenic effects *in vitro*, confirming its role as an important virulence factor of this emerging human pathogen (Young et al., 2000).

Type 6 Secretion System (T6SS)

T6SS is a newly identified secretion system in gram negative bacteria encoded in pathogenicity islands (Bingle et al., 2008). The T6SS is composed of 13 core components and displays structural similarities with the tail-tube of bacteriophages. The phage uses a tube and a puncturing device to penetrate the cell envelope of target bacteria and inject DNA. It is proposed that the T6SS creates a specific path in the bacterial cell envelope to drive effectors and toxins to the surface. T6SS device can also perforate other cells with which the bacterium is in contact, thus injecting the effectors into these targets. The tail tube and puncturing device of the T6SS are composed of *Hcp* and *VgrG* proteins, respectively (Hachani et al., 2013). *Hcp* and *VgrG* are T6SS effector proteins, the presence of which is considered a prerequisite for T6SS function. Both *Hcp* and *VgrG* are extracellular components forming a needle like projection

that makes contact with the host cell. Hcp forms a hexametric ring that is believed to stack into a tubular structure. On the other hand, VgrG proteins can in fact have an effector function. So called evolved *VgrG* has a sizeable C-terminal containing effector domain (Pukatzki et al., 2007). The existence of a putative functional T6SS in *H. pullorum* was proposed by Sirianni in 2013 (Sirianni et al., 2013). Genomic analysis of three out of four chicken isolates depicted the presence of T6SS genes (Borges et al., 2015). Certain structural similarities between Hcp and endocytic vesicle coat proteins suggest its role in cellular invasion via interaction with endocytic vesicles of host, although *in vitro* validation of this *in silico* study is lacking. In addition, the T6SS is associated with a more severe form of diarrhea and bacteremia during *C. jejuni* infection supporting the contribution of the system to the virulence of this pathogen (Bleumink-Pluym et al., 2013).

It has been shown that the T6SS effector proteins, vgrG and Hcp also play a major role in host cell pathogenesis, although their role in *H. pullorum* infection has not been completely understood. VgrG proteins form a trimeric, needle-like structure and puncture host cell membrane. Hcp is involved in induction of actin cytoskeleton rearrangement and production of IL-6 and IL-8. Furthermore, two proteins suggested to be 1-phosphatidylinositol-4- phosphate 5-kinases were found to be involved in the regulation of the actin cytoskeleton and were also identified in proximity to the T6SS proteins (Sirianni et al., 2013).

IMMUNE RESPONSE AND IMMUNOGENIC PROTEINS

Immunogenic cell surface proteins of *H. pullorum*, along with other EHS compared to *H. pylori* were characterized via two dimensional electrophoresis and immunoblotting using immunized rabbit antisera. Twenty-One specific immunogenic proteins were identified, with proteins of *H. pylori* and *H. pullorum* showing similarities in their protein profiles (Kornilovs'ka et al., 2002).

ANTIBIOTIC RESISTANCE AND RESISTANCE MECHANISMS

Although *H. pullorum* infection has been associated with gastroenteritis and vibrionic hepatitis, there is no antibiotic recommendation for this organism. Isolates of poultry origin show resistance to ciprofloxacin, gentamycin, erythromycin and tetracyclin and is susceptible to colistin sulfate and ampicillin (Hassan et al., 2014a). Draft genome sequence of *H. pullorum* human isolate, MIT 98-5489 reveal that the bacteria are clarithromycin resistant. This resistance may be mediated by a mutation in the 23S rRNA gene. On the other hand, Rifampin resistance is conferred by four missense mutations in *RpoB*. *H. pullorum* (MIT 98-5489) is also resistant to ciprofloxacin. This is consistent with the finding that individual missense mutations were detected in *gyrA* which is responsible for conferring ciprofloxacin resistance (Shen et al., 2014). Furthermore, a triple-base-pair mutation in 16S rRNA is reported to confer tetracyclin resistance to the bacterium as well (Borges et al., 2015).

On the other hand *H. pullorum* human isolate (16S rRNA sequence accession no. AF334681) shows susceptibility to aminoglycosides and third-generation cephalosporins, β-lactams, and doxycycline (Tee et al., 2001). Keeping this in mind treatment strategies for patients may be recommended. The antibiotic susceptibility may vary according to strain, especially in geographically distinct regions. Intensive farming practices, where antibiotics are routinely fed to livestock as growth promoters and to prevent potential bacterial infections have contributed to increase in drug resistance worldwide, enabling re-emergence of zoonotic infections (Andersson, 2003). Most of these antibiotics have been banned in the European Union which may predict a very different antibiotic susceptibility pattern in isolates from other regions (Roe and Pillai, 2003; Cantas et al., 2013).

CONCLUSION

Zoonotic pathogens are twice as likely to be associated with emerging zoonosis including approximately 12% of human pathogens (Taylor et al., 2001). According to WHO, emerging or reemerging zoonosis are diseases caused by novel or partially new etiological agents or by a microorganism previously known but now occurring in species or places where the disease was unknown (Meslin, 1992). Engering and colleagues describe a comprehensive framework of disease emergence depicting drivers of pathogen emergence including (i) its description in a novel host; (ii) occurrence of a mutant pathogen with novel traits with ability to cause more severe form of the disease; or (iii) presence in a novel geographic region. These factors change the overall pattern of the pathogen–host–environment interactions leading to disease emergence (Engering et al., 2013). Presence of *H. pullorum* in various geographical zones as well as its wide range of hosts may pose a potential health risk. This is further confounded by the *H. pullorum* outbreak reported in mice raised in a barrier facility (Boutin et al., 2010).

Recent epidemiological data shows Salmonellosis and Campylobacteriosis to be the most frequent food-borne bacterial zoonoses in Europe (team Ee, 2013). It is unknown whether *H. pullorum* in humans is acquired by eating uncooked poultry, as is the case with *C. jejuni* acquired zoonotic infection. However, human transmission from poultry seems likely, considering the high prevalence rates reported from various regions. Routine surveillance of the pathogen in poultry as well as clinical samples is necessary. Future studies determining common sequence types in isolates of human and poultry origin as well as description of specific source markers will enable source tracking and infection risk in the population.

AUTHOR CONTRIBUTIONS

FG, KJ, and SJ contributed to the literature review and writing of the manuscript. SJ edited the manuscript and approved final draft. HB contributed to the final edit and critical review of the manuscript.

REFERENCES

Ananieva, O., Nilsson, I., Vorobjova, T., Uibo, R., and Wadström, T. (2002). Immune responses to bile-tolerant *Helicobacter* species in patients with chronic liver diseases, a randomized population group, and healthy blood donors. *Clin. Diagn. Lab. Immunol.* 9, 1160–1164. doi: 10.1128/cdli.9.6.1160-1164.2002

Andersson, D. I. (2003). Persistence of antibiotic resistant bacteria. *Curr. Opin. Microbiol.* 6, 452–456. doi: 10.1016/j.mib.2003.09.001

Apostolov, E., Al-Soud, W. A., Nilsson, I., Kornilovska, I., Usenko, V., Lyzogubov, V., et al. (2005). *Helicobacter pylori* and other *Helicobacter* species in gallbladder and liver of patients with chronic cholecystitis detected by immunological and molecular methods. *Scand. J. Gastroenterol.* 40, 96–102. doi: 10.1080/00365520410009546

Bauer, S., Endres, M., Lange, M., Schmidt, T., Schumbrutzki, C., Sickmann, A., et al. (2013). Novel function assignment to a member of the essential HP1043 response regulator family of *epsilon-proteobacteria*. *Microbiology (Reading Engl).* 159(Pt 5), 880–889. doi: 10.1099/mic.0.066548-0

Beren, K. B., and Seyyal, A. K. (2013). *Investigation of Helicobacter pullorum Occurence in Chicken in the Marmara Region of Turkey*. Istanbul Üniversity Veteriner Fakultesi Dergisi. 39, 63–66.

Bingle, L. E., Bailey, C. M., and Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Curr. Opin. Microbiol.* 11, 3–8. doi: 10.1016/j.mib.2008.01.006

Bleumink-Pluym, N. M., van Alphen, L. B., Bouwman, L. I., Wösten, M. M., and van Putten, J. P. (2013). Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity. *PLoS Pathog.* 9:e1003393. doi: 10.1371/journal.ppat.1003393

Bohr, U. R., Annibale, B., Franceschi, F., Roccarina, D., and Gasbarrini, A. (2007). Extragastric manifestations of *Helicobacter pylori* infection – other *Helicobacters*. *Helicobacter* 12(Suppl. 1), 45–53. doi: 10.1111/j.1523-5378.2007.00533.x

Bohr, U. R., Glasbrenner, B., Primus, A., Zagoura, A., Wex, T., and Malfertheiner, P. (2004). Identification of enterohepatic *Helicobacter* species in patients suffering from inflammatory bowel disease. *J. Clin. Microbiol.* 42, 2766–2768. doi: 10.1128/JCM.42.6.2766-2768.2004

Borges, V., Santos, A., Correia, C. B., Saraiva, M., Ménard, A., Vieira, L., et al. (2015). *Helicobacter pullorum* isolated from fresh chicken meat: antibiotic resistance and genomic traits of an emerging foodborne pathogen. *Appl. Environ. Microbiol.* 81, 8155–8163. doi: 10.1128/AEM.02394-15

Boutin, S. R., Shen, Z., Roesch, P. L., Stiefel, S. M., Sanderson, A. E., Multari, H. M., et al. (2010). *Helicobacter pullorum* outbreak in C57BL/6NTac and C3H/HeNTac barrier-maintained mice. *J. Clin. Microbiol.* 48, 1908–1910. doi: 10.1128/JCM.02531-09

Burnens, A. P., Stanley, J., Morgenstern, R., and Nicolet, J. (1994). Gastroenteritis associated with *Helicobacter pullorum*. *Lancet* 344, 1569–1570. doi: 10.1016/S0140-6736(94)90376-X

Burnens, A. P., Stanley, J., and Nicolet, J. (1996). "Possible association of *Helicobacter pullorum* with lesions of vibrionic hepatitis in poultry," in *Campylobacters, Helicobacters and Related Organisms*, Vol. 2, eds D. G. Newell, J. M. Ketley, and R. A. Feldman (New York, NY: Springer), 291–293.

Cacioppo, L. D., Turk, M. L., Shen, Z., Ge, Z., Parry, N., Whary, M. T., et al. (2012). Natural and experimental *Helicobacter pullorum* infection in Brown Norway rats. *J. Med. Microbiol.* 61(Pt 9), 1319–1323. doi: 10.1099/jmm.0.042374-0

Cantas, L., Shah, S. Q., Cavaco, L. M., Manaia, C. M., Walsh, F., Popowska, M., et al. (2013). A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Front. Microbiol.* 4:96. doi: 10.3389/fmicb.2013.00096

Ceelen, L., Decostere, A., Devriese, L. A., Ducatelle, R., and Haesebrouck, F. (2005b). *In vitro* susceptibility of *Helicobacter pullorum* strains to different antimicrobial agents. *Microb. Drug Resist.* 11, 122–126. doi: 10.1089/mdr.2005.11.122

Ceelen, L., Decostere, A., Verschraegen, G., Ducatelle, R., and Haesebrouck, F. (2005a). Prevalence of *Helicobacter pullorum* among patients with gastrointestinal disease and clinically healthy persons. *J. Clin. Microbiol.* 43, 2984–2986. doi: 10.1128/JCM.43.6.2984-2986.2005

Ceelen, L. M., Decostere, A., Ducatelle, R., and Haesebrouck, F. (2006b). Cytolethal distending toxin generates cell death by inducing a bottleneck in the cell cycle. *Microbiol. Res.* 161, 109–120. doi: 10.1016/j.micres.2005.04.002

Ceelen, L. M., Decostere, A., Van den Bulck, K., On, S. L., Baele, M., Ducatelle, R., et al. (2006a). *Helicobacter pullorum* in chickens, Belgium. *Emerg. Infect. Dis.* 12, 263–267. doi: 10.3201/eid1202.050847

Chen, D. F., Hu, L., Yi, P., Liu, W. W., Fang, D. C., and Cao (2007). *H pylori* are associated with chronic cholecystitis. *World J. Gastroenterol.* 13, 1119–1122. doi: 10.3748/wjg.v13.i7.1119

Engering, A., Hogerwerf, L., and Slingenbergh, J. (2013). Pathogen-host-environment interplay and disease emergence. *Emerg. Microbes Infect.* 2:e5. doi: 10.1038/emi.2013.5

Fox, J. G., Dewhirst, F. E., Shen, Z., Feng, Y., Taylor, N. S., Paster, B. J., et al. (1998). Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 114, 755–763. doi: 10.1016/S0016-5085(98)70589-X

Fukuda, K., Kuroki, T., Tajima, Y., Tsuneoka, N., Kitajima, T., Matsuzaki, S., et al. (2002). Comparative analysis of *Helicobacter* DNAs and biliary pathology in patients with and without hepatobiliary cancer. *Carcinogenesis* 23, 1927–1931. doi: 10.1093/carcin/23.11.1927

Hachani, A., Lossi, N. S., and Filloux, A. (2013). A visual assay to monitor T6SS-mediated bacterial competition. *J. Vis. Exp.* e50103. doi: 10.3791/50103

Hameed, K. G. A., and Sender, G. (2011). Prevalence of *Helicobacter pullorum* in Egyptian hen's eggs and *in vitro* susceptibility to different antimicrobial agents. *Anim. Sci. Papers Rep.* 29, 257–264. Available online at: http://www.ighz.edu.pl/uploaded/FSiBundleContentBlockBundleEntityTranslatableBlockTranslatableFilesElement/ filePath/448/pp257-268.pdf

Hassan, A., Shahata, M., Refaie, E., and Ibrahim, R. (2014a). Pathogenicity testing and antimicrobial susceptibility of *Helicobacter pullorum* isolates from chicken origin. *Int. J. Vet. Sci. Med.* 2, 72–77. doi: 10.1016/j.ijvsm.2013.12.001

Hassan, A., Shahata, M., Refaie, E., and Ibrahim, R. (2014b). Detection and identification of *Helicobacter pullorum* in poultry species in upper Egypt. *J. Adv. Vet. Res.* 4, 42–48. Available online at: http://www.advetresearch.com/index.php/avr/article/view/253

Hynes, S. O., Ferris, J. A., Szponar, B., Wadström, T., Fox, J. G., O'Rourke, J., et al. (2004). Comparative chemical and biological characterization of the lipopolysaccharides of gastric and enterohepatic *Helicobacters*. *Helicobacter* 9, 313–323. doi: 10.1111/j.1083-4389.2004.00237.x

Hynes, S. O., McGuire, J., Falt, T., and Wadström, T. (2003). The rapid detection of low molecular mass proteins differentially expressed under biological stress for four *Helicobacter* spp. using ProteinChip technology. *Proteomics* 3, 273–278. doi: 10.1002/pmic.200390040

Jamshidi, A., Bassami, M. R., Salami, H., and Mohammadi, S. (2014). Isolation and identification of *Helicobacter pullorum* from caecal content of broiler chickens in Mashhad, Iran. *Iran. J. Vet. Res.* 15, 179–182. Available online at: http://ijvr.shirazu.ac.ir/article_2369_fd08efd47ed8f4ece62c318e7e5056f4.pdf

Jervis, A. J., Langdon, R., Hitchen, P., Lawson, A. J., Wood, A., Fothergill, J. L., et al. (2010). Characterization of N-linked protein glycosylation in *Helicobacter pullorum*. *J. Bacteriol.* 192, 5228–5236. doi: 10.1128/JB.00211-10

Kaakoush, N. O., Sodhi, N., Chenu, J. W., Cox, J. M., Riordan, S. M., and Mitchell, H. M. (2014). The interplay between *Campylobacter* and *Helicobacter* species and other gastrointestinal microbiota of commercial broiler chickens. *Gut Pathog.* 6:18. doi: 10.1186/1757-4749-6-18

Karagin, P. H., Stenram, U., Wadström, T., and Ljungh, A. (2010). *Helicobacter* species and common gut bacterial DNA in gallbladder with cholecystitis. *World J. Gastroenterol.* 16, 4817–4822. doi: 10.3748/wjg.v16.i38.4817

Kornilovs'ka, I., Nilsson, I., Utt, M., Ljungh, A., and Wadström, T. (2002). Immunogenic proteins of *Helicobacter pullorum, Helicobacter bilis* and *Helicobacter hepaticus* identified by two-dimensional gel electrophoresis and immunoblotting. *Proteomics* 2, 775–783. doi: 10.1002/1615-9861(200206)2:6<775::AID-PROT775>3.0.CO;2-R

Lutay, N., Nilsson, I., Wadström, T., and Ljungh, A. (2011). Effect of heparin, fucoidan and other polysaccharides on adhesion of enterohepatic *Helicobacter* species to murine macrophages. *Appl. Biochem. Biotechnol.* 164, 1–9. doi: 10.1007/s12010-010-9109-7

Manfreda, G., Parisi, A., Lucchi, A., Zanoni, R. G., and De Cesare, A. (2011). Prevalence of *Helicobacter pullorum* in conventional, organic, and free-range broilers and typing of isolates. *Appl. Environ. Microbiol.* 77, 479–484. doi: 10.1128/AEM.01712-10

Meslin, F. X. (1992). Surveillance and control of emerging zoonoses. *World Health Stat. Q.* 45, 200–207.

Mohamed, M., Ragab, I., Shahata, M., and El-Refaie, M. (2010). *Helicobacter pullorum* among Poultry in Assiut-Egypt: genetic characterization, virulence and MIC. *Int. J. Poult. Sci.* 9, 521–526. doi: 10.3923/ijps.2010.521.526

Mulatu, G., Beyene, G., and Zeynudin, A. (2014). Prevalence of *Shigella, Salmonella* and *Campylobacter* species and their susceptibility patters among under five children with diarrhea in Hawassa town, south Ethiopia. *Ethiop. J. Health Sci.* 24, 101–108. doi: 10.4314/ejhs.v24i2.1

Murata, H., Tsuji, S., Tsujii, M., Fu, H. Y., Tanimura, H., Tsujimoto, M., et al. (2004). *Helicobacter bilis* infection in biliary tract cancer. *Aliment. Pharmacol. Ther.* 20(Suppl. 1), 90–94. doi: 10.1111/j.1365-2036.2004.01972.x

Parente, M. R., Monteiro, J. T., Martins, G. G., and Saraiva, L. M. (2016). *Helicobacter pullorum* induces nitric oxide release in murine macrophages that promotes phagocytosis and killing. *Microbiology (Reading Engl).* 162, 503–512. doi: 10.1099/mic.0.000240

Ponzetto, A., Pellicano, R., Leone, N., Cutufia, M. A., Turrini, F., Grigioni, W. F., et al. (2000). *Helicobacter* infection and cirrhosis in hepatitis C virus carriage: is it an innocent bystander or a troublemaker? *Med. Hypotheses* 54, 275–277. doi: 10.1054/mehy.1999.0987

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., and Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15508–15513. doi: 10.1073/pnas.0706532104

Roe, M. T., and Pillai, S. D. (2003). Monitoring and identifying antibiotic resistance mechanisms in bacteria. *Poult. Sci.* 82, 622–626. doi: 10.1093/ps/82.4.622

Shahram, B., Javadi, A., and Mahdi, G. R. (2015). *Helicobacter pullorum* prevalence in patients with gastroenteritis in humans and chicken in the province of Ardabil in 2014. *Indian J. Fundam. Appl. Life Sci.* 5, 87–94. Available online at: http://www.cibtech.org/J-LIFE-SCIENCES/PUBLICATIONS/2015/Vol-5-No-2/13-JLS-012-%20FSHIN-HELICOBACTER.pdf

Shen, Z., Sheh, A., Young, S. K., Abouelliel, A., Ward, D. V., Earl, A. M., et al. (2014). Draft genome sequences of six enterohepatic *Helicobacter* species isolated from humans and one from *Rhesus macaques*. *Genome Announc.* 2:e00857-14. doi: 10.1128/genomeA.00857-14

Sirianni, A., Kaakoush, N. O., Raftery, M. J., and Mitchell, H. M. (2013). The pathogenic potential of *Helicobacter pullorum*: possible role for the type VI secretion system. *Helicobacter* 18, 102–111. doi: 10.1111/hel.12009

Stanley, J., Linton, D., Burnens, A. P., Dewhirst, F. E., On, S. L., Porter, A., et al. (1994). *Helicobacter pullorum* sp. nov.-genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiology* 140(Pt 12), 3441–3339. doi: 10.1099 13500872-140-12-3441

Steinbrueckner, B., Haerter, G., Pelz, K., Weiner, S., Rump, J. A., Deissler, W., et al. (1997). Isolation of *Helicobacter pullorum* from patients with enteritis. *Scand. J. Infect. Dis.* 29, 315–318. doi: 10.3109/0036554970 9019053

Taylor, L. H., Latham, S. M., and Woolhouse, M. E. (2001). Risk factors for human disease emergence. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356, 983–989. doi: 10.1098/rstb.2001.0888

team Ee, R. (2013). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011 has been published. *Euro Surveill.* 18, 20449.

Tee, W., Montgomery, J., and Dyall-Smith, M. (2001). Bacteremia caused by a *Helicobacter pullorum*-like organism. *Clin. Infect. Dis.* 33, 1789–1791. doi: 10.1086/323983

Turk, M. L., Cacioppo, L. D., Ge, Z., Shen, Z., Whary, M. T., Parry, N., et al. (2012). Persistent *Helicobacter pullorum* colonization in C57BL/6NTac mice: a new mouse model for an emerging zoonosis. *J. Med. Microbiol.* 61(Pt 5), 720–728. doi: 10.1099/jmm.0.040055-0

Van den Bulck, K., Decostere, A., Baele, M., Marechal, M., Ducatelle, R., and Haesebrouck, F. (2006). Low frequency of *Helicobacter* species in the stomachs of experimental rabbits. *Lab. Anim.* 40, 282–287. doi: 10.1258/002367706777611424

Varon, C., Duriez, A., Lehours, P., Ménard, A., Layé, S., Zerbib, F., et al. (2009). Study of *Helicobacter pullorum* proinflammatory properties on human epithelial cells *in vitro*. *Gut.* 58, 629–635. doi: 10.1136/gut.2007.144501

Varon, C., Mocan, I., Mihi, B., Péré-Védrenne, C., Aboubacar, A., Moraté, C., et al. (2014). *Helicobacter pullorum* cytolethal distending toxin targets vinculin and cortactin and triggers formation of lamellipodia in intestinal epithelial cells. *J. Infect. Dis.* 209, 588–599. doi: 10.1093/infdis/jit539

Wai, S., Saleha, A., Zunita, Z., Hassan, L., and Jalila, A. (2012). Occurrence of Co-Infection of *Helicobacter pullorum* and *Campylobacter* spp. in Broiler and Village (Indigenous) Chickens. *Pak. Vet. J.* 32, 503–506.

Wainø, M., Bang, D. D., Lund, M., Nordentoft, S., Andersen, J. S., Pedersen, K., et al. (2003). Identification of *campylobacteria* isolated from Danish broilers by phenotypic tests and species-specific PCR assays. *J. Appl. Microbiol.* 95, 649–655. doi: 10.1046/j.1365-2672.2003.01996.x

Wedderkopp, A., Rattenborg, E., and Madsen, M. (2000). National surveillance of *Campylobacter* in broilers at slaughter in Denmark in 1998. *Avian Dis.* 44, 993–999. doi: 10.2307/1593078

Yanagisawa, N., Geironson, L., Al-Soud, W. A., and Ljungh, S. (2005). Expression of matrix metalloprotease-2, -7 and -9 on human colon, liver and bile duct cell lines by enteric and gastric *Helicobacter* species. *FEMS Immunol. Med. Microbiol.* 44, 197–204. doi: 10.1016/j.femsim.2004.11.009

Young, V. B., Chien, C. C., Knox, K. A., Taylor, N. S., Schauer, D. B., and Fox, J. G. (2000). Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. *J. Infect. Dis.* 182, 620–623. doi: 10.1086/315705

Zanoni, R. G., Piva, S., Rossi, M., Pasquali, F., Lucchi, A., De Cesare, A., et al. (2011). Occurrence of *Helicobacter pullorum* in turkeys. *Vet. Microbiol.* 149, 492–496. doi: 10.1016/j.vetmic.2010.11.013

Zanoni, R. G., Rossi, M., Giacomucci, D., Sanguinetti, V., and Manfreda, G. (2007). Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. *Int. J. Food Microbiol.* 116, 168–173. doi: 10.1016/j.ijfoodmicro.2006.12.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Javed, Gul, Javed and Bokhari. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 April 2017
doi: 10.3389/fmicb.2017.00626

Biosynthesis of Silver Nanoparticles from *Protium serratum* and Investigation of their Potential Impacts on Food Safety and Control

Yugal K. Mohanta[1], Sujogya K. Panda[2], Akshaya K. Bastia[1]* and Tapan K. Mohanta[3]**

[1] Biochemistry Laboratory, Department of Botany, North Orissa University, Baripada, India, [2] Department of Zoology, North Orissa University, Baripada, India, [3] Free Major of Natural Sciences, College of Basic Studies, Yeungnam University, Gyeongsan, South Korea

Silver nanoparticles play an integral part in the evolution of new antimicrobials against the broad ranges of pathogenic microorganisms. Recently, biological synthesis of metal nanoparticles using plant extracts has been successfully consummated. In the present study, the biosynthesis of silver nanoparticles (AgNPs) was conducted using the leaf extract of plant *Protium serratum*, having novel ethnomedicinal. The synthesized AgNPs were characterized using UV-Visible spectroscopy, dynamic light scattering spectroscopy (DLS), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy. The DLS study revealed the surface charge of the resulted nanoparticles that was highly negative, i.e., −25.0 ± 7.84 mV and the size was 74.56 ± 0.46 nm. The phytochemical and FTIR analysis confirmed the role of water-soluble phyto-compounds for the reduction of silver ions to silver nanoparticles. The potential antibacterial activity of AgNPs was studied against the food borne pathogens viz. *Pseudomonas aeruginosa* (IC_{50} = 74.26 ± 0.14 μg/ml), *Escherichia coli* (IC_{50} = 84.28 ± 0.36 μg/ml), *Bacillus subtilis* (IC_{50} = 94.43 ± 0.4236 μg/ml). The *in vitro* antioxidant potential of AgNPs was evaluated using 1, 1-diphenyl-2-picryl-hydrazil (IC_{50} = 6.78 ± 0.15 μg/ml) and hydroxyl radical assay (IC_{50} = 89.58 ± 1.15 μg/ml). In addition, the cytotoxicity of AgNPs was performed against fibroblast cell line L-929 to evaluate their biocompatibility. The overall results of the present investigation displayed the potential use of *P. serratum* leaf extract as a good bio-resource for the biosynthesis of AgNPs and their implementation in diverse applications, specifically as antibacterial agent in food packaging and preservation to combat against various food borne pathogenic bacteria along with its pharmaceutical and biomedical applications.

Keywords: silver nanoparticles, *Protium serratum*, antimicrobial activity, antioxidant capacity, normal fibroblast cell line (L-929), food borne pathogens

OPEN ACCESS

Edited by:
Rosanna Tofalo,
University of Teramo, Italy

Reviewed by:
Soner Soylu,
Mustafa Kemal University, Turkey
Fatih Ozogul,
Çukurova University, Turkey

****Correspondence:***
Yugal K. Mohanta
ykmohanta@gmail.com
Akshaya K. Bastia
bastianou@gmail.com
Tapan K. Mohanta
nostoc.tapan@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *16 February 2017*
Accepted: *28 March 2017*
Published: *18 April 2017*

Citation:
Mohanta YK, Panda SK, Bastia AK and Mohanta TK (2017) Biosynthesis of Silver Nanoparticles from Protium serratum and Investigation of their Potential Impacts on Food Safety and Control. Front. Microbiol. 8:626. doi: 10.3389/fmicb.2017.00626

INTRODUCTION

The phyto-mediated synthesis is a rapid, suitable and most acceptable biosynthetic method for synthesis of metal nanoparticles. Now a days, various plant parts like bark, leaf, fruit, stem and seed extracts have been successfully used for the synthesis of metal nanoparticles (Mittal et al., 2013). Among different metal nanoparticles, silver (Ag) nanoparticles have been used enormously

due to their potential anti-bacterial (Mohanta et al., 2016a,b), anti-fungal and anti-proliferative activity (Kim et al., 2007; Nayak et al., 2015). Due to the excellent antimicrobial properties, the silver (Ag) nanoparticles have been extensively used in food packaging, food and seed preservation, biofertilizers, cosmetics and medicines (Marambio-Jones and Hoek, 2010; Dipankar and Murugan, 2012). Besides these applications, the silver nanoparticles were found to be implemented widely in the field of high sensitive bio-molecular detection, diagnostics, catalysis and micro-electronics (Mohanta and Behera, 2014).

A number of standard approaches by means of physical and chemical methods have been used for the synthesis of silver nanoparticles (AgNPs) viz. reduction in solutions, chemical and photochemical reactions in reverse micelles, thermal decomposition of silver compounds, radiation assisted, electro chemical, sono-chemical, microwave-assisted process, and most recently using green chemistry technology (Song and Kim, 2009). The green synthesis approach provides most advantages over the chemical and physical method as it is cost effective, eco- friendly and easy to scaled up for large-scale synthesis without applying energy, high pressure, temperature and toxic chemicals (Albrecht et al., 2006).

There are several reports on synthesis of green silver nanoparticles (AgNPs) using various plant products. However, there is still a need for economically stable, commercially viable and environmentally clean route to synthesize AgNPs by using new plant sources (Chung et al., 2016). During recent years, the use of plants and plant based product in the synthesis of various metal nanoparticles has been broadly investigated (Arunachalam et al., 2013). A number of ethno medicinal plants are industrially used for production of different herbal medicine as well bioactive compounds for healthcare and nutritional product. Among them, *P. serratum* is widely used throughout the world for its active pharmaceutical compounds curing of different gastrointestinal disease and strong antimicrobial agents. Different parts of the *P. serratum* plant have been effectively utilized as fruit, food and as potential therapeutic agents in traditional medicines (Panda, 2014; Panda et al., 2016). A number of phytocompounds, such as polyphenols, flavonoids, tannin, sugars, alkaloids and triterpenoids/steroids have been reported to be present in the *P. serratum* plant and its various parts including leaves, roots, fruits, seeds and others., which are responsible for potential antioxidant, anti-inflammatory, larvicidal and other medicinal properties (Tanamatayarat, 2016). Thus we used the leaves of *P. serratum* for the synthesis of metal nanoparticles which would be a lucrative, cost-effective and an eco-friendly approach.

Now a day, food spoilage is very common problem throughout the world due to the notorious activity of food borne pathogens (Soylu et al., 2009; Newell et al., 2010; Tajkarimi et al., 2010; Negi, 2012). Moreover, the development of new resistant pathogenic strains of bacteria to current available antibiotics has become a serious threat to the public health, which triggers the immediate development of strong new generation bactericides (Rai et al., 2009). As the food is very indispensable materials to the living beings, it is urgent necessary to think about the food safety, quality and increase the shelf life of it by unraveling new antimicrobials and antioxidant agents. There are many positive influences of AgNPs to be utilized as efficient antimicrobial agents. They are highly competent against a broad range of pathogenic microbes and parasites, with low systemic toxicity toward human (AshaRani et al., 2009; Abbasi et al., 2016). Besides, AgNPs have been proclaimed to be employed and tested for numerous biomedical and industrial applications including avoidance of bacterial colonization and eradication of microbes on different metal and non-metal medical devices, disinfectant agent in wastewater treatment plants, and in silicone rubber gaskets for preserving and transporting food and textile fabrics materials (Patra and Baek, 2017). As silver has long been known to exhibit a strong toxicity to a wide range of microorganisms, it is a great advantage to utilize silver based compound for antimicrobial applications against food borne pathogens as well as antioxidants to maintain the food quality.

The present study reported the biological synthesis of AgNPs using the cell-free aqueous leaf extract of *P. serratum* and evaluation of its potential application as an antibacterial agent against two Gram negative (*Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 739) and two Gram positive (*Bacillus subtilis* MTCC 736, *Staphylococcus aureus* MTCC 2940) food borne pathogenic bacteria along with antioxidant potentials in terms of DPPH and OH radical scavenging activity. Moreover, the cytotoxicity test against L-929 cell line (normal fibroblast) was carried out to evaluate the biocompatibility as well as multifunctionality for potential pharmaceutical and biomedical applications. Usage of these plant materials in the green synthesis of metal nanoparticles could proficiently prove the cost effective approach.

MATERIALS AND METHODS

Collection and Preparation of Plant Extract

Healthy leaves of *P. serratum* were collected from forest of Similipal Biosphere Reserve (21°–28′ and 22°–08′ North latitude and 86°–04′ and 86°–37′ East longitude), Mayurbhanj, Odisha, India during the months of January to March 2015. The identified plant specimen was deposited in the Department of Botany, North Orissa University. The shed dried leaves were powdered and sieved using a 20-mm mesh in order to maintain a uniform size. To make aqueous leaf extract, 5 g of leaf powder was mixed with 50 ml of sterile distilled water and sonicated for 15–20 min. The sonicated aqueous extract was purified by repeated centrifugation. The purified extract was filtered through Whatman filter paper no. 40 and the filtrate was stored at 4°C for further use.

Biosynthesis of Silver Nanoparticles (AgNPs)

For the biosynthesis of silver nanoparticles, the suitable reaction mixture was prepared by adding 1 ml of aqueous leaf extract and 9 ml of 1 mM $AgNO_3$ solution in a clean 25 ml Erlenmeyer flask. On the contrary, same experimental set up of 1 ml of aqueous leaf extracts with 9 ml distilled water was kept as

control. Both flasks were incubated for 2–4 h in the rotary shaker under dark conditions at 25°C. Later, the synthesized silver nanoparticles (AgNPs) were separated and purified by continuous centrifugation (9000 rpm; 20 min; 10°C) with sterile miliQ water. The dried AgNPs were kept at 4°C for further characterization and bioactivity study (Mohanta et al., 2016a).

Characterization of Silver Nanoparticles

The biosynthesis of the silver (Ag) nanoparticles (bio reduction of the Ag^+ ions) in aqueous solution was monitored periodically in UV-Vis spectrophotometer (Lambda 35® PerkinElmer, USA) within the range of 400–600 nm. The UV–visible spectra of the resulting reaction solution was monitored as a function of reaction time at a resolution of 1 nm room temperature (25°C). The average size and surface charge of the silver (Ag) nanoparticles were analyzed by Zetasizer (ZS 90, Malvern, UK). The purified samples were 10-folds diluted with the phosphate buffer saline PBS (0.15M, pH 7.2). The aliquots were later sampled in dynamic light scattering (DLS) cuvettes and examined for equivalent diameters, size distribution and zeta potential. The particle diameters were assessed at scattering angle of 90° at room temperature (25°C). Fourier Transform Infra-Red spectra of the silver (Ag) nanoparticles were studied in FT-IR spectrophotometer (8400S, Shimadzu, Japan) in transmission (%) mode with a 200 scans. The AgNPs were pelletized with potassium bromide (KBr) having 1% sample concentration (w/w) and was analyzed against the background of pure KBr pellet.

The nano-scale size of silver particles were confirmed by analysis of morphological structure under scanning electron microscope (Jeol 6480LV JSM, USA) performed at acceleration voltage of 15 KV (Mohanta and Behera, 2014; Nayak et al., 2015).

Antibacterial Activity against Food Borne Pathogens

Microbial Strains

Common food borne pathogens viz. *B. subtilis* (MTCC 736), *S. aureus* (MTCC 2940), *P. aeruginosa* (MTCC 2453), and *E. coli* (MTCC 739) were used for the tests of antibacterial assay. All strains were procured from Microbial Type Culture Collection, Chandigarh, India.

Agar Well Diffusion and Micro Broth Dilution Methods

A single colony of each bacterial strain was inoculated from an agar slant in 1 mL Muller Hinton broth medium (0.2% beef extract, 0.015% soluble starch and 1.75% casamino acids) under aseptic conditions. The reaction tubes were incubated overnight (200 rpm; 37°C).

The antibacterial activities of AgNPs were investigated against bacterial species using well diffusion method on Muller Hinton Agar. To test the antibacterial activity, Muller Hinton Broth culture (100 μl) of each test organisms were seeded over the Muller Hinton Agar plates. Wells were made of approximately 5 mm in diameter and 2.5 mm deep. Each well was filled with 50 μl of AgNPs. Simultaneously, 50 μl of $AgNO_3$ solution was kept to serve as control while standard antibiotic Gentamicin was used as a reference. The plates were incubated at 37°C for 24 h. After the incubation period, the diameter of the growth inhibition zones was measured. The AgNPs with the zone of inhibition greater or equal to 8-mm diameter were regarded as the positive activity.

Further, the confirmatory antibacterial activity was observed through micro broth dilution method along with calculation of the minimum inhibitory concentration (MIC) of AgNPs on bacterial strains (Panda et al., 2016). The percentage of inhibition more than 90% in micro broth dilution method was considered as potential activity and further experiments were conducted to calculate the MIC. Briefly, for MIC calculation, the test inoculum (190 μL; $A_{600} = 0.1$) with different concentrations of AgNPs (10 μL) ranges from 500 to 31.25 μg/ml (twofold dilution) were taken until the percentage of inhibition was found to be <50%. The micro broth dilution study was conducted in 96-well plates and the microbial growth or inhibition was measured in Microplate Reader (Biorad, USA) at 600 nm. The MIC was calculated by IC_{50}/IC_{90} Laboratory Excel Calculation Tools and expressed as IC_{50}. All the experiments were conducted in triplicates and the zone and percentage of inhibitions were expressed in mean ± SD.

Qualitative Phytochemical Analysis

The qualitative phytochemical analysis of *P. serratum* extract was performed following the standard method (Parekh and Chanda, 2008; Arunachalam et al., 2012). The obtained results were qualitatively expressed as positive (+) or negative (−) Guruvaiah et al., 2012). The chemicals and reagents used for the study were purchased from Sigma–Aldrich (India).

Quantitative Phytochemical Analysis and *In vitro* Antioxidant Properties

Total Phenolic Content Determination

Total phenolic quantity in the leaf extract was measured using Folin–Ciocalteu method with slight modifications (McDonald et al., 2001). All the experiments were performed in triplicates. The TPC was expressed as gallic acid equivalent (GAE) in mg/g sample.

Total Flavonoids Content Determination

Total amount of flavonoids were estimated by a modified aluminum chloride method (Chang et al., 2002). All estimations were carried out in triplicate. The TFC was expressed as GAE in mg/g sample.

DPPH Radical Scavenging Activity

Potential antioxidant activity was determined using 1, 1-diphenyl-2-picryl-hydrazil (DPPH) assay with sufficient modification wherever it seemed necessary (McDonald et al., 2001). Various concentrations, such as 5, 10, 15, and 20 μg/ml of AgNPs were taken for study of DPPH scavenging capacity. The MIC was calculated and results were presented IC50 value. The results were expressed as percentage (%) radical scavenging activity. The equivalent concentrations of ascorbic acid were taken as a positive control.

Hydroxyl Radical Scavenging Activity

The method was adapted with slight modification as reported by Tanamatayarat (2016). Fifty percent of the inhibitory concentration (IC_{50}) was calculated from the percentage of scavenging capacity. Ascorbic acid was taken as a positive control. Different concentrations such as 20, 40, 60, 80, 100, 120, and 140 μg/ml of AgNPs and Ascorbic acid were taken for OH scavenging capacity and MIC determination.

Biocompatibility Study

The biocompatibility of AgNPs was evaluated by calculating % of viability of cells by treating AgNPs on L-929 normal fibroblast cell line. The L-929 cells were seeded in flask with Dulbecco's Modified Eagle's Medium (DMEM) and M-199 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C (5% CO_2) for 24 h. Following the incubation period, the attached cells were trypsinized for 3–5 min to get the individual cells and centrifuged (800 rpm, 10 min.). The cells were counted and distributed in 96 well Enzyme-linked immunosorbent assays (ELISA) plate with 5000 cells in each well and incubated for 24 h to form ∼70 to 80% confluence as a monolayer (Nayak et al., 2015). The AgNPs have the capacity to strongly reduce the Adenosine Triphosphate (ATP) content of the cell which ultimately cause mitochondrial damage and increase the production of reactive oxygen species (ROS) in a dose-dependent manner (Nayak et al., 2016). Hence the toxicity of AgNPs was determined at different concentrations ranges from 100 to 700 μg/ml in triplicates. To detect the cell viability, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution 200 μl was added to each well and left for incubation (4–5 h). Later, the MTT solution was discarded and 200 μl of DMSO solvent was added to each well under dark followed by 15–20 min. of incubation and later the optical density (OD) of the formazan product was measured at 595 nm in a micro-triter plate reader (Biorad, USA) (AshaRani et al., 2009). The media, antibiotics and other chemicals used in these experiments were purchased from Sigma–Aldrich (India).

Statistical Analysis

Each activity assay was performed in triplicates in order to determine their reproducibility. The antioxidant results were expressed as percentage of inhibition whereas the cytotoxicity results were represented as percentage of viability with respect to control values. The values of antioxidant and cytotoxicity assays results were compared by Student's *t*-test with their control values. The antibacterial data were subjected to analysis of one way ANOVA and Duncan's Multiple Range Test using the SPSS statistics program (IBM SPSS statistics 19). A significant difference was considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Biosynthesis and UV-vis Spectra Analysis of AgNPs

The UV–vis spectroscopy is an indirect method to examine the bioreduction of Ag nanoparticles from aqueous $AgNO_3$ solution. Initially 9 ml of 1mM $AgNO_3$ solution was taken for the bioreduction of silver by aqueous leaf extract. Two hours post-addition of leaf extract to the $AgNO_3$ solution, a visible color change was observed from pale yellow to dark brown. The intensity of the color increased with increase in incubation time due to the excitation of surface plasmon vibrations in the metal nanoparticles (Jain et al., 2007). The AgNPs synthesized by *P. serratum* extract exhibited characteristic peak at 432 nm. Previous studies reported that the silver ions give absorption in between 430 and 440 nm due to its surface plasmon resonance (Chung et al., 2016). The AgNPs from *P. serratum* extract has shown peak at 432 nm which confirms the biosynthesis Ag nanoparticles (**Figure 1**). In the present study, the Ag nanoparticles was observed to be very stable in the solution, even after 6 months of their synthesis, which strongly validates the use of aqueous leaf extract of *P. serratum* in synthesis of AgNPs. The *P. serratum* leaf is rich in flavonoids, sugar, phenolic compounds, tannins and terpenoids, which contribute to its distinct aroma (Tanamatayarat, 2016). The terpenoids were believed to play an important role in biosynthesis of AgNPs through the reduction of Ag ions to its elemental form. Shankar et al. (2003) reported about the possible role of terpenoids from *Geranium* leaf in the synthesis of nano-sized Ag particles (Shankar et al., 2003). Polyols such as terpenoids, flavones and polysaccharides in the *Cinnamomum camphora* leaf were reported to be the main cause of the bioreduction of silver and chloroaurate ions (Huang et al., 2007). A similar mechanism might have operated in the present case as well where the flavonoids and phenolic compounds extracted from *P. serratum* leaf might have act as capping and stabilizing agents. To summarize these results, the water-soluble fractions comprised of complex polyols (Sharma et al., 2009) in the biomass were believed to have played a major role in the bioreduction of Ag ions.

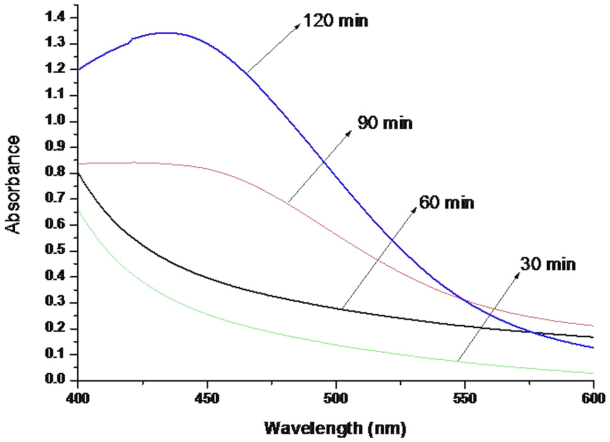


FIGURE 1 | UV-Vis spectra of AgNPs synthesized by *P. serratum*.

DLS Analysis

The size distribution and surface charge of the AgNPs were determined using DLS in aqueous solution. It was found that

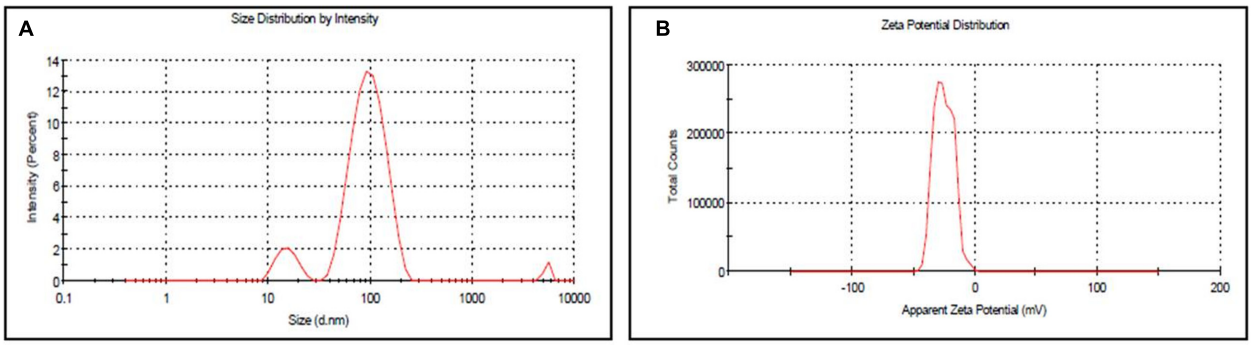


FIGURE 2 | (A) Size distribution of synthesized AgNPs **(B)** Zeta potential of synthesized AgNPs by DLS analysis.

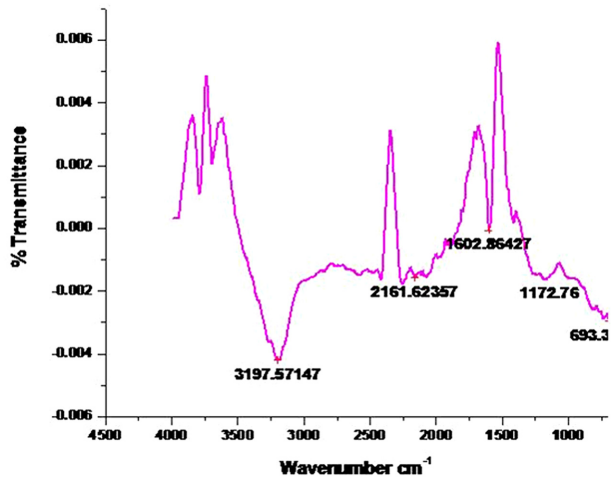


FIGURE 3 | Fourier transform infrared spectroscopy result analysis of AgNPs synthesized by *P. serratum leaf*. *X*-axis represents the spectra (cm^{-1}) and *Y*-axis represents percent (%) transmission.

the average size and charge of the AgNPs were 74.56 ± 0.46 nm and −25.0 ± 7.84 mV, respectively (**Figures 2A,B**). The average size and potential contribute a strong characteristic of AgNPs to be used in biomedical sciences. The size of the particle is very important in cellular transportation. Smaller the size, it is easier to pass through the plasma membrane of the cell. So the nano size particle <100 nm was considered to be useful particles for different applications in drug delivery as well as in development of biosensors (Mukherjee et al., 2014). Besides the size of the AgNPs, the surface charge of the nanoparticles was considered to be important for interaction with different macromolecules as well as biochemical pathways present in the cell (Nayak et al., 2015).

FTIR Spectroscopic Analysis

The FTIR spectra of the AgNPs was recorded in order to identify the functional groups of the biomolecules present in the aqueous extract of *P. serratum* leaf involved in the synthesis and stabilization of the nanoparticles. The interaction of nanoparticles with phytochemicals of *P. serratum* showed intense peaks at 3197.57; 2161.62; 1602.86; 1172.76 and 693.3 cm^{-1} (**Figure 3**). A strong absorption peak was found at 3197.57 cm^{-1} strongly suggested the binding of silver ion with hydroxyl group and the broad spectrum at 2161.62 cm^{-1} was referred as the strong stretching of -OH group. The other three bands ∼1602.86 cm^{-1}, ∼1172.76, and ∼693.3 cm^{-1} were due to stretching vibrations of $C = O$, C-C, C-N and O-H functional group, respectively. The $C = O$ and C-N stretching are generally found in the proteins involve in the reduction of the metal ions. The observations suggested that the hydroxyl and carbonyl groups might be responsible for the synthesis of AgNPs.

Overall, it can be concluded that the *P. serratum* proteins adsorb as a layer over the green-synthesized silver nanoparticles, which stabilized them. It is well known that the proteins can bind to the silver nanoparticles via free amine groups or through cysteine residues in the saponins, phenolic and quinones from *P. serratum*, thereby stabilizing the nanoparticles formed through the surface-bound proteins. The present result is strongly supported by previous reports as well (Pant et al., 2012; Saranyaadevi et al., 2014).

SEM Study

The morphology along with spherical shape and monodispersity nature of the synthesized nanoparticles capped with its biomoities were confirmed by the SEM micrograph (**Figure 4**). Almost all nanoparticles were irregularly spherical with smooth edge. The shape of the nanoparticles had profound impact during the conjugation with specific drug molecules and target to the cells (Dauthal and Mukhopadhyay, 2016).

Antibacterial Activity of Silver Nanoparticles

Preliminary screening of antibacterial activity was evaluated by agar well diffusion method against four pathogenic bacteria reported in **Table 1**. In agar cup method, zone of inhibition was found against Gram positive bacteria *B. subtilis* and Gram-negative bacteria and *E. coli,* and *P. aeruginosa* (**Figure 5**) while no zone of inhibition was found against *S. aureus*.

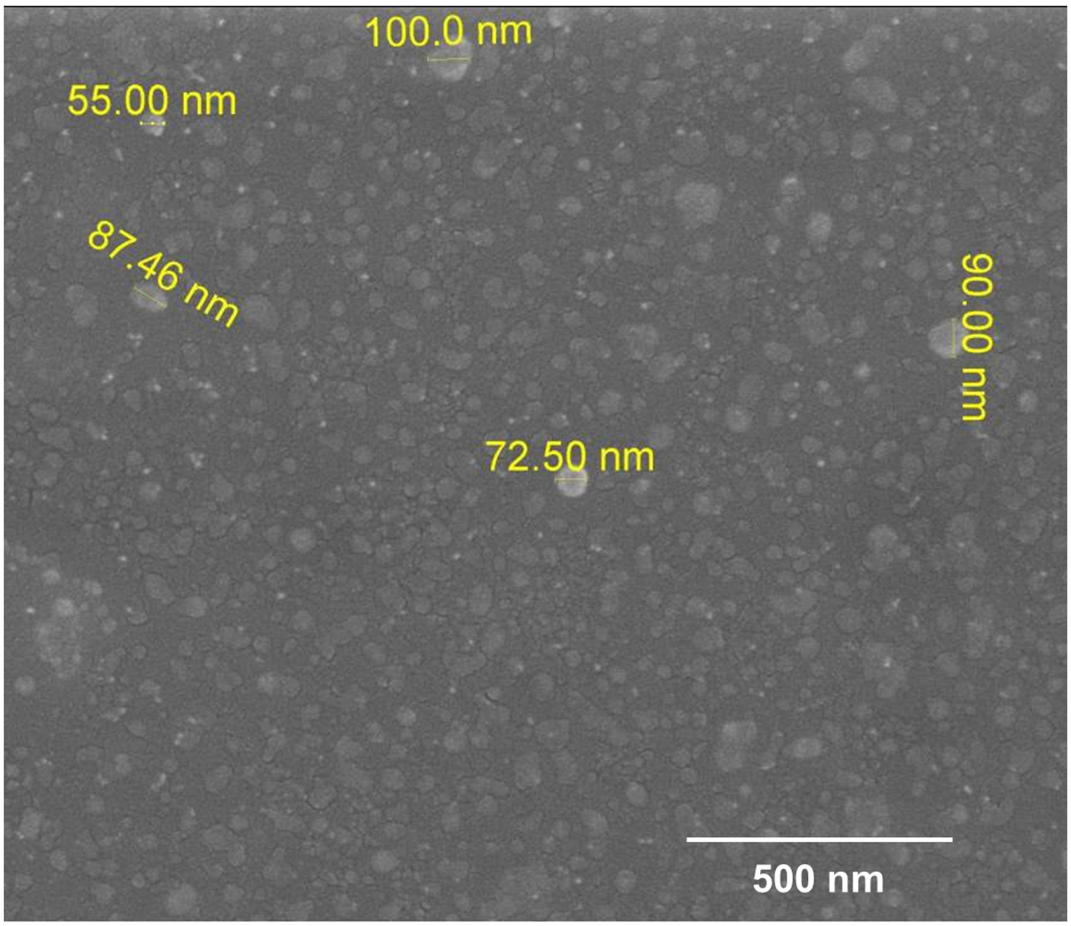


FIGURE 4 | Scanning electron microscopy image of biosynthesized AgNPs.

TABLE 1 | Antimicrobial activity of AgNPs by agar-well diffusion method.

	Mean zone of inhibition ± SD (in mm)		
Name of the test strain	***Silver nanoparticles (500 μg/mL)***	***Gentamicin (10 μg/mL)***	***Silver nitrate (500 μg/mL)***
Bacillus subtilis	13 ± 0.50	20.8 ± 0.59	0
Staphylococcus aureus	0	16.3 ± 0.22	0
Escherichia coli	13 ± 1.00	13.13 ± 0.13	0
Pseudomonas aeruginosa	22 ± 1.15	15.3 ± 0.22	0

The micro broth dilution assay was followed to verify the antibacterial activity of AgNPs and the percentage (%) of inhibition and MIC of each strain is reported in **Table 2** (Supplementary Data Sheet 1). All the three strains showed growth inhibition above 99%. The MIC was calculated in terms of IC_{50} value and found to be effective against *P. aeruginosa* (74.26 ± 0.14 μg/ml), *E. coli* (84.28 ± 0.36 μg/ml) and *B. subtilis* (94.43 ± 0.42 μg/ml) (**Table 2**). The specific mechanism of nanoparticle-mediated antibacterial activity is not clearly understood till date. However, various probable mechanisms were explained in the literature regarding the antibacterial effect of nanoparticles. Sondi and Salopek-Sondi (2004) proposed that nanoparticles penetrate the cell wall of the bacteria due to their anchoring ability which ultimately responsible for the structural changes of the membrane and finally force to cell death (Sondi and Salopek-Sondi, 2004). There are several probable prospective mechanisms are exists for the decisive antibacterial activity of AgNPs which comprise the enzyme degradation, inactivation of major cellular proteins and impairment of genetic materials (Guzman et al., 2009, 2012; Patra and Baek, 2017). Numerous bacterial enzymes are inactivated due to firm interaction of Ag ions, released from silver nanoparticles with –SH groups which is the major structural part of the enzyme

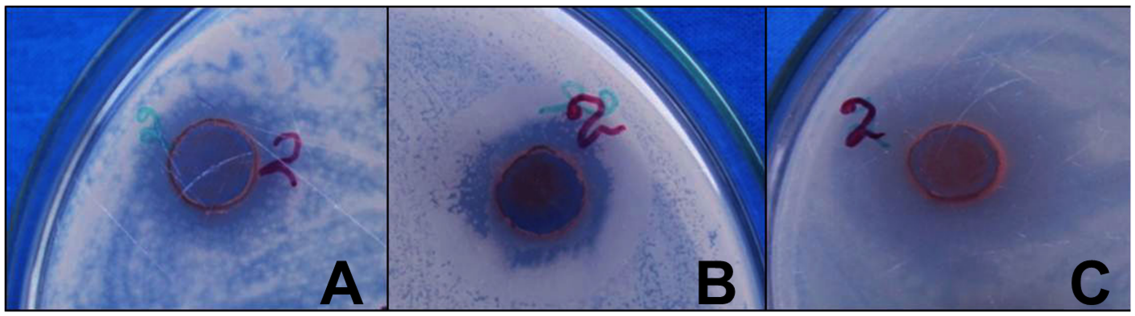


FIGURE 5 | Antibacterial activity of AgNPs synthesized by *P. serratum* against (A) *B. subtilis* **(B)** *E. coli* **(C)** *P. aeruginosa* (where 2 = PS-AgNPs).

TABLE 2 | Antimicrobial activity of AgNPs by micro broth dilution method.

Antibacterial activity of AgNPs [Percentage of inhibition (%) ± SD]						
Name of the test strain	**500 μg/ml**	**250 μg/ml**	**125 μg/ml**	**62.5 μg/ml**	**31.2 μg/ml**	**IC_{50} (μg/ml)**
Bacillus subtilis	99.6 ± 0.16^{a}	99.4 ± 0.04^{a}	60.5 ± 0.70^{b}	34.5 ± 0.61^{c}	1.67 ± 0.47^{d}	94.43 ± 0.42
Escherichia coli	99.3 ± 0.08^{a}	99.4 ± 0.08^{a}	69.87 ± 0.33^{b}	34.9 ± 0.04^{c}	3.0 ± 0.81^{d}	84.28 ± 0.36
Pseudomonas aeruginosa	99.3 ± 0.08^{a}	99.3 ± 0.16^{a}	65.1 ± 0.08^{b}	45.0 ± 0.08^{c}	5.6 ± 2.1^{d}	74.26 ± 0.14

The data are expressed as a percentage inhibition of bacteria and represent the mean ± SD (n = 3). Antimicrobial activity: exponentially growing cells were treated with different concentrations of AgNPs for 24 h and cell growth inhibition was analyzed through broth dilution assay. In each row, mean values followed with different superscripts significantly differ from each other according to Duncan's Multiple Range Test ($P < 0.05$). IC_{50} is defined as the concentration, which results in a 50% reduction in cell numbers as compared with that of the control cultures (AgNPs). The values represent the mean ± SD of three individual observations.

conformation (Yousefzadi et al., 2014; Swamy et al., 2015). Frequent interaction of AgNPs with the sulfur and phosphorus groups, intrude in the DNA replication and subsequently disintegrate microbial system (Singh et al., 2014; Ramesh et al., 2015). However, continuous in depth research is highly needed to prove the exact mechanisms about the antibacterial activity of nanoparticles. Such antibacterial properties signify AgNPs as possible candidate for the pharmaceutical industries in advancement of contemporary antimicrobial products. Moreover, the AgNPs could be convenient for formulating the polymeric materials for food packaging and other useful durable antimicrobial proof materials.

Qualitative and Quantitative Assessment of Phytochemicals and Corresponding Anti-oxidative Activities

Qualitative and quantitative phytochemical examinations of the aqueous leaf extracts has summarized in **Tables 3**, **4**. The phytochemical analysis revealed the existence of flavonoids, tannins, phenolic, sugars and triterpenoids whereas glycoside, steroids and sterols were found to be absent. The phytochemical study of the leaf extract of *P. serratum* showed that flavonoids, tannins, phenolic compounds, sugars were present in the extract which may be the principal chemicals constituents responsible for the synthesis of AgNPs. Shankar et al. (2003) reported the possible of role of terpenoids from *Geranium* leaf in the synthesis of nano-sized Ag particles (Shankar et al., 2003). Polyols such as terpenoids, flavones and polysaccharides in the *C. camphora* leaf were reported to be the main cause of the bioreduction of silver and chloroaurate ions (Huang et al., 2007).

The current work does not report the presence of glycosides, steroids and sterols which might be an outcome of selective qualitative test performed, and/or extraction procedures. As long as the hypothetical mechanism of AgNPs' biosynthesis is concerned, cascades of complex antioxidant enzymes might be involved in the biosynthesis of Ag nanoparticles (Prasad, 2014).

Antioxidant potential result for *P. serratum* exhibits a positive response toward the possible involvement of antioxidant molecules from the leaf extract during the biogenic synthesis

TABLE 3 | Qualitative phytochemical screening of aqueous extract of *P. serratum*.

Name of the phytoconstituents	**Observation**
Alkaloids	–
Tannins and phenolic compounds	+++
Glycoside	–
Flavonoids	+++
Steroids and sterols	–
Triterpenoids	+
Sugars	+++

"+++", Highly present; "+", Less present; '–', Absent.

TABLE 4 | Quantitative phytochemical constituents of aqueous extract of *P. serratum*.

Phytochemical constituent	**mg/100 g dry weight (Mean ± SD)**
TPC	530.57 ± 26.00
TFC	810.76 ± 25.10

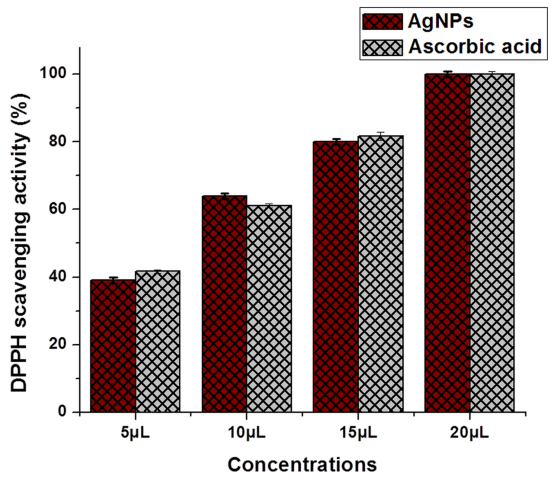


FIGURE 6 | 1, 1-diphenyl-2-picryl-hydrazil radical scavenging assay of AgNPs. The data are presented in the form of a bar graph and plotted using mean ± SD of individual replicates (n = 3). The P-value for significantly different mean, $P > 0.05$ versus positive control (ascorbic acid).

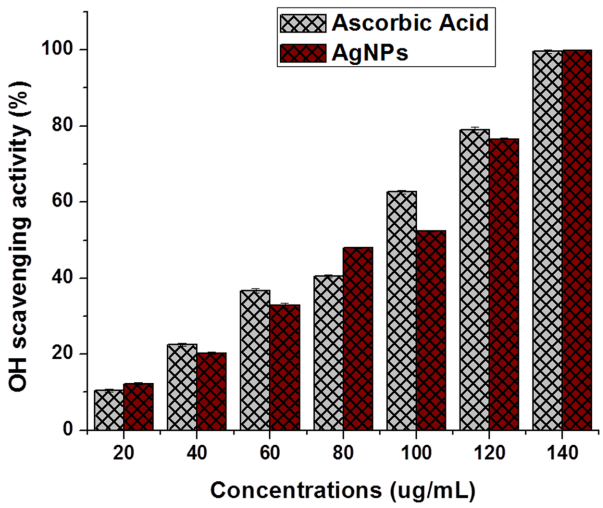


FIGURE 7 | Hydroxyl radical scavenging assay of AgNPs. The data are presented in the form of a bar graph and plotted using mean ± SD of individual replicates (n = 3). The P-values for significantly different mean values, $P > 0.05$ versus positive control (ascorbic acid).

of AgNPs. It is known that, the plants have a large collection of phenolics and flavonoids which might have possess super anti-oxidative capabilities and considered strong free radical scavengers. Significant anti-oxidant activity was also observed by DPPH and hydroxyl radical scavenging assays (**Figures 6**, **7**). The antioxidant capacity was found to be due to DPPH scavenging activity (IC_{50} = 6.78 ± 0.15 µg/ml) and hydroxyl radical assay (IC_{50} = 89.58 ± 1.15 µg/ml). The presence of moderate concentration of total phenolics and flavonoids in *P. serratum* leaves indicated a notable anti-oxidant activity. The high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging activity of bioactive compounds (Hagerman et al., 1998). Recently, Pratap Chandran et al. (2013) reported *in vitro* anti-oxidant potential of methanolic and aqueous extracts of *A. solanacea* Roxb. leaf through DPPH radical scavenging assay that strongly supports our present result. Luximon-Ramma et al. (2002) investigated the entire phenolic, proanthocyanidin, flavonoids and the anti-oxidant activities of vegetative and reproductive parts (Luximon-Ramma et al., 2002). The anti-oxidant activities were highly correlated with total phenolic levels. However, the result showed that the anti-oxidant activities of reproductive parts surpassed the anti-oxidant activities of the vegetative organs, including the pods that have the highest total phenolic and flavonoid contents (Abdel-Aziz et al., 2014). Related findings were also reported in the present experiments.

It is also essential to evaluate the anti-oxidant potential as some of the plant molecules are still remain with Ag nanoparticles after purification as a capping agent, which should not be harmful to cells during cellular application of the nanoparticles. Thus, the anti-oxidant potential of *P. serratum* established the green synthesis process of silver nanoparticles to be highly safe for biological applications.

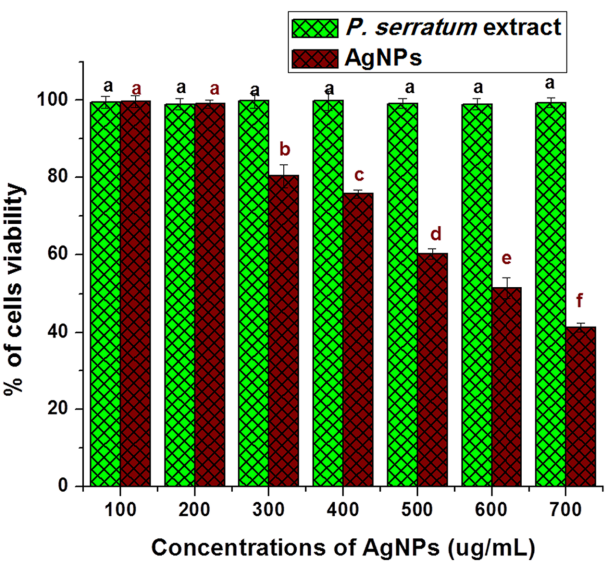


FIGURE 8 | Biocompatibility activity study of AgNPs on normal fibroblast cell line (L-929). The data are expressed as a percentage viability of cells and represent the mean ± SD (n = 3). In all individual experiments, control (without treatment) was taken as 100% viable. Bars with different superscripts significantly differ from each other ($P < 0.05$). Bars with the same superscripts ($P > 0.05$) are not significantly differing from each other.

Cytotoxic Activity/Biocompatibility Study

It is very pivotal to understand the biocompatibility of AgNPs for its successful implication in biomedicine and its direct use by human beings as food additives. The cytotoxicity of AgNPs was also tested against normal fibroblast cell lines L-929 to check their biocompatibility. The safety use of AgNPs is a major concern

along with toxicity against normal cell lines which can impact on the biological applications. In the present study, AgNPs have not been observed of inhibition against L-929 cell line at lower concentrations. The percentage of cell viability of normal fibroblast cells is declined with an increase in concentration of AgNPs (**Figure 8**). The IC_{50} value of AgNPs against normal L-929cell lines was calculated as 600.28 ± 0.75 μg/mL. The IC_{50} value indicates the high biological compatibility and safe use of AgNPs in human body. The plant extract did not show any toxicity against L-929cell line and proved its potential use in synthesis of AgNPs. The AgNPs were also previously studied for its biocompatibility against Chinese hamster ovary (CHO) cell line (Netala et al., 2016). The study of Netala et al. (2016) corroborated with our present findings. In fact, the AgNPs should be thoroughly studied for its safety and biocompatibility before its practical application as product.

CONCLUSION

Silver nanoparticles exhibited enormous antibacterial potency against three food borne pathogens. Such positive results highly recommend that AgNPs can be used in food packaging materials and also as disinfectant and cleaning agents. Further, the antioxidant activity of AgNPs revealed the protection from oxidation due to external factors as well as radical activity. The AgNPs were also very much stable and biocompatible to the human cell lines. Ethno-medicinal report suggests that *P. serratum* extract is not harmful to the human body and oral administration of its leaf extract is highly effective against gastrointestinal disorders and also stomach ulcer (Panda et al., 2016). Hence the present research highlights the potential involvement of nanoscience in food industry.

AUTHOR CONTRIBUTIONS

YM carried out all the experiment and wrote the manuscript. SP edited the manuscript. AB revised the manuscript. TM English editing and revised the manuscript.

ACKNOWLEDGMENT

North Orissa University is highly acknowledged for providing research facility.

REFERENCES

Abbasi, E., Milani, M., Aval, S. F., Kouhi, M., Akbarzadeh, A., Nasrabadi, H. T., et al. (2016). Silver nanoparticles: synthesis methods, bio- applications and properties. *Crit. Rev. Microbiol.* 42, 173–180. doi: 10.3109/1040841X.2014.912200

Abdel-Aziz, M. S., Shaheen, M. S., El-Nekeety, A. A., and Abdel-Wahhab, M. A. (2014). Antioxidant and antibacterial activity of silver nanoparticles biosynthesized using *Chenopodium murale* leaf extract. *J. Saudi Chem. Soc.* 18, 356–363. doi: 10.1016/j.jscs.2013.09.011

Albrecht, M. A., Evans, C. W., and Raston, C. L. (2006). Green chemistry and the health implications of nanoparticles. *Green Chem.* 8, 417–432.

Arunachalam, K. D., Annamalai, S. K., and Hari, S. (2013). One-step green synthesis and characterization of leaf extract-mediated biocompatible silver and gold nanoparticles from *Memecylon umbellatum*. *Int. J. Nanomed.* 8, 1307–1315. doi: 10.2147/IJN.S36670

Arunachalam, K. D., Suhashani, S., and Sathesh, K. A. (2012). Wound healing and Antigenotoxic activities of *Aegle marmelos* with relation to its antioxidant properties. *J. Pharm. Res.* 5, 1492–1502.

AshaRani, P., Low Kah Mun, G., Hande, M., and Valiyaveettil, S. (2009). Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS Nano* 3, 279–290. doi: 10.1021/nn800596w

Chang, C.-C., Yang, M.-H., Wen, H.-M., and Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* 10, 178–182.

Chung, I.-M., Park, I., Seung-Hyun, K., Thiruvengadam, M., and Rajakumar, G. (2016). Plant-mediated synthesis of silver nanoparticles: their characteristic properties and therapeutic applications. *Nanoscale Res. Lett.* 11, 40. doi: 10.1186/s11671-016-1257-4

Dauthal, P., and Mukhopadhyay, M. (2016). Noble metal nanoparticles?: plant-mediated synthesis, mechanistic aspects of synthesis, and applications. *Ind. Eng. Chem. Res.* 55, 9557–9577. doi: 10.1021/acs.iecr.6b00861

Dipankar, C., and Murugan, S. (2012). The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from *Iresine herbstii* leaf aqueous extracts. *Colloids Surf. B Biointerfaces* 98, 112–119. doi: 10.1016/j.colsurfb.2012.04.006

Guruvaiah, P., Arunachalam, A., and Velan, L. P. T. (2012). Evaluation of phytochemical constituents and antioxidant activities of successive solvent extracts of leaves of *Indigofera caerulea* Roxb using various in vitro antioxidant assay systems. *Asian Pac. J. Trop. Dis.* 2, S118–S123. doi: 10.1016/S2222-1808(12)60136-4

Guzman, M., Dille, J., and Godet, S. (2009). Antibacterial activity of silver nanoparticles prepared by a chemical reduction method. *Int. J. Chem. Biomol. Eng.* 2, 104–111. doi: 10.1007/s11814-010-0067-0

Guzman, M., Dille, J., and Godet, S. (2012). Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. *Nanomed. Nanotechnol. Biol. Med.* 8, 37–45. doi: 10.1016/j.nano.2011.05.007

Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W., et al. (1998). High molecular weight plant polyphenolics (Tannins) as biological antioxidants. *J. Agric. Food Chem.* 46, 1887–1892. doi: 10.1021/jf970975b

Huang, J., Li, Q., Sun, D., Lu, Y., Su, Y., Yang, X., et al. (2007). Biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaf. *Nanotechnology* 18:105104.

Jain, P. K., Huang, X., El-Sayed, I. H., and El-Sayed, M. A. (2007). Review of some interesting surface plasmon resonance-enhanced properties of noble metal nanoparticles and their applications to biosystems. *Plasmonics* 2, 107–118.

Kim, J. S., Kuk, E., Yu, K. N., Kim, J.-H., Park, S. J., Lee, H. J., et al. (2007). Antimicrobial effects of silver nanoparticles. *Nanomed. Nanotechnol. Biol. Med.* 3, 95–101. doi: 10.1016/j.nano.2006.12.001

Luximon-Ramma, A., Bahorun, T., Soobrattee, M. A., and Aruoma, O. I. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassia fistula*. *J. Agric. Food Chem.* 50, 5042–5047. doi: 10.1021/jf0201172

Marambio-Jones, C., and Hoek, E. M. V. (2010). A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *J. Nanopart. Res.* 12, 1531–1551. doi: 10.1007/s11051-010-9900-y

McDonald, S., Prenzler, P. D., Antolovich, M., and Robards, K. (2001). Phenolic content and antioxidant activity of olive extracts. *Food Chem.* 73, 73–84. doi: 10.1016/S0308-8146(00)00288-0

Mittal, A. K., Chisti, Y., and Banerjee, U. C. (2013). Synthesis of metallic nanoparticles using plant extracts. *Biotechnol. Adv.* 31, 346–356. doi: 10.1016/j.biotechadv.2013.01.003

Mohanta, Y., Panda, S., Biswas, K., Tamang, A., Bandyopadhyay, J., De, D., et al. (2016a). Biogenic synthesis of silver nanoparticles from *Cassia fistula* (Linn.):

in vitro assessment of their antioxidant, antimicrobial and cytotoxic activities. *IET Nanobiotechnol.* 10, 438–444. doi: 10.1049/iet-nbt.2015.0104

Mohanta, Y., Singdevsachan, S., Parida, U., Panda, S., Mohanta, T., and Bae, H. (2016b). Green synthesis and antimicrobial activity of silver nanoparticles using wild medicinal mushroom *Ganoderma applanatum* (Pers.) pat. from similipal biosphere reserve. Odisha, India. *IET Nanobiotechnol.* 10, 184–189. doi: 10.1049/iet

Mohanta, Y. K., and Behera, S. K. (2014). Biosynthesis, characterization and antimicrobial activity of silver nanoparticles by *Streptomyces* sp. SS2. *Bioprocess Biosyst. Eng.* 37, 2263–2269. doi: 10.1007/s00449-014-1205-6

Mukherjee, S., Chowdhury, D., Kotcherlakota, R., Patra, S., and Vinothkumar, B. (2014). Potential theranostics application of bio-synthesized silver nanoparticles (4-in- 1 System). *Theranostics* 4, 316–335. doi: 10.7150/thno.7819

Nayak, D., Ashe, S., Rauta, P. R., Kumari, M., and Nayak, B. (2016). Bark extract mediated green synthesis of silver nanoparticles: evaluation of antimicrobial activity and antiproliferative response against osteosarcoma. *Mater. Sci. Eng. C Mater. Biol. Appl.* 58, 44–52. doi: 10.1016/j.msec.2015.08.022

Nayak, D., Pradhan, S., Ashe, S., Rauta, P. R., and Nayak, B. (2015). Biologically synthesised silver nanoparticles from three diverse family of plant extracts and their anticancer activity against epidermoid A431 carcinoma. *J. Colloid Interface Sci.* 457, 329–338. doi: 10.1016/j.jcis.2015.07.012

Negi, P. (2012). Plant extracts for the control of bacterial growth: efficacy stability and safety issues for food application. *Int. J. Food Microbiol.* 156, 7–17. doi: 10.1016/j.ijfoodmicro.2012.03.006

Netala, V. R., Bethu, M. S., Pushpalatha, B., Baki, V. B., Aishwarya, S., and Rao, J. V. (2016). Biogenesis of silver nanoparticles using endophytic fungus *Pestalotiopsis microspora* and evaluation of their antioxidant and anticancer activities. *Int. J. Nanomed.* 11, 5683–5696. doi: 10.2147/IJN.S112857

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., et al. (2010). Food-borne diseases - The challenges of 20years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* 139, S3–S15. doi: 10.1016/j.ijfoodmicro.2010.01.021

Panda, S. K. (2014). Ethno-medicinal uses and screening of plants for antibacterial activity from similipal biosphere reserve. Odisha, India. *J. Ethnopharmacol.* 151, 158–175. doi: 10.1016/j.jep.2013.10.004

Panda, S. K., Mohanta, Y. K., Padhi, L., Park, Y. H., Mohanta, T. K., and Bae, H. (2016). Large scale screening of ethnomedicinal plants for identification of potential antibacterial compounds. *Molecules* 21, 1–20. doi: 10.3390/molecules21030293

Pant, G., Nayak, N., and Prasuna, G. (2012). Enhancement of antidandruff activity of shampoo by biosynthesized silver nanoparticles from *Solanum trilobatum* plant leaf. *Appl. Nanosci.* 3, 431–439. doi: 10.1007/s13204-012-0164-y

Parekh, J., and Chanda, S. V. (2008). In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants. *Turk. J. Biotechnol.* 31, 53–58.

Patra, J. K., and Baek, K.-H. (2017). Antibacterial activity and synergistic antibacterial potential of biosynthesized silver nanoparticles against foodborne pathogenic bacteria along with its anticandidal and antioxidant effects. *Front. Microbiol.* 8:167. doi: 10.3389/fmicb.2017.00167

Prasad, R. (2014). Synthesis of silver nanoparticles in photosynthetic plants. *J. Nanopart.* 2014, 1–8. doi: 10.1155/2014/963961

Pratap Chandran, R., Manju, S., Vysakhi, M. V., Shaji, P. K., and Achuthan Nair, G. (2013). In vitro antioxidant potential of methanolic and aqueous extracts of *Ardisia solanacea* Roxb. leaf. *J. Pharm. Res.* 6, 555–558. doi: 10.1016/j.jopr.2013.04.038

Rai, M., Yadav, A., and Gade, A. (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnol. Adv.* 27, 76–83. doi: 10.1016/j.biotechadv.2008.09.002

Ramesh, P., Kokila, T., and Geetha, D. (2015). Plant mediated green synthesis and antibacterial activity of silver nanoparticles using *Emblica officinalis* fruit extract. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 142, 339–343. doi: 10.1016/j.saa.2015.01.062

Saranyaadevi, K., Subha, V., Ravindran, R. S. E., and Renganathan, S. (2014). Green synthesis and characterization of silver nanoparticle using leaf extract of *Capparis zeylanica*. *Asian J. Pharm. Clin. Res.* 7, 44–48.

Shankar, S. S., Ahmad, A., and Sastry, M. (2003). Geranium leaf assisted biosynthesis of silver nanoparticles. *Biotechnol. Prog.* 19, 1627–1631. doi: 10.1021/bp034070w

Sharma, V. K., Yngard, R. A., and Lin, Y. (2009). Silver nanoparticles: green synthesis and their antimicrobial activities. *Adv. Colloid Interface Sci.* 145, 83–96. doi: 10.1016/j.cis.2008.09.002

Singh, K., Panghal, M., Kadyan, S., Chaudhary, U., and Yadav, J. (2014). Antibacterial activity of synthesized silver nanoparticles from *Tinospora cordifolia* against multi drug resistant strains of *Pseudomonas aeruginosa* isolated from burn patients. *J. Nanomed. Nanotechnol.* 5:192.

Sondi, I., and Salopek-Sondi, B. (2004). Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *J. Colloid Interface Sci.* 275, 177–182. doi: 10.1016/j.jcis.2004.02.012

Song, J. Y., and Kim, B. S. (2009). Rapid biological synthesis of silver nanoparticles using plant leaf extracts. *Bioprocess Biosyst. Eng.* 32, 79–84. doi: 10.1007/s00449-008-0224-6

Soylu, S., Soylu, E. M., and Evrendilek, G. A. (2009). Chemical compositions and antibacterial activities of bitter fennel *Foeniculum vulgare* Mill var vulgare and dill *Anethum graveolens* L essential oils against the growth of food borne and seed borne plant pathogenic bacteria. *Ital. J. Food Sci.* 21, 347–355.

Swamy, M., Mohanty, S., Jayanta, K., and Subbanarasiman, B. (2015). The green synthesis, characterization, and evaluation of the biological activities of silver nanoparticles synthesized from *Leptadenia reticulata* leaf extract. *Appl. Nanosci.* 5, 73–81. doi: 10.1007/s13204-014-0293-6

Tajkarimi, M. M., Ibrahim, S. A., and Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control* 21, 1199–1218. doi: 10.1016/j.foodcont.2010.02.003

Tanamatayarat, P. (2016). Antityrosinase, antioxidative activities, and brine shrimp lethality of ethanolic extracts from *Protium searratum* (Wall. ex Colebr.) Engl. *Asian Pac. J. Trop. Biomed.* 6, 1050–1055. doi: 10.1016/j.apjtb.2016.10.001

Yousefzadi, M., Rahimi, Z., and Ghafori, V. (2014). The green synthesis, characterization and antimicrobial activities of silver nanoparticles synthesized from green alga *Enteromorpha flexuosa* (wulfen). J. Agardh. *Mater. Lett.* 137, 1–4. doi: 10.1016/j.matlet.2014.08.110

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Mohanta, Panda, Bastia and Mohanta. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

OPINION
published: 24 May 2017
doi: 10.3389/fmicb.2017.00930

A Highlight for Non-*Escherichia coli* and Non-*Salmonella* sp. *Enterobacteriaceae* in Dairy Foods Contamination

Angelo M. B. Amorim[1,2] *and Janaína dos Santos Nascimento*[2]*

[1] *Laboratory of Microbiology, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rio de Janeiro, Brazil,*
[2] *Department of Quality Control, Instituto de Tecnologia em Imunobiológicos Bio-Manguinhos, Fiocruz, Rio de Janeiro, Brazil*

Keywords: enterobacteriaceae, dairy foods, multidrug resistance, virulence factors, biofilm formation, antimicrobial substances, health of consumers, enterobacter

OPEN ACCESS

Edited by:
Maria Schirone,
University of Teramo, Italy

Reviewed by:
Mirella Luciani,
Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Italy
Hongxia Wang,
University of Alabama at Birmingham, United States

***Correspondence:**
Janaína dos Santos Nascimento
janaina.nascimento@ifrj.edu.br

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 December 2016*
Accepted: *08 May 2017*
Published: *24 May 2017*

Citation:
Amorim AMB and Nascimento JS (2017) A Highlight for Non-Escherichia coli and Non-Salmonella sp. Enterobacteriaceae in Dairy Foods Contamination. Front. Microbiol. 8:930. doi: 10.3389/fmicb.2017.00930

BACKGROUND

Dairy products are the base of the diet of many families in all social classes worldwide. However, the high content of milk nutrients and their derivatives, in addition to the near neutral pH and high water activity, provide an ideal environment for the growth of many microorganisms in dairy products (Oladipo and Omo-Adua, 2011). Moreover, during its manufacturing, processing, storage, distribution, and marketing, dairy products may be subject to inadequate hygiene conditions, which can promote spoilage and contamination with pathogenic microorganisms, including *Enterobacteriaceae* (Cusato et al., 2013; Freitas et al., 2013).

Several studies have reported the contamination of dairy products by *Salmonella* sp. and *Escherichia coli*; however, few studies have described the presence of *Enterobacter*, *Klebsiella*, and *Serratia* species, particularly with regard to the presence of these genera in foods and milk products. In addition, although most studies performed with these bacteria have focused on quantification and identification, some studies have also explored antimicrobial resistance (Samaržija et al., 2012; Zhang et al., 2015) and other virulence factors, such as biofilm (Cherif-Antar et al., 2016), proteolytic enzymes (Chove et al., 2013), and lipolytic enzymes (Masiello et al., 2016).

Cheese is a major dairy product that can be manufactured using nonpasteurized milk; this process is often carried out at farms and small establishments, increasing the potential for contamination by microorganisms, including *Enterobacteriaceae* (Zhang et al., 2015). Even in products subjected to the pasteurization process, the absence of contaminants is not guaranteed because of possible flaws in the process or after pasteurization. In Brazil, the most commonly consumed fresh cheese, "Minas frescal," has undergone changes in its composition, particularly after campaigns pushing for sodium reduction. However, such changes in composition can exacerbate problems with contamination because sodium is effective in controlling some pathogenic and deteriorative microorganisms, including *Enterobacteriaceae* family members, directly affecting the shelf life of the product and potentially altering its rheological and sensory features (Doyle and Glass, 2010; Cruz et al., 2011; Damaceno et al., 2015).

Contamination in dairy products is not restricted to fresh products. Infant milk formulas and milk powder, which are subjected to various moisture removal processes, have also been reported to be contaminated with representatives of this family. Isolates of *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter cloacae*, and other members of the *E. cloacae* complex have been found in these foods (Oonaka et al., 2010; Sani and Yi, 2011; Yao et al., 2012).

RESISTANCE TO ANTIBIOTICS

Antibiotics are often used indiscriminately in animals with the goals of prevention and treatment of clinical diseases as well as enhancing growth and development (Fleming et al., 2010; Bosco et al., 2012; Murphy et al., 2016). This routine may be affecting various aspects of the food industry, since antibiotic-resistant microorganisms from animals can be disseminated in different food products (Rolain, 2013).

The use of antibiotics targeting animal growth is still controversial; some researchers argue that consumption of products derived from these animals is not a risk to human health, whereas others have questioned the possibility that bacterial strains with resistance genes may be transmitted by food derived from these animals. For example, Marshall and Levy (2011) reported a study on people working on a farm at which antibiotics were used for animal development; these workers harbored resistant bacteria in their intestinal microbiota, and the same bacterial profile was observed in the animals at the farm.

Notably, such gene transfer can occur, even in the lumen of human and animal intestines, influencing the evolution of newly pathogenic strains (Grotiuz et al., 2006). In addition, milk is considered an excellent culture medium for gene transfer by conjugation and has been reported to have an efficiency that is 10 times higher than that of laboratory culture medium (Verraes et al., 2013).

Some studies have described the occurrence of multidrug resistance (MDR) in members of the *Enterobacteriaceae* family, isolated from dairy products, which do not belong to the species *E. coli* and *S. enterica,* considered classical foodborne pathogens. For example, Chauhan et al. (2013) described the isolation of MDR *K. pneumoniae* from raw milk samples. MDR isolates from the genera *Enterobacter*, *Citrobacter*, and *Klebsiella*, all of which showed resistance to imipenem, were also described by Fakruddin et al. (2014) in different food samples, including milk powder. More recently, our group has isolated MDR *Enterobacter* spp. from "Minas frescal" cheese and pasteurized milk (Damaceno et al., 2015; Amorim, 2016).

Additionally, studies have shown that *E. cloacae* strains are often isolated from dairy products. However, studies of the virulence factors of these strains and their MDR potential are still scarce.

Among the factors related to acquisition of antimicrobial resistance by representatives of the *Enterobacteriaceae* family, the factor that is most concerning to the scientific community is the ability of these bacteria to produce extended spectrum β-lactamase (ESBL; Thenmozhi et al., 2014; Tekiner and Özpinar, 2016). Inhibitors of ESBL are widely used for the treatment of bacterial infections, particularly for gram-negative bacteria. Therefore, ESBL production can confer resistance to many classes of antibiotics.

The *Enterobacteriaceae* family, which are the greatest producers of ESBL, includes *K. pneumoniae* and *E. coli* strains; these strains have high clinical relevance (Munoz-Price and Weinstein, 2008; Saito et al., 2010) and are unrelated to *Acinetobacter* spp. (*Moraxellaceae* family).

Foods with certain characteristics may facilitate the spread of ESBL bacteria. For example, Calbo et al. (2011) described the transmission of an ESBL-producing *K. pneumoniae* strain by food consumption at a health facility; after obtaining negative results for surfaces and professionals in the ward, they determined that the propagation occurred via food, affecting 14% of the food handlers and 35% of hospital kitchen surfaces.

However, the results of various studies have been controversial. Some studies have shown that milk is not a good disseminator of ESBL-producing bacteria, whereas other studies have indicated that milk can facilitate the dissemination of these bacteria. Moreover, most studies showing that ESBL-producing bacteria were present in milk were performed in developing countries, as India, Brazil, and Indonesia, and the results of these studies contrasted with those of studies in developed countries, such as Switzerland (Chauhan et al., 2013; Dahmen et al., 2013; Sudarwanto et al., 2015; Amorim, 2016).

OTHER VIRULENCE FACTORS

Biofilm Production

Biofilm formation in the dairy industry can occur within a few hours after processing (Mogha et al., 2014). Milk, obviously a major component of dairy products, has characteristics that may promote or present biofilm production on surfaces. Its composition is rich in lipids, proteins, and certain divalent cations, e.g., calcium, which favors the formation of biofilm (Teh et al., 2014; Flint et al., 2015).

Some studies have reported the presence of *Enterobacteriaceae* biofilm producers associated with industrial dairy production plants. Cherif-Antar et al. (2016) found distinct gram-negative bacteria, including *K. pneumoniae*, *Serratia marcescens*, and *Enterobacter* spp., attached to the stainless steel surfaces of pipes of a milk processing plant.

Notably, these bacteria can be resistant to cleaning products, as described by Malek et al. (2012), who collected samples from farms producing pasteurized milk and skimmed milk powder in Algeria. The entire production line was constantly subjected to sanitization using ammonia- and peracetic acid-based products. Despite this attempt at sanitization, representatives of the *Enterobacter* sp. were still found.

Proteolytic and Lipolytic Activity

Enterobacteriaceae capable of synthesizing proteolytic and lipolytic enzymes are largely responsible for the deterioration of milk and dairy products, which may cause various issues in the dairy industry (Zajác et al., 2015; Masiello et al., 2016).

In cheese production, for example, these enzymes destabilize casein micelles and may modify or even prevent the coagulation of milk, what can directly affect the formation of the product (Caldera et al., 2015). Another major problem is that these bacteria can cause off-flavor, i.e., can considerably affect the sensory properties of the foods, such as color, odor, flavor, and texture (Böhme et al., 2013; Caldera et al., 2015). Such changes can directly affect the acceptance or rejection of the product by the consumer.

Bacterial synthesis of lipolytic enzymes has also shown to be important for the food industry due the direct influence of these enzymes on sensory properties, particularly flavor and texture. Lipolysis may lead to a process called hydrolytic rancidity, wherein the product develops a sour taste and an unpleasant odor (Carpiné et al., 2010; Krewinkel et al., 2016).

Recently, Masiello et al. (2016) isolated lipolytic representatives of the *Enterobacteriaceae* family (genera *Serratia*, *Enterobacter*, and *Raoutella*) from pasteurized milk samples. The authors indicated that diverse bacteria found in pasteurized milk, exhibiting phenotypic characteristics such as production of lipolytic and proteolytic enzymes, can result in milk spoilage.

PRODUCTION OF ANTIMICROBIAL SUBSTANCES

Some pathogenic bacteria, such as representatives of the *Enterobacteriaceae* family, are capable of producing biologically active compounds known as antimicrobials, acting in a competition niche against its competitors.

These compounds can be purified and used by the food industry as tools to protect against bacteria that cause deterioration in their products, thus increasing their shelf life (Verraes et al., 2013; Damaceno et al., 2015) and maintenance of product characteristics, since they are bactericidal or bacteriostatic without altering the sensory properties of food. These substances have shown to be essential for the food industry since antibiotics cannot be used in foods (Fleming et al., 2010).

Bacteriocins are major antimicrobial substance produced by bacteria; when they are produced by commensal bacteria in the intestinal tract of animals, they may have an important role in elimination of MDR microorganisms, without major changes in intestinal flora (Kommineni et al., 2015), since they are degraded by enzymes of the digestive system and have probiotic properties (Rosa et al., 2016).

In a previous work carried out by our research group, two representatives of *Enterobacter* sp. and nine other representatives of the *Enterobacteriaceae* family were found to produce antimicrobial substances against strains *E. coli* and *S. enterica* used as indicators (Damaceno et al., 2015). These two species of bacteria are among the major causes of foodborne illnesses, and this previous work suggested that classical *Enterobacteriaceae* pathogens can be inhibited by other representatives of the same family, which could justify their absence or low levels in some dairy foods.

CONCLUSIONS

Dairy products are potential vehicles for microorganisms from the *Enterobacteriaceae* family, which can exhibit MDR to available antimicrobials, reduced susceptibility phenotypes to carbepenens (KPC), and ESBL production and produce biofilm, proteolytic enzymes, lipolytic enzymes, and antimicrobial substances, providing advantages for the bacteria in a competitive niche. All these factors represent potential risks to the health of consumers of dairy products, particularly immunocompromised consumers.

In the supply chain of dairy products, including all stages (e.g., production lines, transport, and storage), good manufacturing practices and hygiene, as well as best practices in commercialization, must be followed mainly for products consumed without any prior processing. Additionally, the absence of classic pathogens, such as *Salmonella* sp. and *E. coli*, does not indicate that the product is fit for consumption, since other potentially pathogenic bacteria of the same family may be present in the food. Thus, testing for *Enterobacteriaceae*, including species that are not yet assessed according to regulator standards, may offer a better view of the quality, sanitary conditions, and safety of dairy foods.

AUTHOR CONTRIBUTIONS

AA and JN wrote this article.

ACKNOWLEDGMENTS

This research was supported by grants from Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro (IFRJ), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

REFERENCES

Amorim, A. M. B. (2016). *Avaliação da Multirresistência a Drogas e da Produção de Outros Fatores de Virulência por Isolados de Enterobacteriaceae Provenientes de Produtos Lácteos*. Master's thesis, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rio de Janeiro.

Böhme, K., Fernández-No, I. C., Barros-Velázquez, J., Gallardo, J. M., Calo-Mata, P., and Cañas, B. (2013). Species identification of food spoilage and pathogenic bacteria by MALDI-TOF mass fingerprinting. *J. Prot. Res.* 9, 3169–3183. doi: 10.1021/pr100047q

Bosco, K. J., Kaddu-Mulindwa, D. H., and Asiimwe, B. B. (2012). Antimicrobial drug resistance and plasmid profiles of Salmonella isolates from humans and foods of animal origin in Uganda. *Adv. Infect. Dis.* 2, 151–155. doi: 10.4236/aid.2012.24025

Calbo, E., Freixas, N., Xercavins, M., Riera, M., Nicolás, C., Monistrol, O., et al. (2011). Foodborne nosocomial outbreak of SHV1 and CTX-M-15–producing *Klebsiella pneumoniae*: epidemiology and control. *Clin. Infect. Dis.* 52, 743–749. doi: 10.1093/cid/ciq238

Caldera, L., Arioli, S., Stuknyte, M., Scarpellini, M., and Franzetti, L. (2015). Setup of a rapid method to distinguish among dead, alive, and viable but not cultivable cells of *Pseudomonas* spp. in mozzarella cheese. *J. Dairy Sci.* 98, 8368–8374. doi: 10.3168/jds.2015-9677

Carpiné, D., Dagostin, J. L. A., Santa, H. S. D., Alvarez, D. C., Terra, N. N., and Santa, O. R. D. (2010). Atividade proteolítica e lipolítica de bactérias lácticas isoladas de salames artesanais. *Ambiência* 6, 125–132.

Chauhan, S., Farooq, U., Singh, V., and Kumar, A. (2013). Determination of prevalence and antibacterial activity of ESBL (Extended Spectrum Beta-lactamases) producing *Klebsiella* species isolated from raw milk of Doon Valley in India. *Int. J. Pharm. Biol. Sci.* 4, 417–423.

Cherif-Antar, A., Moussa-Boudjemâa, B., Didouh, S., Medjahdi, K., Mayo, B., and Flórez, A. B. (2016). Diversity and biofilm-forming capability of bacteria recovered from stainless steel pipes of a milk-processing dairy plant. *Dairy Sci. Technol.* 96, 27–38. doi: 10.1007/s13594-015-0235-4

Chove, L. M., Issa-Zacharia, A., Grandison, A. S., and Lewis, M. J. (2013). Proteolysis of milk heated at high temperatures by native enzymes analysed by trinitrobenzene sulphonic acid (TNBS) method. *Afr. J. Food Sci.* 7, 232–237. doi: 10.5897/AJFS13.0987

Cruz, A. G., Faria, J. A. F., Pollonio, M. A. R., Bolini, H. M. A., Celeghini, R. M. S., Granato, D., et al. (2011). Cheeses with reduced sodium content: effects on functionality, public health benefits and sensory properties. *Trends Food Sci. Tech.* 22, 276–291. doi: 10.1016/j.tifs.2011.02.003

Cusato, S., Gameiro, A. H., Corassin, C. H., Sant'ana, A. S., Cruz, A. G., Faria, J. A., et al. (2013). Food safety systems in a small dairy factory: implementation, major challenges, and assessment of systems performances. *Foodborne Pathog. Dis.* 10, 6–12. doi: 10.1089/fpd.2012.1286

Dahmen, S., Métayer, V., Gay, E., Madec, J.-Y., and Haenni, M. (2013). Characterization of extended-spectrum beta-lactamase (ESBL)-carrying plasmids and clones of Enterobacteriaceae causing cattle mastitis in France. *Vet. Microbiol.* 162, 793–799. doi: 10.1016/j.vetmic.2012.10.015

Damaceno, H. F. B., Freitas Junior, C. V., Marinho, I. L., Cupertino, T. R., Costa, L. E. O., and Nascimento, J. S. (2015). Antibiotic resistance versus antimicrobial substances production by gram-negative foodborne pathogens isolated from minas frescal cheese: heads or tails? *Foodborne Pathog. Dis.* 12, 297–301. doi: 10.1089/fpd.2014.1876

Doyle, M. E., and Glass, K. A. (2010). Sodium reduction and its effect on food safety, food quality, and human health. *Comp. Rev. Food Sci. Food Safety* 9, 44–56. doi: 10.1111/j.1541-4337.2009.00096.x

Fakruddin, Md., Rahaman, M., Ahmed, M. M., and Hoque, M. (2014). Antimicrobial resistance and virulence factors of Enterobacteriaceae isolated from food samples of Bangladesh. *Int. J. Microbiol. Immunol. Res.* 3, 12–18.

Fleming, L. R., Bolzan, D. N., and Nascimento, J. S. (2010). Antimicrobial substances produced by coliform strains active against foodborne pathogens. *Foodborne Pathog. Dis.* 7, 243–247. doi: 10.1089/fpd.2009.0333

Flint, S., Jamaludin, N. M., Somerton, B., Palmer, J., and Brooks, J. (2015). "The effect of milk composition on the development of biofilms," in *Biofilms in the Dairy Industry*, eds K. H. Teh, S. Flint, J. Brooks, and G. Knight (Chichester: John Wiley & Sons), 36–48. doi: 10.1002/9781118876282.ch3

Freitas, R., Brito, M. A., Nero, L. A., and De Carvalho, A. F. (2013). Microbiological safety of Minas Frescal Cheese (MFC) and tracking the contamination of *Escherichia coli* and *Staphylococcus aureus* in MFC processing. *Foodborne Pathog. Dis.* 10, 951–955. doi: 10.1089/fpd.2013.1525

Grotiuz, G., Sirok, A., Gadea, P., Varela, G., and Schelotto, F. (2006). Shiga toxin 2-producing Acinetobacter haemolyticus associated with a case of bloody diarrhea. *J. Clin. Microbiol.* 44, 3838–3841. doi: 10.1128/JCM.00407-06

Kommineni, S., Bretl, D. J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., et al. (2015). Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526, 719–722. doi: 10.1038/nature15524

Krewinkel, M., Baur, C., Kranz, B., Von Neubeck, M., Wenning, M., Scherer, S., et al. (2016). A sensitive and robust method for direct determination of lipolytic activity in natural milk environment. *Food Anal. Method* 9, 646–655. doi: 10.1007/s12161-015-0233-4

Malek, F., Moussa-Boudjemâa, B., Khaouani-Yousfi, F., Kalai, A., and Kihel, M. (2012). Microflora of biofilm on Algerian dairy processing lines: an approach to improve microbial quality of pasteurized milk. *Afr. J. Microbiol. Res.* 6, 3836–3844. doi: 10.5897/AJMR11.1120

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/CMR.00002-11

Masiello, S. N., Martin, N. H., Trmčić, A., Wiedmann, M., and Boor, K. J. (2016). Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk. *J. Dairy Sci.* 99, 130–140. doi: 10.3168/jds.2015-9728

Mogha, K. V., Shah, N. P., Prajapati, J. B., and Chaudhari, A. R. (2014). Biofilm - A threat to dairy industry. *Indian J. Dairy Sci.* 67:6.

Munoz-Price, L. S., and Weinstein, R. A. (2008). Acinetobacter infection. *N.Engl. J. Med.* 358, 1271–1281. doi: 10.1056/NEJMra070741

Murphy, C. P., Fajt, V. R., Scott, H. M., Foster, M. J., Wickwire, P., and McEwen, S. A. (2016). Scoping review to identify potential non-antimicrobial interventions to mitigate antimicrobial resistance in commensal enteric bacteria in North American cattle production systems. *Epidemiol. Infect.* 144, 1–18. doi: 10.1017/S0950268815000722

Oladipo, I. C., and Omo-Adua, R. O. (2011). Antibiotics resistance among bacteria isolated from evaporated milk. *Asian J. Biol. Sci.* 4, 77–83. doi: 10.3923/ajbs.2011.77.83

Oonaka, K., Furuhata, K., Hara, M., and Fukuyama, M. (2010). Powder infant formula milk contaminated with *Enterobacter sakazakii*. *Jpn. J. Infect. Dis.* 63, 103–107.

Rolain, J. (2013). Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Front. Microbiol.* 4:173. doi: 10.3389/fmicb.2013.00173

Rosa, L. J. B., Esper, L. M. R., Cabral, J. P. L. G., Franco, R. M., and Cortez, M. A. S. (2016). Viability of probiotic micro-organism *Lactobacillus acidophilus* in dairy chocolate dessert and its action against foodborne pathogens. *Ciênc. Rural* 46, 368–374. doi: 10.1590/0103-8478cr20141864

Saito, R., Koyano, S., Nagai, R., Okamura, N., and Koike, K. (2010). Evaluation of a Chromogenic medium for extended-spectrum beta-lactamase-producing Enterobacteriaceae. *Lett. Appl. Microbiol.* 51, 704–706. doi: 10.1111/j.1472-765X.2010.02945.x

Samaržija, D., Zamberlin, S., and Pogačić, T. (2012). Psychrotrophic bacteria and their negative effects on milk and dairy products quality. *Mljekarstvo* 62, 77–95.

Sani, N. A., and Yi, L. Y. (2011). Enterobacteriaceae, Cronobacter (Enterobacter) sakazakii and microbial population in infant formula products in the Malaysian market. *Sains Malays.* 40, 345–351.

Sudarwanto, M., Akinedem, O., Odenthal, S., Gross, M., and Usleber, E. (2015). Extended-spectrum β-lactamase (ESBL)–producing *Klebsiella pneumoniae* in bulk tank milk from dairy farms in Indonesia. *Foodborne Pathog. Dis.* 12, 585–590. doi: 10.1089/fpd.2014.1895

Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., and Lindsay, D. (2014). Biofilm — An unrecognised source of spoilage enzymes in dairy products? *Int. Dairy J.* 34, 32–40. doi: 10.1016/j.idairyj.2013.07.002

Tekiner, I. H., and Özpinar, H. (2016). Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin. *Braz. J. Microbiol.* 47, 444–451. doi: 10.1016/j.bjm.2015.11.034

Thenmozhi, S., Moorthy, K., Sureshkumar, B. T., and Suresh, M. (2014). Antibiotic resistance mechanism of ESBL producing Enterobacteriaceae in clinical field: a review. *Int. J. Pure App. Biosci.* 2, 207–226.

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., et al. (2013). Antimicrobial resistance in the food chain: a review. *Int. J. Environ. Res. Public Health* 10, 2643–2669. doi: 10.3390/ijerph10072643

Yao, K., Zinzendorf, N. Y., Bohoua, G., Kouassi, K. A., Koua, A., Kouadio, L. K., et al. (2012). Occurrence of *Enterobacter sakazakii* (Cronobacter) and Other Enterobacteriaceae in commercial powdered infant formula in Abidjan, Ivory Coast. *Food Nutr. Sci.* 3, 822–226. doi: 10.4236/fns.2012.36110

Zajác, P., Čapla, J., Vietoris, V., Zubrická, S., and Čurlej, J. (2015). Effects of storage on the major constituents of raw milk. *Potravinarstvo* 9, 375–381. doi: 10.5219/518

Zhang, R., Huo, W., Zhu, W., and Mao, S. (2015). Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. *J. Sci. Food Agric.* 95, 1072–1079. doi: 10.1002/jsfa.6800

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Amorim and Nascimento. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 June 2017
doi: 10.3389/fmicb.2017.01030

Detection of Mycobacteria by Culture and DNA-Based Methods in Animal-Derived Food Products Purchased at Spanish Supermarkets

Iker A. Sevilla[1], Elena Molina[1], Maitane Tello[1], Natalia Elguezabal[1], Ramón A. Juste[2] and Joseba M. Garrido[1]*

[1] Animal Health Department, NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Bizkaia Science and Technology Park 812L, Derio, Spain, [2] SERIDA-Servicio Regional de Investigación y Desarrollo Agrario, Carretera de Oviedo, Villaviciosa, Spain

Mycobacteria include obligate and opportunistic pathogens that cause significant human and animal disease. The burden of tuberculosis has been largely reduced in developed territories but remains a huge problem worldwide. The significance of nontuberculous mycobacteria is growing considerably, especially in developed regions with higher life expectancy and more therapy-related immunosuppressed individuals. Due to their robustness mycobacteria can contaminate animal products by direct transmission from infected individuals or by environmental contamination during processing. The situation at market level is poorly known. Most studies analyzing commercially available foods are limited to a small or local scale and mainly focused on a particular mycobacterial species. There is a need to investigate if animal products that have passed the established controls to be for sale at main supermarkets could represent a route of contact with any mycobacteria. Thus, our goal was to study the prevalence of mycobacteria in these foods to assess if this could represent a source of human exposure. Five stores from the main supermarket chains in Spain were selected. 138 dairy and 119 meat products were purchased. All were processed using culture and multiplex real-time PCR methods. Additional molecular methods were used to specifically identify any positive result. *Mycobacterium avium* subsp. *hominissuis* (2), *M. avium* subsp. *avium* (1), and *M. fortuitum* (1) were isolated from powdered infant formula and ground beef, chicken sausage, and mortadella cold cut, respectively. Mycobacterial DNA (*M. avium*, *M. tuberculosis* complex and other nontuberculous mycobacteria) was detected in 15% of dairy products and 2% of meat products. These results show that the prevalence of viable mycobacteria in foods of animal origin obtained at the supermarket was not substantial although a considerable proportion of them contained mycobacterial DNA. Contact with mycobacteria through this route could be ensured over time. Further investigation is necessary to determine the real impact of foodborne mycobacterial exposure on human health and identify critical points in the food production system to enable setting up more stringent control measures.

Keywords: nontuberculous mycobacteria, *Mycobacterium tuberculosis* complex, dairy products, meat products, food contamination, prevalence

OPEN ACCESS

Edited by:
Giovanna Suzzi,
University of Teramo, Italy

Reviewed by:
Benjamin Michael Connor Swift,
University of Nottingham,
United Kingdom
Nigel Cook,
Jorvik Food and Environmental Virology Ltd., United Kingdom
Iva Slana,
Veterinary Research Institute, Czechia

****Correspondence:***
Iker A. Sevilla
isevilla@neiker.eus

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *07 March 2017*
Accepted: *23 May 2017*
Published: *09 June 2017*

Citation:
Sevilla IA, Molina E, Tello M, Elguezabal N, Juste RA and Garrido JM (2017) Detection of Mycobacteria by Culture and DNA-Based Methods in Animal-Derived Food Products Purchased at Spanish Supermarkets. Front. Microbiol. 8:1030. doi: 10.3389/fmicb.2017.01030

INTRODUCTION

The genus *Mycobacterium* includes important obligate and opportunistic infectious microorganisms that cause human and animal disease. *M. tuberculosis* complex together with *M. avium* complex and other nontuberculous mycobacteria (NTM) like *M. kansasii*, *M. malmoense*, *M. chelonae-abscessus* group, and *M. fortuitum* complex stand out among the rest of mycobacterial species in terms of medical and veterinary significance (Biet and Boschiroli, 2014; Falkinham, 2016). *M. tuberculosis* complex encompasses the etiological agents of human and zoonotic animal tuberculosis (TB), diseases of great health and socioeconomic impact as indicated in annual reports of the World Health Organization and manuals of the World Organization for Animal Health. *M. avium* subspecies are responsible for paratuberculosis (PTB), avian TB, infections in swine and other domestic and wild species, and cervical lymphadenitis, respiratory disease and disseminated or focal infections in humans (Biet and Boschiroli, 2014; Falkinham, 2016). *M. kansasii* is responsible for human disseminated infections and lung disease almost identical to typical TB (Griffith et al., 2007) and is commonly found in lymph node and tissue lesions in cattle in some regions (Biet and Boschiroli, 2014). The incidence of some ubiquitous fast growing NTM species (e.g., *M. fortuitum* complex and *M. chelonae-abscessus*) considered opportunistic pathogens for humans (Griffith et al., 2007) and animals (Bercovier and Vincent, 2001; Biet and Boschiroli, 2014) that originate diverse infections, has increased notably during the last decades (García-Martos and García-Agudo, 2012). In regions with developed health care resources the number of individuals susceptible to mycobacterial infection, especially with NTM, is growing due to the aging of the population, the maintenance of natural immune deficiencies and chronic diseases and the use of immunosuppressive therapies (Lake et al., 2016). Many mycobacterial infections originate from airborne exposure. However, other routes are also important, including the oral route.

Reports on foodborne (excluding water) mycobacterial transmission are mainly limited to zoonotic TB cases derived from the consumption of untreated or not properly treated *M. bovis*-contaminated foods. In developed territories infection through this route is not as frequent as in developing ones as a consequence of milk treatment and tuberculosis eradication programs (Pérez-Lago et al., 2014). But other mycobacterial infections can be contracted through oral exposure apart from TB. NTM cervical lymphadenitis affects mainly infants and there is circumstantial evidence indicating that infection originates from oral exposure (Kasperbauer and Huitt, 2013), most likely from water (Falkinham, 2003), and thus contaminated drinks and foods should not be discarded as possible vehicles. In most cases of NTM disseminated infection mycobacteria are introduced through the lungs or the digestive tract (Kasperbauer and Huitt, 2013). An association between NTM lung infection and gastroesophageal disorders has been previously recognized, being aspiration of contaminated water a probable means of bacterial introduction (Thomson et al., 2013). An etiologic role has been attributed to *M. avium* subsp. *paratuberculosis* in Crohn's disease but it remains controversial (Sechi and Dow, 2015). If this link is finally demonstrated, consumption of contaminated milk and dairy products could represent the most likely source of *M. avium* subsp. *paratuberculosis* (Grant, 2010). In ruminants the oral route is the main route of entry of this bacterium as is the case of *M. avium* subsp. *avium* in avian TB (Tell et al., 2001), and thus it could also represent a means of contact for humans.

The term environmental for NTM is determined by their presence in water, pipe systems, soil, dust and other ecosystems (Falkinham, 2016). Tuberculous mycobacteria and also NTM can contaminate foods of animal origin passing directly from infected individuals (including also their fluids and feces) to the product, but environmental contamination during the processing and storage is also possible (Grant, 2010; Ghodbane et al., 2014; Klanicova-Zalewska and Slana, 2014). In addition, as a result of their robustness mycobacteria can resist adverse conditions and survive to some of the disinfection or decontamination procedures applied to foods and drinks. In terms of microbiological safety and hygiene the presence of mycobacteria in foods represents a potential biological hazard that should be prevented. There is a lack of information with reference to supermarket foods that have passed the established controls. Many studies report on the detection of mycobacteria in animal tissues and raw milk and thus support that foods of animal origin may act as vehicles of mycobacteria transmission to humans (Kaneene et al., 2014; Klanicova-Zalewska and Slana, 2014; Pérez-Lago et al., 2014; Waddell et al., 2016). Several surveys have found mycobacterial DNA and viable cells in commercially available foodstuffs but the number of such reports is limited and most of them are mainly focused on the detection of *M. avium* subspecies or *M. bovis* (Grant, 2010; Klanicova et al., 2011; Faria et al., 2014; Lorencova et al., 2014; Pereira-Suarez et al., 2014; Savi et al., 2015; Cezar et al., 2016). According to the bibliography, animal products in which mycobacteria have been identified include raw and pasteurized milk, milk powder, powdered infant milk formula, cheese, meat, ground meat, fermented meat, ham, salami and sausages obtained from the main livestock species. Under these circumstances and taking into account the apparent increasing vulnerability of developed populations to some mycobacterial infections, we centered our attention on retail animal products available at the supermarket to determine whether they could represent a source of contact with mycobacteria for humans. For this purpose, processed and raw dairy and meat products purchased at different supermarkets were screened for the presence of members of the genus *Mycobacterium* using culture and PCR-based methods.

MATERIALS AND METHODS

Dairy and Meat Products

Five stores belonging to 5 of the main supermarket chains operating in Spain were selected. A total of 138 dairy and 119 meat products of different types and brands were collected at two sampling times. Samples covered top-selling brands along with own-label brands. Dairy foodstuffs included milk, milk powder, powdered infant formula, cream, butter, cheese and yogurt, and meat products consisted of packed ground meat,

hamburger patties, fresh and cooked sausages, cold cuts, fresh Spanish chorizo and pâté. Most products were collected at both sampling times, thus two different batches of the same brand could be analyzed. All of them are frequently consumed products produced, processed, bottled or packed in Spain (89.9%), France (6.6%), Ireland (0.8%), Italy (0.4%), Germany (0.8%), Netherlands (0.8%), and Switzerland (0.8%). The number and type of products analyzed is summarized in **Table 1**. Brand names are not indicated to preserve the anonymity of the survey.

Culture

All samples were cultured in Lowenstein-Jensen with pyruvate (Difco, Francisco Soria Melguizo SA, Madrid, Spain), Coletsos (Difco), modified Middlebrook 7H10 (Becton, Dickinson and Company, MD, USA) with egg yolk and mycobactin J (IDvet, Grabels, France) prepared as previously described (Whittington et al., 2011) and in BBL Mycobacteria growth indicator tubes (MGIT) supplemented with BACTEC MGIT growth supplement and BBL MGIT PANTA (Becton, Dickinson and Company). Cultures on solid media were incubated at 37°C for 6 months and periodically inspected for the presence of colonies. MGIT cultures were incubated for 3 months in a BACTEC MGIT 960 System and time to detection (TTD) values recorded. Colonies on slants and MGIT cultures with a TTD value were submitted to PCR for verification and identification purposes. Different culture procedures were performed according to the sample type.

Milk, Milk Powder, and Powdered Infant Formula

Powdered material was reconstituted with sterile distilled water following manufacturer's instructions and then treated like liquid milk. For solid cultures 30 ml of milk was centrifuged at 8,000 × g for 20 min. The pelleted material and the cream were homogenized and resuspended in 12 ml of 0.75% (wt/vol) RonaCare Cetylpyridinium Chloride (Merck, Darmstadt, Germany). Tubes were incubated on a rotating wheel at room temperature for 5 h (Donaghy et al., 2008). Samples were then centrifuged at 3,500 × g for 15 min. The intermediate liquid phase was discarded and the pellet and the

TABLE 1 | List of food types and number of brands and items analyzed.

Product		Obtained from (country)	Number of brands tested			
			First sampling	Second sampling	Both[a]	Total products
Dairy products	Sterilized whole milk	Cattle (Spain)	1	1	1	2
	UHT whole milk	Cattle (Spain)	12	12	11	24
	Pasteurized whole milk	Cattle, sheep (Spain)	4	7	3	11
	Evaporated whole milk	Cattle (Spain)	0	1	0	1
	Milk powder (whole/skim)	Cattle (Spain)	4	4	4	8
	Powdered infant formula	Cattle (Spain, Netherlands, Switzerland)	5	5	5	10
	UHT milk cream	Cattle (Spain)	4	4	4	8
	Butter	Cattle (Spain, France, Germany)	7	7	7	14
	Pasteurized milk fresh cheese	Cattle (Spain, Italy)	12	13	12	25
	Pasteurized milk soft cheese	Cattle (Spain, France)	4	3	3	7
	Processed cheese	Cattle (Spain, France)	5	5	5	10
	Spreadable cheese	Cattle, goat (France)	2	2	2	4
	Pasteurized milk Greek style yogurt	Cattle (Spain, France)	7	7	7	14
	Total dairy products		67	71	64	138
Meat products	Ground meat	Cattle, pig, Iberian pig, chicken, turkey (Spain)	16	14	12	30
	Hamburger	Cattle, pig, Iberian pig, chicken, ostrich, horse, rabbit (Spain, Ireland)	9	13	7	22
	Fresh sausage	Pig, Iberian pig, chicken (Spain)	2	3	2	5
	Cooked sausage	Pig, Iberian pig, chicken (Spain)	3	4	3	7
	Mortadella cold cut	Pig (Spain)	3	3	3	6
	Headcheese cold cut	Iberian pig (Spain)	0	2	0	2
	Fresh Spanish chorizo	Pig, Iberian pig (Spain)	8	10	8	18
	Pâté	Pig; Iberian pig, wild boar, deer, duck, goose (Spain, France)	14	15	14	29
	Total meat products		55	64	49	119
All products	Total		122	135	113	257

The country of production, processing, bottling or packing indicated on the product is included.

[a] *Number of products tested in both sampling time points represented with two independent batches.*

remaining upper creamy fraction thoroughly vortexed with 1.5 ml of phosphate-buffered saline (PBS) containing 0.05% (wt/vol) Tween 20 (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA). Solid media tubes were inoculated with 0.2 ml of this suspension. An additional 30 ml aliquot of samples was used for liquid culture. Following centrifugation at 8,000 × g for 20 min, the pellet and cream fractions were thoroughly homogenized in 10 ml of PBS-Tween 20. Afterwards, samples were processed with BD BBL MycoPrep kit (Becton, Dickinson and Company) following the instructions of the manufacturer and the resulting material was inoculated in appropriately supplemented MGIT tubes.

Cream, Butter, Cheese, and Yogurt

Ninety ml of pre-warmed diluent containing 0.5% (wt/vol) sodium chloride, 2% (wt/vol) sodium citrate and 1% (wt/vol) Bacto Casitone (Difco, Becton, Dickinson and Company) were added to either 10 g of butter, 10 g of cheese or 10 g of yogurt contained in filter stomacher bags. For cream 30 ml of this product and 60 ml of diluent were used. Filter bags were introduced in a stomacher lab blender and suspensions homogenized for 3 min at high speed. After incubation at 56°C for 1 h on an orbital shaker platform, the homogenization step in the stomacher was repeated. Sixty ml of these suspensions were processed for solid (30 ml) and liquid (30 ml) culture following the steps previously described for milk samples. Ten milliliter of the same suspensions were used for DNA extraction.

Meat Products

Filter stomacher bags were used to weigh 2 g of meat product. Following addition of freshly prepared RonaCare Cetylpyridinium Chloride (38 ml), samples were thoroughly homogenized in a lab blender and the suspension transferred to 50 ml centrifuge tubes. Tubes were then spun at 100 × g for 1 min and 15 ml of the supernatant containing no gross material was transferred to new centrifuge tubes. After an overnight decontamination, samples were centrifuged at 3,500 × g for 15 min and pellets resuspended in 1.5 ml of PBS-Tween 20. This was the suspension used to inoculate solid media tubes. An additional portion of 2 g was homogenized in a filter stomacher bag containing 10 ml of sterile distilled water. The homogenate was collected in 50 ml tubes and treated for MGIT culture as indicated before.

DNA Extraction

Dairy Products

Adiapure milk kit (Adiagèn, bioMérieux, Marcy l'Etoile, France) was used as per manufacturer's instructions to extract DNA from milk and dairy product suspensions previously prepared for culture purposes (Donaghy et al., 2011). Briefly, a mixture consisting of 10 ml of sample and lysis buffer was vortexed and magnetic beads added. Tubes were gently mixed on a rotating wheel during 30 min for mycobacteria capture. Magnetic beads were separated from the liquid phase using a 15/50 ml tube magnet (Qiagen GmbH, Hilden, Germany), resuspended in the appropriate reagent and disrupted with glass beads for 10 min at maximum speed in a Tissue Lyser II (Qiagen) to lyse cells bound to magnetic beads. Beads were precipitated by centrifugation and supernatants treated with the buffer containing proteinase K.

Meat Products

A modified protocol of the Speedtools Tissue DNA extraction kit (Biotools B&M Labs S. A., Madrid, Spain) was used (Sevilla et al., 2015). Meat product samples (2 g) were stomached with 10 ml of sterile distilled water in stomacher filter bags to give a homogeneous suspension. The material suspended in 1.25 ml was pelleted and the supernatant removed. Zirconia/silica beads of 0.1 mm diameter (Bio Spec Products Inc., Bartlesville, OK, USA), lysis buffer and proteinase K was added to tubes. Samples were incubated at 56°C with agitation in a ThermoMixer (Eppendorf, San Diego, CA, USA) until the suspension was completely clear. Incubation was extended with additional fresh proteinase K if this was not achieved at the first attempt. Subsequent processing was as the standard protocol of the kit indicated except for the inclusion of one bead-beating step in a Tissue Lyser II (Qiagen).

Positive Cultures

One milliliter of MGIT broth was centrifuged at 16,000 × g for 3 min and the pellets washed with sterile distilled water. To extract DNA from these pellets and from the colonies grown on solid media the modified Speedtools Tissue DNA extraction kit (Biotools B&M Labs S. A.) protocol described above was employed.

All DNA extracts were stored at −20°C if not used immediately.

Tetraplex Real-Time PCR Screening

DNA from food products and cultures was tested using an improved version of a real-time PCR able to simultaneously detect the genus *Mycobacterium*, the *M. avium* subspecies, the *M. tuberculosis* complex and an internal amplification control (Sevilla et al., 2015). Modifications involved oligonucleotide changes in *M. avium* and *M. tuberculosis* complex PCR components to increase performance and accommodate the method to the use of different PCR mastermixes and platforms. The new protocol is currently under evaluation by an external party with commercial interests. Using the unmodified assay would yield similar results if performed under the same conditions reported earlier for the original method (Sevilla et al., 2015). Samples were tested in duplicate and all assays were validated based on the results obtained for internal amplification control, DNA extraction negative and positive controls, no template PCR controls and PCR positive controls. Amplification was carried out in a 7,500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA) under universal TaqMan assay conditions and SDS software v. 1.5.1 (Applied Biosystems) was used to calculate valid threshold cycle (C_T) and baseline. C_T values can be used as a rough reference of the estimated numbers of mycobacteria that could be present in samples (Sevilla et al., 2015).

Confirmation and Further Identification of Samples and Cultures Positive to the Tetraplex Real-Time PCR Screening

Samples Positive to *M. avium*

Samples positive for *M. avium* were submitted to IS*900*-IS*Map02* real-time PCR (Sevilla et al., 2014) and other conventional (Bartos et al., 2006) or real-time (Slana et al., 2010) PCR methods

to detect IS*1245*, IS*901,* and IS*901*-flanking region (FR300). *M. avium* subspecies were identified on the basis of the presence (+) or absence (−) of the specified genomic targets as follows: *M. avium* subsp. *paratuberculosis* is IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, and FR300+; *M. avium* subsp. *avium* is IS*900*−, IS*Map02*−, IS*1245*+, IS*901*+ and FR300−; *M. avium* subsp. *silvaticum* is IS*900*−, IS*Map02*−, IS*1245*+, IS*901*+, and FR300+, *M. avium* subsp. *hominissuis* is IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, and FR300+ (Bartos et al., 2006).

Samples Positive to *M. tuberculosis* Complex

To confirm the positive results and distinguish between the species grouped in the *M. tuberculosis* complex, these DNA samples were analyzed by spoligotyping (Kamerbeek et al., 1997) and a panel of conventional singleplex PCR assays using primers and conditions previously described to detect the regions of difference (RD) 1, 4, 9, and 12 of *M. tuberculosis* (Halse et al., 2011). The RD signature patterns used to differentiate between *M. tuberculosis* complex members were those specified earlier (Halse et al., 2011). Briefly, *M. tuberculosis* is positive to all RDs; *M. canettii* is RD1+, RD4+, RD9+, and RD12−; *M. africanum* and *pinnipedii* are RD1+, RD4+, RD9−, and RD12+; *M. microti* is RD1−, RD4+, RD9−, and RD12+; *M. caprae* is RD1+, RD4+, RD9−, and RD12−; *M. bovis* is RD1+, RD4−, RD9−, and RD12−.

Samples Positive to *Mycobacterium* sp. but Negative to *M. avium* and *M. tuberculosis* Complex

Species or complex identification and confirmation of samples only positive to the genus *Mycobacterium* was attempted by PCR and sequence analysis of 16S-23S rRNA internal transcribed spacer (ITS) (Richter et al., 1999) or by sequencing the short amplicon obtained for the ITS component (genus) in the screening PCR (Sevilla et al., 2015). Sequencing primers were the same used for amplification in both cases. Sequencing reactions were carried out using BigDye Terminator chemistry on a 3,130 Genetic Analyzer (Applied Biosystems). Sequences were inspected, edited and aligned with Sequencing Analysis 5.2 (Applied Biosystems) and Vector NTI (Informax Inc., Bethesda, MD, USA) software assistance and compared with other publicly available sequences using online BLAST (NCBI, NLM, Bethesda, MD, USA) analysis.

RESULTS

There was no agreement between PCR and culture positive results, no sample tested positive for both. Four dairy products that were positive in the direct PCR were also positive to the same PCR component when DNA extracted from MGIT culture was used, but viability could not be demonstrated as explained below.

Culture Results and Identification of Isolates

Colonies grown on solid media and MGIT cultures with positive TTD readouts on the BACTEC instrument were screened for the presence of mycobacterial DNA using the tetraplex PCR. All but six MGIT and two Middlebrook 7H10 cultures tested negative (see **Table 2**). However, four of these MGIT cultures yielded high C_T values (**Table 2**) and subculture attempts failed to demonstrate actual cell viability.

Identification of isolates is shown in **Table 3**. *M. avium* subsp. *hominisssuis* was isolated from a powdered infant formula (one colony on 7H10) as well as from one ground beef sample (one colony on 7H10). *M. avium* subsp. *avium* and *M. fortuitum* complex grew in MGIT cultures from a fresh chicken sausage and one mortadella sample, respectively. The ITS sequence obtained for the *M. fortuitum* complex isolate displayed a homology of 99% with *M. senegalense* strain MF-417 (82% query coverage) in BLAST analysis. No viable bacteria belonging to the *M. tuberculosis* complex were isolated.

Direct PCR Detection in Products and Identification of Positive Samples

Mycobacterial DNA was detected in a total of 23 samples that accounted for the 15.22% of dairy products and 1.68% of meat products analyzed (**Table 2**). According to the tetraplex PCR results, 34.78% of positives corresponded to *M. avium*, 30.43% to *M. tuberculosis* complex and the remaining 34.78% to mycobacteria other than *M. avium* and *M. tuberculosis* complex. In meat foods only *M. avium* was detected. All but one product PCR positive samples displayed C_T values above 36 indicating the presence of very low amounts of detectable mycobacterial DNA. Regardless of these high C_T values, all positive results could be confirmed by the repetition of the screening PCR as well as by PCR testing of *M. avium* insertion sequences or *M. tuberculosis* complex RDs and mycobacterial ITS amplicon sequencing (see **Table 3**).

In this survey *M. tuberculosis* complex DNA was identified only in dairy products, including whole milk powder, infant formula, butter, cheese and yogurt. Samples recorded as *M. tuberculosis* complex positive were inspected for the presence of RD 1, 4, 9, and 12, and only RD1 was detected in all cases. This signature was suggestive of the presence of *M. bovis*. Spoligotyping failed to confirm this due to the absence of any perceptible hybridization signal.

DNA from *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* was identified in 6 dairy foods (pasteurized whole milk, infant formula, cream and fresh cheese) and 2 meat products (hamburger and sausage), as assessed by the presence or absence of the insertion sequences investigated. Samples deemed *Mycobacterium* sp. positive in the tetraplex PCR were finally classified as mycobacteria compatible with *M. fortuitum* complex (5), *M. terrae* complex (2) and *M. gordonae* (1) according to the sequences obtained.

With regard to foods represented by two product batches, only two displayed a positive result for both. However, the mycobacteria identified differed between batches. One powdered infant formula resulted positive to *M. tuberculosis* complex (*M. bovis*) in the first sampling and positive to *M. avium* (subsp. *paratuberculosis*) in the second. The other product was a fresh chicken sausage with batches positive to *M. avium* subsp. *hominissuis* DNA in one case and to *M. avium* subsp. *avium* culture in the other.

TABLE 2 | Culture and screening tetraplex real-time PCR results of positive samples (detection of *M. avium*, *M. tuberculosis* complex and genus *Mycobacterium*).

Product	From	Sample ID	Product culture (isolation medium)[a]	Product direct PCR (C_T)[a]	Culture PCR (C_T)[a]
Pasteurized whole milk	Cattle	1	N	*Mycobacterium* (32.64)	ND
Whole milk powder	Cattle	2	N	*Mycobacterium* (43.62)	N
Pasteurized milk fresh cheese	Cattle	3	N	*Mycobacterium* (37.62)	N
Pasteurized milk fresh cheese	Cattle	4	N	*Mycobacterium* (36.42)	*Mycobacterium* (42.28)
Pasteurized milk fresh cheese	Cattle	5	N	*Mycobacterium* (38.31)	ND
Pasteurized milk fresh cheese	Cattle	6	N	*Mycobacterium* (38.98)	*Mycobacterium* (41.64)
Pasteurized milk soft cheese	Cattle	7	N	*Mycobacterium* (37.21)	ND
Pasteurized milk greek style yogurt	Cattle	8	N	*Mycobacterium* (38.46)	ND
Mortadella cold cut	Pig	9	P (MGIT)	N	*Mycobacterium* (25.6)
Pasteurized whole milk	Cattle	10	N	*M. avium* (39.1)	ND
Powdered infant formula	Cattle	11	N	*M. avium* (36.79)	*M. avium* (42.01)
Powdered infant formula	Cattle	12	P (M7H10)	N	*M. avium* (25.14)
UHT milk cream	Cattle	13	N	*M. avium* (38.77)	ND
Pasteurized milk fresh cheese	Cattle	14	N	*M. avium* (37.8)	ND
Pasteurized milk fresh cheese	Cattle	15	N	*M. avium* (36.74)	ND
Pasteurized milk fresh cheese	Cattle	16	N	*M. avium* (38.77)	N
Ground meat	Cattle	17	P (M7H10)	N	*M. avium* (14.34)
Hamburger	Horse	18	N	*M. avium* (37)	N
Fresh sausage	Chicken	19	N	*M. avium* (39)	N
Fresh sausage	Chicken	20	P (MGIT)	N	*M. avium* (14.90)
Whole milk powder	Cattle	21	N	*M. tuberculosis* complex (40)	N
Powdered infant formula	Cattle	22	N	*M. tuberculosis* complex (38.29)	N
Butter	Cattle	23	N	*M. tuberculosis* complex (40.03)	ND
Butter	Cattle	24	N	*M. tuberculosis* complex (38.08)	ND
Pasteurized milk fresh cheese	Cattle	25	N	*M. tuberculosis* complex (38.25)	N
Pasteurized milk soft cheese	Cattle	26	N	*M. tuberculosis* complex (40.12)	N
Pasteurized milk greek style yogurt	Cattle	27	N	*M. tuberculosis* complex (39.86)	*M. tuberculosis* complex (38.45)

[a] *P, positive; N, negative; ND, not done; C_T, threshold cycle; M7H10, modified Middlebrook 7H10; MGIT, Mycobacterial Growth Indicator Tube.*

DISCUSSION

Direct PCR revealed the presence of mycobacteria in 9% of products, a proportion increased in dairy foodstuffs (15%) if compared with meat products (2%). All samples displayed very high C_T values close to or above those obtained at the limit of detection of the unmodified technique with artificially inoculated samples (100 CFU per gram) (Sevilla et al., 2015). The different nature of samples as well as the use of the modified method might account for a slightly different association between C_T values and limit of detection. In spite of this, the high C_T values were not PCR artifacts as was demonstrated by the confirmatory tests performed and therefore, low bacterial concentrations were to be expected in samples. The lack of agreement observed between PCR and culture can be explained by the heterogeneous distribution and low concentration of viable mycobacteria (probably below the limit of detection of PCR) in the products and the fact that DNA detection does not necessarily imply viability. Other factors that could account for PCR negative but culture positive results include a lower sensitivity of the DNA extraction and detection method or the presence of cell clumps.

M. avium subsp. *paratuberculosis* DNA was present in 4% of dairy foods. This figure is consistent with the lowest percentages of positives reported elsewhere (Grant, 2010; Waddell et al., 2016). In spite of this, we found less *M. avium* subsp. *paratuberculosis* than we anticipated based on the high prevalence and spread of PTB. In comparison to other reports on the culture of viable *M. avium* subsp. *paratuberculosis* from raw and pasteurized milk products (Waddell et al., 2016), culture did not produce any isolates. Our survey did not include any raw milk products and the number of pasteurized liquid milk samples was reduced. The sensitivity of DNA extraction and culture could represent an additional limitation that may be improved using novel methods (Botsaris et al., 2016). Despite this, a colony of *M. avium* subsp. *hominissuis* was isolated from a powdered infant formula. Little is known about the prevalence of this subspecies in dairy products. Among other infections, it is responsible for cervical lymphadenitis in infants and the most likely route of infection is oral (Falkinham, 2003; Kasperbauer and Huitt, 2013). Water is considered the main source of human exposure to this microbe (Falkinham, 2015) but infant formula could also represent a way of contact for children.

TABLE 3 | Confirmation and identification of samples positive to either culture or screening tetraplex real-time PCR.

Product	From	Sample ID	Method used[a]	Results[a,b]	Identified as
Pasteurized whole milk	Cattle	1	ITS sequencing[e]	*M. fortuitum* str.: 86–97%, 96–100%	*M. fortuitum* complex
Whole milk powder	Cattle	2	ITS sequencing[d]	*M. fortuitum* str.: 99%, 97%	*M. fortuitum* complex
Pasteurized milk fresh cheese	Cattle	3	ITS sequencing[d]	*M. terrae*/*M. arupense* str.: 100%, 100%	*M. terrae* complex
Pasteurized milk fresh cheese	Cattle	4	ITS sequencing[d]	*M. fortuitum* str.: 100%, 100%	*M. fortuitum* complex
Pasteurized milk fresh cheese	Cattle	5	ITS sequencing[d]	*M. gordonae* str.: 96%, 98%	*M. gordonae*
Pasteurized milk fresh cheese	Cattle	6	ITS sequencing[d]	*M. fortuitum* str.: 99%, 100%	*M. fortuitum* complex
Pasteurized milk soft cheese	Cattle	7	ITS sequencing[d]	*M. fortuitum* str.: 100%, 100%	*M. fortuitum* complex
Pasteurized milk greek style yogurt	Cattle	8	ITS sequencing[d]	*M. terrae*/*M. arupense* str.: 100%, 100%	*M. terrae* complex
Mortadella cold cut	Pig	9[c]	ITS sequencing[e]	*M. senegalense* str.: 99%, 82%	*M. fortuitum* complex
Pasteurized whole milk	Cattle	10	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Powdered infant formula	Cattle	11	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Powdered infant formula	Cattle	12[c]	IS screening	IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, FR300+	*M. avium* subsp. *hominissuis*
UHT milk cream	Cattle	13	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Pasteurized milk fresh cheese	Cattle	14	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Pasteurized milk fresh cheese	Cattle	15	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Pasteurized milk fresh cheese	Cattle	16	IS screening	IS*900*+, IS*Map02*+, IS*1245*−, IS*901*−, FR300+	*M. avium* subsp. *paratuberculosis*
Ground meat	Cattle	17[c]	IS screening	IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, FR300+	*M. avium* subsp. *hominissuis*
Hamburger	Horse	18	IS screening	IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, FR300+	*M. avium* subsp. *hominissuis*
Fresh sausage	Chicken	19	IS screening	IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, FR300+	*M. avium* subsp. *hominissuis*
Fresh sausage	Chicken	20[c]	IS screening	IS*900*−, IS*Map02*−, IS*1245*+, IS*901*−, FR300−	*M. avium* subsp. *avium*
Whole milk powder	Cattle	21	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Powdered infant formula	Cattle	22	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Butter	Cattle	23	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Butter	Cattle	24	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Pasteurized milk fresh cheese	Cattle	25	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Pasteurized milk soft cheese	Cattle	26	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*
Pasteurized milk greek style yogurt	Cattle	27	RD screening	RD1+, RD4−, RD9−, RD12−	*M. bovis*

[a] *+, positive; −, negative; ITS, 16S-23S rRNA internal transcribed spacer; IS, insertion sequences of M. avium; RD, regions of difference of M. tuberculosis complex; Str., strain.*
[b] *BLAST results displaying highest identity and coverage percentages for sequenced ITS amplicons: identity %, coverage %.*
[c] *DNA extracted from the isolate. Otherwise, the DNA was obtained directly from the product.*
[d] *Sequenced ITS amplicon corresponded to the tetraplex real-time PCR (Sevilla et al., 2015).*
[e] *Sequenced ITS amplicon corresponding to the PCR described by Richter et al. (1999).*

On the other hand, the detection of *M. tuberculosis* complex DNA in 5% of dairy products was completely unexpected. All cases were identified as *M. bovis* by RD screening. Spoligotyping was negative presumably because very small amounts and proportion of target DNA was present in DNA specimens obtained from food products. DNA extracted from these complex matrices can harbor a high concentration of nontarget DNA and other elements that may interfere with spoligotyping. The performance of spoligotyping can be limited also when applied directly to clinical samples (Milián Suazo et al., 2010; Ereqat et al., 2013). Our results did not seem to reflect the current PTB and TB situation in developed countries with high PTB prevalence and ongoing TB eradication and surveillance programs (Lombard, 2011; Schiller et al., 2011). It can be speculated that contaminated milk or byproducts imported from regions with high TB burden was used to produce these foods. *M. bovis* has been detected in milk, cheese and other products (Kaneene et al., 2014; Pereira-Suarez et al., 2014; Pérez-Lago et al., 2014; Cezar et al., 2016), mainly in regions where animal TB still represents a major issue.

All samples positive only to *Mycobacterium* sp. DNA corresponded to dairy products. This could be related to a higher exposure of milk to environmental NTM due to the way it is collected, transported and processed prior to being commercialized. The mycobacterial DNA detected displayed sequences highly compatible with *M. fortuitum* complex, *M. terrae* complex, and *M. gordonae*. These NTM are only occasionally considered pathogenic (Griffith et al., 2007). *M. fortuitum* complex is the only rapid growing mycobacterial group identified in our survey. However, it was detected in 4% of dairy samples, including one pasteurized milk with the highest estimated bacterial concentration ($C_T = 32.64$). The detection of these mycobacterial groups in unpasteurized milk is not unusual, but only few studies report on the isolation from pasteurized

milk (Sgarioni et al., 2014), consistent with thermal inactivation profiles of *M. fortuitum* in milk (Grant et al., 1996).

PCR and further methods revealed *M. avium* subsp. *hominissuis* DNA in 2% of meat foodstuffs. Culture retrieved one *M. avium* subsp. *avium* isolate from a fresh chicken sausage, one *M. avium* subsp. *hominissuis* isolate from ground beef and one *M. senegalense* isolate from mortadella. These data diverged from other surveys on *M. avium* subspecies detection showing quite high proportions of PCR positives but no isolate recovery (Klanicova et al., 2011; Lorencova et al., 2014). The outcome of our market situation study may fit best with other reports (Jaravata et al., 2007; Savi et al., 2015). *M. avium* isolates were grown from uncooked meat products. *M. senegalense* strain was isolated from a cold cut originated from cooked pork meat and fat, suggesting an environmental contamination during further processing. *M. avium* subsp. *avium* is an obligate pathogen rarely isolated from environmental samples unrelated to birds (Turenne et al., 2008). The subspecies *avium* is the main cause of typical avian TB in domestic birds and can infect cattle, deer and wild boar, and humans more sporadically (Dvorska et al., 2004). Since these infections are more common in older animals, we find more likely that this sausage contained meat from culled laying hens instead of young broilers specifically bred for meat and deduce that improved control measures are desirable. Raw meats are generally intended for being consumed cooked, but eating undercooked meats is quite common and mycobacterial inactivation during cooking is time and temperature dependent (Hammer et al., 2013). In addition, cooked products can become cross-contaminated through direct or indirect contact with raw meat as a consequence of improper food handling. Meat could still pose a risk of exposure to viable *M. avium* subspecies and other mycobacteria if not produced hygienically and cooked properly (Klanicova-Zalewska and Slana, 2014).

Mycobacteria are present infecting animals and in our environment, water and food being likely means of contact for humans. Zoonotic TB and NTM infection have been of great importance in human health over time. The prevalence of zoonotic TB was dramatically reduced owing to the implementation of abattoir inspection, milk pasteurization and other measures, but the problem is still considerable in many regions (Kaneene et al., 2014). With respect to NTM, much effort has been devoted to the understanding of human infection and thus different mechanisms leading to susceptibility and predisposition consisting in immunological flaws and conditions that compromise physical barriers to infection have been identified (Lake et al., 2016). Exposure to attenuated or inactivated mycobacteria can modulate the immune response resulting in a favorable effect (Beltran-Beck et al., 2014; Cardona et al., 2015; Kaufmann et al., 2015; Chambers et al., 2017). On the other hand, genetic susceptibility to chronic inflammatory disorders coupled with heavy exposure to *M. avium* subsp. *paratuberculosis* has been pointed out as a potential etiological factor in Crohn's and other chronic inflammatory diseases (Sechi and Dow, 2015). Moreover, it has been hypothesized that it could drive an autoimmune response by molecular mimicry (Sechi and Dow, 2015). Unfortunately, a consensus opinion on the potential role of this bacterium in Crohn's disease has not yet been reached (Van Kruiningen, 2011; Waddell et al., 2016). Since few bacteria are detected in Crohn's disease patients it could be speculated that *M. avium* subsp. *paratuberculosis* might not need to be active to trigger a deleterious response. A latent, dormant or even dead condition could be sufficient as showed by the necrotizing colitis induced in mice after transanal injection of *M. avium* subsp. *paratuberculosis* antigens (Momotani et al., 2012). However, our results do not indicate that a heavy foodborne exposure occurs in populations where the main source of food of animal origin is processed food.

In summary, our results show that the prevalence of viable mycobacteria in packed products of animal origin available at Spanish supermarkets was not substantial although a considerable proportion of them contained mycobacterial DNA. These figures are probably translatable to other European countries as a result of market globalization and common food safety legislation. This survey indicates that viable mycobacteria and mycobacterial components are present in a range of products and at a frequency that could ensure repeated exposure over time during the lifetime of any individual. Consequently, setting up more stringent control measures should be considered. Further research is necessary to identify critical points in the food production system and determine the real impact of foodborne mycobacterial exposure on human health either directly, causing infection, or indirectly, modifying individual's immune status and susceptibility to other diseases.

AUTHOR CONTRIBUTIONS

IS, RJ, and JG conceived of the study. EM, MT, IS, and NE carried out the laboratory work and compiled and analyzed the data. IS collated the results and wrote the original draft. IS, EM, MT, NE, RJ, and JG participated in the review and the editing of the original draft. All authors read and approved the final manuscript.

FUNDING

The work of IS, EM, MT, NE, and JG was supported by the Department of Economic Development and Competitiveness of the Basque Government. MT holds a fellowship from the same Department. We also acknowledge this Department and the Department of Education, Universities and Research of the Basque Government for funding Research Projects 32-2016-00030 and PI2011-50.

ACKNOWLEDGMENTS

This work was included in the Food Safety Research Plan of the Basque Country (2011-2016) conceived by the Directorate for Food Quality Control and Industries (Department of Economic Development and Competitiveness) of the Basque Government.

REFERENCES

Bartos, M., Hlozek, P., Svastova, P., Dvorska, L., Bull, T., Matlova, L., et al. (2006). Identification of members of *Mycobacterium avium* species by Accu-Probes, serotyping, and single IS900, IS901, IS1245 and IS901-flanking region PCR with internal standards. *J. Microbiol. Methods* 64, 333–345. doi: 10.1016/j.mimet.2005.05.009

Beltran-Beck, B., de la Fuente, J., Garrido, J. M., Aranaz, A., Sevilla, I., Villar, M., et al. (2014). Oral vaccination with heat inactivated *Mycobacterium bovis* activates the complement system to protect against tuberculosis. *PLoS ONE* 9:e98048. doi: 10.1371/journal.pone.0098048

Bercovier, H., and Vincent, V. (2001). Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum*, *M. fortuitum*, *M. chelonae, M. porcinum, M. farcinogenes, M. smegmatis, M. scrofulaceum, M. xenopi, M. kansasii, M. simiae* and *M. genavense. Rev. Sci. Tech.* 20, 265–290. doi: 10.20506/rst.20.1.1269

Biet, F., and Boschiroli, M. L. (2014). Non-tuberculous mycobacterial infections of veterinary relevance. *Res. Vet. Sci.* 97, S69–S77. doi: 10.1016/j.rvsc.2014.08.007

Botsaris, G., Swift, B. M., Slana, I., Liapi, M., Christodoulou, M., Hatzitofi, M., et al. (2016). Detection of viable *Mycobacterium avium* subspecies *paratuberculosis* in powdered infant formula by phage-PCR and confirmed by culture. *Int. J. Food Microbiol.* 216, 91–94. doi: 10.1016/j.ijfoodmicro.2015.09.011

Cardona, P., Marzo-Escartin, E., Tapia, G., Diaz, J., Garcia, V., Varela, I., et al. (2015). Oral administration of heat-killed *Mycobacterium manresensis* delays progression toward active tuberculosis in C3HeB/FeJ mice. *Front. Microbiol.* 6:1482. doi: 10.3389/fmicb.2015.01482

Cezar, R. D., Lucena-Silva, N., Borges, J. M., Santana, V. L., and Pinheiro Junior, J. W. (2016). Detection of *Mycobacterium bovis* in artisanal cheese in the state of Pernambuco, Brazil. *Int. J. Mycobacteriol.* 5, 269–272. doi: 10.1016/j.ijmyco.2016.04.007

Chambers, M. A., Aldwell, F., Williams, G. A., Palmer, S., Gowtage, S., Ashford, R., et al. (2017). The effect of oral vaccination with *Mycobacterium bovis* BCG on the development of tuberculosis in captive European badgers (*Meles meles*). *Front. Cell Infect. Microbiol.* 7:6. doi: 10.3389/fcimb.2017.00006

Donaghy, J. A., Johnston, J., and Rowe, M. T. (2011). Detection of *Mycobacterium avium* ssp. *paratuberculosis* in cheese, milk powder and milk using IS900 and f57-based qPCR assays. *J. Appl. Microbiol.* 110, 479–489. doi: 10.1111/j.1365-2672.2010.04905.x

Donaghy, J. A., Rowe, M. T., Rademaker, J. L., Hammer, P., Herman, L., De Jonghe, J. V., et al. (2008). An inter-laboratory ring trial for the detection and isolation of *Mycobacterium avium* subsp. paratuberculosis from raw milk artificially contaminated with naturally infected faeces. *Food Microbiol.* 25, 128–135. doi: 10.1016/j.fm.2007.06.007

Dvorska, L., Matlova, L., Bartos, M., Parmova, I., Bartl, J., Svastova, P., et al. (2004). Study of *Mycobacterium avium* complex strains isolated from cattle in the Czech Republic between 1996 and 2000. *Vet. Microbiol.* 99, 239–250. doi: 10.1016/j.vetmic.2004.01.008

Ereqat, S., Nasereddin, A., Levine, H., Azmi, K., Al-Jawabreh, A., Greenblatt, C. L., et al. (2013). First-time detection of *Mycobacterium bovis* in livestock tissues and milk in the West Bank, Palestinian Territories. *PLoS Negl. Trop. Dis.* 7:e2417. doi: 10.1371/journal.pntd.0002417

Falkinham, J. O. III. (2016). Current epidemiologic trends of the nontuberculous *Mycobacteria* (NTM). *Curr. Environ. Health Rep.* 3, 161–167. doi: 10.1007/s40572-016-0086-z

Falkinham, J. O. (2003). The changing pattern of nontuberculous mycobacterial disease. *Can. J. Infect. Dis.* 14, 281–286. doi: 10.1155/2003/323058

Falkinham, J. O. III. (2015). Environmental sources of nontuberculous mycobacteria. *Clin. Chest Med.* 36, 35–41. doi: 10.1016/j.ccm.2014.10.003

Faria, A. C., Schwarz, D. G., Carvalho, I. A., Rocha, B. B., De Carvalho Castro, K. N., Silva, M. R., et al. (2014). Short communication: viable *Mycobacterium avium* subspecies *paratuberculosis* in retail artisanal Coalho cheese from Northeastern Brazil. *J. Dairy Sci.* 97, 4111–4114. doi: 10.3168/jds.2013-7835

García-Martos, P., and García-Agudo, L. (2012). Infections due to rapidly growing mycobacteria. *Enferm. Infecc. Microbiol. Clin.* 30, 192–200. doi: 10.1016/j.eimc.2011.09.017

Ghodbane, R., Mba, M. F., Lepidi, H., Nappez, C., and Drancourt, M. (2014). Long-term survival of tuberculosis complex mycobacteria in soil. *Microbiology* 160, 496–501. doi: 10.1099/mic.0.073379-0

Grant, I. R. (2010). "*Mycobacterium avium* subsp. paratuberculosis in animal-derived foods and the environment," in *Paratuberculosis: Organism, Disease, Control*, eds M. A. Behr and D. M. Collins (Oxfordshire: CABI), 29–39.

Grant, I. R., Ball, H. J., and Rowe, M. T. (1996). Thermal inactivation of several *Mycobacterium* spp. in milk by pasteurization. *Lett. Appl. Microbiol.* 22, 253–256. doi: 10.1111/j.1472-765X.1996.tb01154.x

Griffith, D. E., Aksamit, T., Brown-Elliott, B. A., Catanzaro, A., Daley, C., Gordin, F., et al. (2007). An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am. J. Respir. Crit. Care Med.* 175, 367–416. doi: 10.1164/rccm.200604-571ST

Halse, T. A., Escuyer, V. E., and Musser, K. A. (2011). Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the *Mycobacterium tuberculosis* complex in clinical specimens. *J. Clin. Microbiol.* 49, 262–2567. doi: 10.1128/JCM.00467-11

Hammer, P., Walte, H. G., Matzen, S., Hensel, J., and Kiesner, C. (2013). Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* during cooking of hamburger patties. *J. Food Prot.* 76, 1194–1201. doi: 10.4315/0362-028X.JFP-12-474

Jaravata, C. V., Smith, W. L., Rensen, G. J., Ruzante, J., and Cullor, J. S. (2007). Survey of ground beef for the detection of *Mycobacterium avium paratuberculosis*. *Foodborne. Pathog. Dis.* 4, 103–106. doi: 10.1089/fpd.2006.54

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., et al. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* 35, 907–914.

Kaneene, J. B., Miller, R., Steele, J. H., and Thoen, C. O. (2014). Preventing and controlling zoonotic tuberculosis: a One Health approach. *Vet. Ital.* 50, 7–22. doi: 10.12834/VetIt.1302.08

Kasperbauer, S., and Huitt, G. (2013). Management of extrapulmonary nontuberculous mycobacterial infections. *Semin. Respir. Crit Care Med.* 34, 143–150. doi: 10.1055/s-0033-1333576

Kaufmann, S. H. E., Bloom, B., Brosch, R., Cardona, P. J., Dockrell, H., Fritzell, B., et al. (2015). Developing whole mycobacteria cell vaccines for tuberculosis: workshop proceedings, Max Planck Institute for Infection Biology, Berlin, Germany, July 9, 2014. *Vaccine* 33, 3047–3055. doi: 10.1016/j.vaccine.2015.03.056

Klanicova, B., Slana, I., Vondruskova, H., Kaevska, M., and Pavlik, I. (2011). Real-time quantitative PCR detection of *Mycobacterium avium* subspecies in meat products. *J. Food Prot.* 74, 636–640. doi: 10.4315/0362-028X.JFP-10-332

Klanicova-Zalewska, B., and Slana, I. (2014). Presence and persistence of *Mycobacterium avium* and other nontuberculous mycobacteria in animal tissues and derived foods: a review. *Meat Sci.* 98, 835–841. doi: 10.1016/j.meatsci.2014.08.001

Lake, M. A., Ambrose, L. R., Lipman, M. C., and Lowe, D. M. (2016). "Why me, why now?" Using clinical immunology and epidemiology to explain who gets nontuberculous mycobacterial infection. *BMC Med.* 14:54. doi: 10.1186/s12916-016-0606-6

Lombard, J. E. (2011). Epidemiology and economics of paratuberculosis. *Vet. Clin. North Am. Food Anim Pract.* 27, 525–535. doi: 10.1016/j.cvfa.2011.07.012

Lorencova, A., Vasickova, P., Makovcova, J., and Slana, I. (2014). Presence of *Mycobacterium avium* subspecies and hepatitis E virus in raw meat products. *J. Food Prot.* 77, 335–338. doi: 10.4315/0362-028X.JFP-13-252

Milián Suazo, F., Harris, B., Arriaga-Díaz, C., Thomsen, B., Stuber, T., González-Suárez, D., et al. (2010). Sensitivity and specificity of nested PCR and spoligotyping as quick diagnostic tests for bovine tuberculosis in fresh tissue. *Rev. Mex. Cienc. Pecu.* 1, 403–415. doi: 10.22319/rmcpv%251494 . Available online at: http://cienciaspecuarias.inifap.gob.mx/index.php/Pecuarias/article/view/1494

Momotani, E., Ozaki, H., Hori, M., Yamamoto, S., Kuribayashi, T., Eda, S., et al. (2012). *Mycobacterium avium* subsp. paratuberculosis lipophilic antigen causes Crohn's disease-type necrotizing colitis in mice. *Springerplus* 1:47. doi: 10.1186/2193-1801-1-47

Pereira-Suarez, A. L., Estrada-Chavez, Y., Zuniga-Estrada, A., Lopez-Rincon, G., Hernandez, D. U., Padilla-Ramirez, F. J., et al. (2014). Detection of *Mycobacterium tuberculosis* complex by PCR in fresh cheese from local markets in Hidalgo, Mexico. *J. Food Prot.* 77, 849–852. doi: 10.4315/0362-028X.JFP-13-389

Pérez-Lago, L., Navarro, Y., and García-de-Viedma, D. (2014). Current knowledge and pending challenges in zoonosis caused by *Mycobacterium bovis*: a review. *Res. Vet. Sci.* 97, S94–S100. doi: 10.1016/j.rvsc.2013.11.008

Richter, E., Niemann, S., Rusch-Gerdes, S., and Hoffner, S. (1999). Identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. *J. Clin. Microbiol.* 37, 964–970.

Savi, R., Ricchi, M., Cammi, G., Garbarino, C., Leo, S., Pongolini, S., et al. (2015). Survey on the presence of *Mycobacterium avium* subsp. paratuberculosis in ground beef from an industrial meat plant. *Vet. Microbiol.* 177, 403–408. doi: 10.1016/j.vetmic.2015.03.013

Schiller, I., Waters, W. R., Vordermeier, H. M., Jemmi, T., Welsh, M., Keck, N., et al. (2011). Bovine tuberculosis in Europe from the perspective of an officially tuberculosis free country: trade, surveillance and diagnostics. *Vet. Microbiol.* 151, 153–159. doi: 10.1016/j.vetmic.2011.02.039

Sechi, L. A., and Dow, C. T. (2015). *Mycobacterium avium* ss. paratuberculosis Zoonosis - the hundred year war - beyond crohn's disease. *Front Immunol.* 6:96. doi: 10.3389/fimmu.2015.00096

Sevilla, I. A., Garrido, J. M., Molina, E., Geijo, M. V., Elguezabal, N., Vázquez, P., et al. (2014). Development and evaluation of a novel multicopy-element-targeting triplex PCR for detection of *Mycobacterium avium* subsp. paratuberculosis in feces. *Appl. Environ. Microbiol.* 80, 3757–3768. doi: 10.1128/AEM.01026-14

Sevilla, I. A., Molina, E., Elguezabal, N., Perez, V., Garrido, J. M., and Juste, R. A. (2015). Detection of mycobacteria, *Mycobacterium avium* subspecies, and *Mycobacterium tuberculosis* complex by a novel tetraplex real-time PCR assay. *J. Clin. Microbiol.* 53, 930–940. doi: 10.1128/JCM.03168-14

Sgarioni, S. A., Hirata, R. D., Hirata, M. H., Leite, C. Q., de Prince, K. A., de Andrade Leite, S. R., et al. (2014). Occurrence of *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM) in raw and pasteurized milk in the northwestern region of Parana, Brazil. *Braz. J. Microbiol.* 45, 707–711. doi: 10.1590/S1517-83822014000200046

Slana, I., Kaevska, M., Kralik, P., Horvathova, A., and Pavlik, I. (2010). Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and IS*901* and IS*1245* quantitative real time PCR. *Vet. Microbiol.* 144, 437–443. doi: 10.1016/j.vetmic.2010.02.024

Tell, L. A., Woods, L., and Cromie, R. L. (2001). Mycobacteriosis in birds. *Rev. Sci. Tech.* 20, 180–203. doi: 10.20506/rst.20.1.1273

Thomson, R., Tolson, C., Sidjabat, H., Huygens, F., and Hargreaves, M. (2013). *Mycobacterium abscessus* isolated from municipal water - a potential source of human infection. *BMC Infect. Dis.* 13:241. doi: 10.1186/1471-2334-13-241

Turenne, C. Y., Collins, D. M., Alexander, D. C., and Behr, M. A. (2008). *Mycobacterium avium* subsp. paratuberculosis and M. avium subsp. avium are independently evolved pathogenic clones of a much broader group of M. avium organisms. *J. Bacteriol.* 190, 2479–2487. doi: 10.1128/JB.01691-07

Van Kruiningen, H. J. (2011). Where are the weapons of mass destruction - the *Mycobacterium paratuberculosis* in Crohn's disease? *J. Crohns. Colitis.* 5, 638–644. doi: 10.1016/j.crohns.2011.08.004

Waddell, L., Rajic, A., Stark, K., and McEwen, S. A. (2016). *Mycobacterium avium* ssp. paratuberculosis detection in animals, food, water and other sources or vehicles of human exposure: a scoping review of the existing evidence. *Prev. Vet. Med.* 132, 32–48. doi: 10.1016/j.prevetmed.2016.08.003

Whittington, R. J., Marsh, I. B., Saunders, V., Grant, I. R., Juste, R., Sevilla, I., et al. (2011). Culture phenotypes of genomically and geographically diverse *Mycobacterium avium* subsp. paratuberculosis isolates from different hosts. *J. Clin. Microbiol.* 49, 1822–1830. doi: 10.1128/JCM.00210-11

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Sevilla, Molina, Tello, Elguezabal, Juste and Garrido. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 June 2017
doi: 10.3389/fmicb.2017.01087

Prevalence and Antimicrobial Susceptibility of *Vibrio parahaemolyticus* Isolated from Short Mackerels (*Rastrelliger brachysoma*) in Malaysia

Chia W. Tan[1], Tan T. H. Malcolm[1], Chee H. Kuan[1], Tze Y. Thung[1], Wei S. Chang[1], Yuet Y. Loo[1], Jayasekara M. K. J. K. Premarathne[1,2], Othman B. Ramzi[1], Mohd F. S. Norshafawatie[1], Nordin Yusralimuna[1], Yaya Rukayadi[1], Yoshitsugu Nakaguchi[3], Mitsuaki Nishibuchi[1] and Son Radu[1,4]*

[1] Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Selangor, Malaysia, [2] Department of Livestock and Avian Science, Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Makandura, Sri Lanka, [3] Center for Southeast Asian Studies, Kyoto University, Kyoto, Japan, [4] Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Selangor, Malaysia

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Learn-Han Lee, Monash University Malaysia, Malaysia
Dapeng Wang, Shanghai Jiao Tong University, China

***Correspondence:**
Chia Wanq Tan
chiawanq@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *11 April 2017*
Accepted: *30 May 2017*
Published: *13 June 2017*

Citation:
Tan CW, Malcolm TTH, Kuan CH, Thung TY, Chang WS, Loo YY, Premarathne JMKJK, Ramzi OB, Norshafawatie MFS, Yusralimuna N, Rukayadi Y, Nakaguchi Y, Nishibuchi M and Radu S (2017) Prevalence and Antimicrobial Susceptibility of Vibrio parahaemolyticus Isolated from Short Mackerels (Rastrelliger brachysoma) in Malaysia. Front. Microbiol. 8:1087. doi: 10.3389/fmicb.2017.01087

Numerous prevalence studies and outbreaks of *Vibrio parahaemolyticus* infection have been extensively reported in shellfish and crustaceans. Information on the quantitative detection of *V. parahaemolyticus* in finfish species is limited. In this study, short mackerels (*Rastrelliger brachysoma*) obtained from different retail marketplaces were monitored with the presence of total and pathogenic strains of *V. parahaemolyticus*. Out of 130 short mackerel samples, 116 (89.2%) were detected with the presence of total *V. parahaemolyticus* and microbial loads of total *V. parahaemolyticus* ranging from <3 to $>10^5$ MPN/g. Prevalence of total *V. parahaemolyticus* was found highest in wet markets (95.2%) followed by minimarkets (89.1%) and hypermarkets (83.3%). Pathogenic *V. parahaemolyticus* strains (*tdh*+ and/or *trh*+) were detected in 16.2% (21 of 130) of short mackerel samples. The density of *tdh*+ *V. parahaemolyticus* strains were examined ranging from 3.6 to $>10^5$ MPN/g and microbial loads of *V. parahaemolyticus* strains positive for both *tdh* and *trh* were found ranging from 300 to 740 MPN/g. On the other hand, antibiotic susceptibility profiles of *V. parahaemolyticus* strains isolated from short mackerels were determined through disc diffusion method in this study. Assessment of antimicrobial susceptibility profile of *V. parahaemolyticus* revealed majority of the isolates were highly susceptible to ampicillin sulbactam, meropenem, ceftazidime, and imipenem, but resistant to penicillin G and ampicillin. Two isolates (2.99%) exhibited the highest multiple antibiotic resistance (MAR) index value of 0.41 which shown resistance to 7 antibiotics. Results of the present study demonstrated that the occurrence of pathogenic *V. parahaemolyticus* strains in short mackerels and multidrug resistance of *V. parahaemolyticus* isolates could be a potential public health concerns to the consumer. Furthermore, prevalence data attained from the current study can be further used to develop a microbial risk assessment model to estimate health risks associated with the consumption of short mackerels contaminated with pathogenic *V. parahaemolyticus.*

Keywords: *Vibrio parahaemolyticus*, finfish, MPN, antibiotic susceptibility, MAR

INTRODUCTION

Vibrio parahaemolyticus is found naturally in the marine environment and may accumulate in seafood especially shellfish at high concentrations. Consumption of foods contaminated with high concentration of total *V. parahaemolyticus* and/or pathogenic *V. parahaemolyticus* can cause gastrointestinal infections. An open wound in skin comes in contact with *V. parahaemolyticus* is suggested as an infection pathway as well. Major syndromes caused by *V. parahaemolyticus* include gastroenteritis, wound infection, and septicaemia. Typical symptoms of the gastroenteritis may include abdominal pain, diarrhea, nausea, and fever. Based on the Foodborne Diseases Active Surveillance Network (FoodNet) data and Morbidity and Mortality Weekly Report (MMWR) published by Centers for Disease Control and Prevention (CDC) in the United States during the year 2016, *V. parahaemolyticus* is the major foodborne pathogen compared to other *Vibrio* isolates and it is estimated *V. parahaemolyticus* causes about 34,664 episodes of domestically acquired foodborne illness annually in the United States (Scallan et al., 2011; Huang et al., 2016).

In term of pathogenicity, thermostable direct hemolysin (TDH) and thermostable direct-related hemolysin (TRH) are the well-known virulence factors in *V. parahaemolyticus*. TDH is a pore-forming and heat stable protein which remains unharmed even heating at 100°C for 10 min (Yanagihara et al., 2010). Two distinct biological characteristics of TDH are the capability of inducing hemolysis and cytotoxicity (Baba et al., 1992; Honda et al., 1992; Fabbri et al., 1998; Raimondi et al., 2000). TRH was first identified and isolated from the gastroenteritis outbreak in Maldives in 1985 (Honda et al., 1988). The isolated *V. parahaemolyticus* strains from the outbreak shown Kanagawa negative on Wagatsuma agar and did not possess the TDH genes (Nishibuchi et al., 1989). Unlike TDH thermostability, TRH is a heat labile protein at 60°C for 10 min (Honda et al., 1988). TRH shares similar biological, immunological, and physicochemical characteristics with TDH have been described (Honda and Iida, 1993). For instance, TRH induces Cl^- secretion in cultured human colonic epithelial cells by a similar mechanism with TDH (Takahashi et al., 2000).

Antimicrobial resistance (AMR) has now been recognized as a significant global threat issue to global public health and food security (FAO, 2016). Many frequently used antibiotics are no longer effective to control infections. Extensive use and misuse of antibiotics in agriculture, aquaculture, and livestock production are considered to be the main factor leads to the emergence and spread of AMR. Multidrug-resistant (MDR) bacterial strain is another emerging challenge when a bacterial cell becomes resistant toward multiple antibiotics. Development of MDR bacterial strains can be achieved through several mechanisms such as chromosomal DNA mutations, enzymatic inactivation, transformation as well as conjugation (Van Hoek et al., 2011). Antimicrobial residues present in the environment may also resultant in selection pressure for AMR bacteria. In aquaculture, antibiotics are used in fish farming to promote the growth of aquatic organisms and control bacterial infections. Administration of antibiotics into feed and water is a usual practice to improve production growth and for the treatment of infection diseases caused by pathogenic bacteria. Several *Vibrio* species are known to cause infections in aquatic fish and extensive use of antibiotics in the past have now led to significant increases in the occurrence of AMR *Vibrio* species (Letchumanan et al., 2015b).

Quinolones, cephalosporins, tetracycline, cefotaxime, ceftazidime, and penicillins are some commonly recommended clinical antibiotics used for the treatment of non-cholerae *Vibrio* spp. infections (Han et al., 2007; Wong et al., 2015). The use of quinolone is generally effective to against all *Vibrio* species; while the use of cephalosporin and tetracycline antibiotics was reported ineffective and associated with higher mortality in patients with vibriosis (Wong et al., 2015). In aquaculture industry, tetracyclines, erythromycin, sulfonamides, oxytetracyclines, chlortetracycline, and amoxicilin are allowed to be used in some of the ASEAN countries including Malaysia, Myanmar, and Philippines; while other antimicrobial drugs such as nitrofurans, chloramphenicol, and dimetridazole/metronidazole are banned in most countries (ASEAN, 2013; Weese et al., 2015). For controlling *Vibrio* spp., sulfonamides sold under the trade name Dimeton is an example of veterinary antibiotic commonly used in hatcheries to control infections (Shariff et al., 2000).

Short mackerel (*Rastrelliger brachysoma*) is an important commercial small pelagic fish species and have a high preference among consumers because of its affordable price and widely available throughout the year in many of the Southeast Asia countries including Malaysia, Thailand, Cambodia, Philippines, and Indonesia. Numerous prevalence studies and outbreaks of *V. parahaemolyticus* infection have been extensively reported in oysters, clams, cockles, mussels, crabs, and shrimps (Newton et al., 2014; Rodgers et al., 2014; Xu et al., 2014; Malcolm et al., 2015). In finfish species, qualitative detection of *V. parahaemolyticus* in the anchovies (*Engraulis* spp.), gray mullet (*Mugil cephalus*), red mullet (*Mullus surmuletus*), sardines (*Sardina* spp.), and Atlantic mackerel (*Scomber scombrus*) have been examined by Baffone et al. (2000) with the used of selective medium and biochemical tests. Hara-kudo et al. (2003) examined 15 horse mackerels purchased from the marketplaces in Japan, for *tdh* positive and total *V. parahaemolyticus* by using PCR and CHROMagar Vibrio (CV) agar. Reports focused on the detection and enumeration of *V. parahaemolyticus* in finfish species such as mackerel is very limited in extent. Available data on the quantitative detection of *V. parahaemolyticus* in different parts of the mackerel's body can only be found in one study reported in a Japanese-language literature (Ohno et al., 1993). Hence, the main purpose of this study was to determine the prevalence of total and pathogenic *V. parahaemolyticus* in short mackerels purchased from different wet markets, hypermarkets, and minimarkets in the state of Selangor of Malaysia by using MPN-PCR methods and to evaluate the antibiotic susceptibility profiles of *V. parahaemolyticus* isolates obtained from the short mackerels.

MATERIALS AND METHODS

Sample Collection

A total of 130 short mackerel (*R. brachysoma*) samples were collected from 67 sampling date within a period of 6 months from Jan 2016 to June 2016 in four wet markets ($n = 42$), five hypermarkets ($n = 42$), and five minimarkets ($n = 46$) in Selangor, Malaysia. A maximum of two short mackerels were collected at each sampling date from the sampling location. All the samples were transported to the laboratory and processed immediately on the day of sampling.

Sample Processing and Most Probable Number (MPN) Method

Flesh (with skin), gills, and intestines excised from each short mackerel were used as the test sample. A total of 390 test samples including of 130 fleshes, 130 gills, and 130 intestines were analyzed in this study. Ten grams of flesh was transferred into 90 ml of alkaline peptone water (APW) (Merck, Darmstadt, Germany) in a sterile stomacher bag. One gram of gills and one gram of intestines, each was transferred into 9 ml of APW in two separate sterile stomacher bags. Samples were homogenized for 1 min using a Stomacher Lab-Blender 400 (Seward Medical, UK). MPN preparation was followed the US FDA Bacteriological Analytical Manual (BAM) three tubes MPN methodology with some modifications (Kaysner and DePaola, 2004). Briefly, a serial dilution was carried out up to 10^{-5} by transferring 1 ml suspension mixture into 9 ml of APW. One milliliters of each dilution sample was pipetted into three microcentrifuge tubes and tubes were incubated at 37°C for 18–24 h.

Genomic DNA Extraction

A number of tubes with growth at each dilution was subjected to genomic DNA extraction through physical cell disruption methods. Samples with growth or turbidity were pelleted by centrifugation at 13,400 × g for 3 min. The supernatant was discarded and the remained pellet was suspended in 200 μl TE buffer. The suspension mixture was heated at 100°C for 15 min in a dry bath (Labnet, USA) and immediately kept at −20°C for another 15 min. After the heat and cold-shock cell lysis, samples were centrifuged at 13,400 × g for 1 min and the supernatant was used as DNA template for multiplex PCR.

Multiplex PCR

Multiplex PCR was performed by using 3 set of primers: (i) toxR (F: 5′-GTCTTCTGACGCAATCGTTG-3′ and R: 5′-ATACGA GTGGTTGCTGTCATG-3′) for species-specific detection of *V. parahaemolyticus* (Kim et al., 1999); (ii) tdh (F: 5′-CCACTACCA CTCTCATATGC-3′ and R: 5′-GGTACTAAATGGCTGACATC-3′) for the detection of pathogenic *tdh* gene (Tada et al., 1992); and (iii) trh (F: 5′-TTGGCTTCGATATTTTCAGTATCT-3′ and R: 5′-CATAACAAACATATGCCCATTTCCG-3′) for the detection of pathogenic *trh* gene (Bej et al., 1999). All primers were synthesized by Sigma-Aldrich (USA) and PCR protocol was carried out according to the method as described by Malcolm et al. (2015). Briefly, PCR reagents (Promega, USA) were prepared by mixing 1.4 × PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 μM of each primer, 2.5U of Taq polymerase, 2 μL of DNA template and top up to the final volume of 25 μL with sterilized ultrapure water. Amplification was performed in a Kyratec SuperCycler Trinity (Australia) with the following thermocycling conditions: initial denaturation at 95°C for 5 min for 1 cycle, 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, extension at 68°C for 1 min, and a final extension at 72°C for 3 min.

Antibiotic Susceptibility Test

One *V. parahaemolyticus* colony from each sampling date was collected for antibiotic susceptibility test. A total of 67 *V. parahaemolyticus* colonies isolated from short mackerel on 67 sampling date were tested for antibiotic susceptibility by using disc diffusion method. Isolates were cultured in 5 mL of Mueller-Hinton broth (Merck, Darmstadt, Germany) supplemented with 3% (w/v) of NaCl (Merck, Darmstadt, Germany) and incubated at 37°C, 120 rpm for 24 h. Inoculums were swabbed with the sterile cotton swab on the entire surface of Mueller-Hinton agar (Merck, Darmstadt, Germany) supplemented with 3% (w/v) of NaCl and left to dry for 3–5 min. Antimicrobial susceptibility test discs (Oxoid, UK) were placed on the inoculated agar plate with a disc dispenser and incubated at 37°C for 24 h. A total of 17 antimicrobial susceptibility test discs impregnated with ampicillin (10 μg), ampicillin sulbactam (20 μg), amikacin (30 μg), amoxicillin/clavulanic acid (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), doxycycline (30 μg), gentamicin (10 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), penicillin G (10 unit), streptomycin (10 μg), and tetracycline (30 μg) were used in this study. *Escherichia coli* ATCC 25922 was used as quality control organism for this antibiotic susceptibility test. After incubation, the diameter of inhibition zone was measured in nearest whole millimeter. Antibiotic susceptibility profile of the isolate was interpreted as sensitivity, intermediate, and resistance based on the Clinical and Laboratory Standards Institute (CLSI) M45 guideline for *Vibrio* spp. (CLSI, 2010). Interpretive criteria for doxycycline, streptomycin and penicillin G not available in the M45 guidelines was referred to CLSI M100 guideline (CLSI, 2016). Multiple antibiotic resistance (MAR) index value was calculated according to Krumperman (1983) by using the formula, a/b, where "a" is the number of antibiotics to which the particular isolate was resistant and "b" is the total number of antibiotics tested.

Statistical Analysis

Statistically significant differences among the sample microbial loads and sampling locations were analyzed with analysis of variance (ANOVA) tests using Minitab statistical package version 16.2 (Minitab Inc., State College, PA). The level of significance was set at $P \leq 0.05$. Significant differences between the microbial concentrations in the flesh, gills, and intestines of short mackerel were examined as well.

RESULTS

Total *V. parahaemolyticus* in Short Mackerels

Prevalence and microbial loads of total *V. parahaemolyticus* in short mackerels are summarized in **Table 1**. DNA fragments of 368 bp in size were produced from the amplification of *V. parahaemolyticus* species-specific gene (*toxR*) indicating the presence of total *V. parahaemolyticus*. Out of 390 short mackerel tested samples, 310 (79.5%) samples were detected with the presence of total *V. parahaemolyticus* (*toxR*) and the microbial loads was between <3 to $>10^5$ MPN/g. Highest prevalence of total *V. parahaemolyticus* was detected in the samples obtained from wet markets (78.6–95.2%), followed by the minimarkets (82.6–89.1%) and hypermarkets (50.0–83.3%). Highest density of total *V. parahaemolyticus* were found in short mackerel gills with mean concentration of 3.66 log MPN/g, followed by intestines with mean concentration of 2.67 log MPN/g and flesh with mean concentration of 1.74 log MPN/g. Besides, 33.9% (105/310) of the samples were detected with high levels ($\geq 10^4$ MPN/g) of total *V. parahaemolyticus*.

Overall, the prevalence of total *V. parahaemolyticus* in short mackerel whole-body was 89.2% (116/130). A total of 95.2% (40/42), 89.1% (41/46), and 83.3% (35/42) of the short mackerel samples obtained from the wet markets, minimarkets, and hypermarkets, respectively, were detected with the presence of total *V. parahaemolyticus*. For statistical analyses, samples collected from the hypermarkets was detected significant lower ($P < 0.05$) from the wet markets and minimarkets. The level of total *V. parahaemolyticus* in short mackerel flesh, gills, and intestines were also showed significantly different ($P < 0.05$) from each other.

Pathogenic *V. parahaemolyticus* Strains in Short Mackerels

Prevalence and microbial loads of pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) strains in short mackerels are shown in **Tables 2, 3**. DNA fragments of 484 and 251 bp in size were produced from the amplification of *V. parahaemolyticus* pathogenic *trh* and *tdh* genes, respectively. A total of 33 out of 390 (8.5%) tested samples were detected positive for the *tdh*

TABLE 1 | Prevalence and microbial loads of total *V. parahaemolyticus* in short mackerels.

Sampling location	Samples	Number of sample	Number of positive sample (%)	Level of total (*toxR*) *V. parahaemolyticus* (MPN/g)				
				<100	10^2	10^3	10^4	$>10^5$
Wet market	Flesh	42	39 (92.9%)	19	6	8	2	4
	Gills	42	40 (95.2%)	1	4	17	8	10
	Intestines	42	33 (78.6%)	1	8	6	9	9
Hypermarket	Flesh	42	23 (54.8%)	17	3	3	–	–
	Gills	42	35 (83.3%)	–	7	19	6	3
	Intestines	42	21 (50.0%)	–	8	10	2	1
Minimarket	Flesh	46	40 (87.0%)	20	11	5	1	3
	Gills	46	41 (89.1%)	–	2	9	12	18
	Intestines	46	38 (82.6%)	1	9	11	6	11
	Total=	390	310 (79.5%)	59	58	88	46	59

TABLE 2 | Prevalence and microbial loads of pathogenic *tdh*+ and *trh*− *V. parahaemolyticus* strains in short mackerels.

Sampling location	Samples	Number of sample	Number of positive sample (%)	Level of (*tdh*+ and *trh*−) *V. parahaemolyticus* (MPN/g)				
				<100	10^2	10^3	10^4	$>10^5$
Wet market	Flesh	42	2 (4.8%)	2	–	–	–	–
	Gills	42	4 (9.5%)	1	1	1	1	
	Intestines	42	3 (7.1%)	–	1	–	–	2
Hypermarket	Flesh	42	3 (7.1%)	2	1	–	–	–
	Gills	42	4 (9.5%)	–	1	2	–	1
	Intestines	42	3 (7.1%)	–	2	–	–	1
Minimarket	Flesh	46	5 (10.9%)	2	–	–	1	2
	Gills	46	5 (10.9%)	–	1	1	1	2
	Intestines	46	4 (8.7%)	–	1	1	–	2
	Total=	390	33 (8.5%)	7	8	5	3	10

TABLE 3 | Prevalence and microbial loads of pathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* strains in short mackerels.

Sampling location	Samples	Number of sample	Number of positive sample (%)	Level of (*tdh*+ and *trh*+) *V. parahaemolyticus* (MPN/g)				
				<100	10^2	10^3	10^4	$>10^5$
Wet market	Flesh	42	0 (0%)	–	–	–	–	–
	Gills	42	0 (0%)	–	–	–	–	–
	Intestines	42	2 (4.8%)	–	2	–	–	–
Hypermarket	Flesh	42	0 (0%)	–	–	–	–	–
	Gills	42	1 (2.4%)	–	1	–	–	–
	Intestines	42	1 (2.4%)	–	1	–	–	–
Minimarket	Flesh	46	0 (0%)	–	–	–	–	–
	Gills	46	0 (0%)	–	–	–	–	–
	Intestines	46	0 (0%)	–	–	–	–	–
	Total=	390	4 (1.0%)	–	4	–	–	–

gene (*tdh*+ and *trh*−) and the microbial loads ranged from 3.6 to $>10^5$ MPN/g. Strains of *V. parahaemolyticus* harbored both the *tdh* and *trh* genes (*tdh*+ and *trh*+) were detected in 4 out of 390 (1.0%) tested samples and the density of *tdh*+ and *trh*+ *V. parahaemolyticus* strains found in the samples ranged from 300 to 740 MPN/g. *V. parahaemolyticus* strain carrying only the *trh* gene (*trh*+ and *tdh*-) was not detected in this study.

Overall, the prevalence of pathogenic *V. parahaemolyticus* strain (*tdh*+ and/or *trh*+) in short mackerel whole-body was 16.2% (21/130). The presence of pathogenic *V. parahaemolyticus* strains can be found in either combination or alone in each part of short mackerel flesh, gills, and intestines. For statistical analyses, the level of pathogenic (*tdh*+ and/or *trh*+) *V. parahaemolyticus* was detected non-significantly different ($P > 0.05$) in various sampling locations and tested samples.

Antibiotics Susceptibility Profile of *V. parahaemolyticus* Isolates

Antibiotic susceptibility profiles of *V. parahaemolyticus* isolated from short mackerel samples are shown in **Table 4**. The majority of isolates tested were susceptible to all of the antibiotics. Isolates tested were highly susceptible to antibiotics such as ampicillin sulbactam (100%), meropenem (100%), ceftazidime (98.5%), and imipenem (98.5%). High level of resistance was observed to penicillin G (92.5%) and ampicillin (82.1%). Of 67 isolates, 60 (89.6%) showed resistant to two or more antibiotics. MAR index value of *V. parahaemolyticus* isolates from short mackerel samples are summarized in **Table 5**. Most of the isolates (40.3%) were detected with MAR index value of 0.12 followed by 16.4 and 13.4% of isolates with MAR index value of 0.29 and 0.24, respectively. Two (3.0%) isolates exhibited the highest MAR index value of 0.41 which shown resistance to 7 types of antibiotics.

TABLE 4 | Antibiotic susceptibility profiles of *V. parahaemolyticus* isolated from short mackerels by disc diffusion method.

Antibiotics	Interpretive category result		
	Resistant (%)	Intermediate (%)	Sensitivity (%)
Ampicillin (10 μg)	55 (82.09)	5 (7.46)	7 (10.45)
Ampicillin sulbactam (20 μg)	–	–	67 (100)
Amikacin (30 μg)	15 (22.39)	8 (11.94)	44 (65.67)
Amoxicillin/clavulanic acid (30 μg)	3 (4.48)	3 (4.48)	61 (91.04)
Ceftazidime (30 μg)	–	1 (1.49)	66 (98.50)
Cefotaxime (30 μg)	1 (1.49)	21 (31.34)	45 (67.16)
Cephalothin (30 μg)	21 (31.34)	14 (20.90)	32 (47.76)
Chloramphenicol (30 μg)	6 (8.96)	–	61 (91.04)
Ciprofloxacin (5 μg)	3 (4.48)	25 (37.31)	39 (58.21)
Doxycycline (30 μg)	2 (2.99)	2 (2.99)	63 (94.03)
Gentamicin (10 μg)	7 (10.45)	10 (14.93)	50 (74.63)
Imipenem (10 μg)	–	1 (1.49)	66 (98.50)
Levofloxacin (5 μg)	1 (1.49)	8 (11.94)	58 (86.57)
Meropenem (10 μg)	–	–	67 (100)
Penicillin G (10 μg)	62 (92.54)	–	5 (7.46)
Streptomycin (10 μg)	36 (53.73)	9 (13.43)	22 (32.84)
Tetracycline (30 μg)	6 (8.96)	5 (7.46)	56 (83.58)

DISCUSSION

High prevalence and microbial loads of total *V. parahaemolyticus* were detected in 89.2% (116/130) of short mackerel samples. Similar results were found in the Hara-kudo et al. (2003) study where 15 out of 17 (88.2%) horse mackerel samples in Japan were detected with the presence of total *V. parahaemolyticus*. However, the incidence rate of total *V. parahaemolyticus* in short mackerels was comparatively higher than many other prevalence studies in other countries. For instance, Baffone et al. (2000) revealed 2.6% (3/114) fish samples including of anchovies, gray mullet, red mullet, sardines, Atlantic mackerel, and other species common to the Adriatic Sea were detected with the presence of *V. parahaemolyticus*. Wong et al. (1999) reported 29.3% of a total 92 fish samples imported from Indonesia were detected with total *V. parahaemolyticus*. Nakaguchi (2013) reported an average of 57.4% fish samples obtained from Asia countries including Vietnam, Indonesia, and Malaysia were

TABLE 5 | Multiple antibiotic resistance (MAR) index value of *V. parahaemolyticus* isolates from short mackerel samples.

MAR index	Antibiotic resistance profiles[a]	Isolates code	Percentage of isolates (%)
0.41	Amp, Ak, Amc, Kf, C, P, S	V8	1.5
	Amp, Ak, Kf, C, Cip, P, S	V11	1.5
0.35	Amp, Ak, Kf, Cn, P, S	V80, V85	3.0
	Amp, Ak, Amc, Kf, C, P	V9	1.5
0.29	Amp, Ak, Kf, P, S	V40, V87, V89	4.5
	Amp, Kf, Cn, P, S	V81, V91	3.0
	Amp, Kf, C, P, Te	V5	1.5
	Amp, Amc, C, P, S	V7	1.5
	Amp, Kf, Cip, P, S	V51	1.5
	Amp, Cn, Lev, P, S	V53	1.5
	Amp, Ak, Cn, P, S	V54	1.5
	Amp, Ak, C, P, S	V54	1.5
0.24	Amp, Kf, P, S	V46, V78, V84, V92	6.0
	Amp, Ak, Kf, P	V39, V58	3.0
	Amp, Kf, P, Te	V25	1.5
	Amp, Ak, P, S	V52	1.5
	Amp, Cn, P, S	V90	1.5
0.18	Amp, P, S	V44, V50, V86	4.5
	Amp, Kf, P	V16	1.5
	Amp, Ak, P	V26	1.5
	Ctx, Kf, P	V38	1.5
	Ak, Cip, S	V57	1.5
0.12	Amp, P	V1, V3, V10, V12, V13, V18, V27-37, V43, V45, V48, VP72-75, V77	37.3
	Do, Te	V22, V23	3.0
0.06	P	V4, V6, V14, V19, V41, V42	9.0
	Te	V20, V21	3.0

[a]*Amp, ampicillin; Ak, amikacin; Amc, amoxicillin/clavulanic acid; Ctx, cefotaxime; Kf, cephalothin; C, chloramphenicol; Cip, ciprofloxacin; Do, doxycycline; Cn, gentamicin; Lev, levofloxacin; P, penicillin G; S, streptomycin; Te, tetracycline.*

found with total *V. parahaemolyticus*. Total *V. parahaemolyticus* were found more prevalently in the samples obtained from wet markets (78.6–97%) followed by minimarkets (82.6–89.1%) and hypermarkets (50.0–83.3%) in this study. Similar study findings reported by Letchumanan et al. (2015a) also found a high level of *V. parahaemolyticus* contamination in shrimp samples purchased from wet markets compared to supermarkets. The reason for these findings may due to the poor storage handling practices by the wet market retailers. For instance, seafood especially cheaper fishes such as short mackerels in the wet markets were usually displayed on a tray with little or no ice left amongst the fish. Additionally, finfish are generally sent directly from the farm to wet markets retailers under minimum temperature controlled.

Microbial loads of total *V. parahaemolyticus* in short mackerel tested samples including flesh, gills, and intestines obtained from the hypermarkets were detected significant lower ($P < 0.05$) from the wet markets and minimarkets. The study findings are in agreement with another study reported the surf clams obtained from hypermarkets were found to contain lower density of *V. parahaemolyticus* (Malcolm et al., 2015). In general, seafood is extremely perishable which needs strictly controlled storage requirements to maintain their freshness and limit the growth of harmful microorganisms. In this study, samples obtained from hypermarkets were found to contain significant lower number of total *V. parahaemolyticus* ($P < 0.05$). This might due to the well-established cold chains and cold storage facilities available in the hypermarkets. Low temperature controlled in supply chains and storage facilities available in the hypermarkets which therefore help reduce the growth of total *V. parahaemolyticus* in short mackerels. Su and Liu (2007) also reported that *V. parahaemolyticus* are cold sensitive thus preserving seafood on low temperature may limit or reduce their growth.

Short mackerel gills were detected with the highest total *V. parahaemolyticus* microbial loads with a mean concentration of 3.66 log MPN/g compared to intestines with a mean concentration of 2.67 log MPN/g and flesh with a mean concentration of 1.74 log MPN/g. Hairy structures of gills provided large surface areas for the bacteria adsorption is suggested one of the reasons where short mackerel gills were detected with the highest density of total *V. parahaemolyticus*. In Malaysia, there is no standard safety limit or minimum allowable level of *V. parahaemolyticus* in seafood. Safety regulations and standards for fish and fishery product established from other countries such as the United States are referred. According to the FDA microbiological safety limits for *V. parahaemolyticus* in ready-to-eat fish products must be $<1 \times 10^4$ per gram (FDA, 2011). In this study, 33.9% of short mackerels exceed the microbiological safety levels but fish evisceration, washing, rinsing, and cooking process done by food handlers during food preparation is suggested capable of achieving certain reductions of *V. parahaemolyticus*. For examples, Watanabe et al. (1994) demonstrated the effectiveness of washing of the horse mackerel eviscerated cavity with clean water resulted in 1.99 log reduction of *V. parahaemolyticus*. Ye et al. (2012) reported mild heat treatment at 50°C for 20 min was capable of reducing the number of *V. parahaemolyticus* to below detection limit (<3 MPN/g).

Most of the *V. parahaemolyticus* clinical isolates exhibit Kanagawa positive or negative possessing the hemolysin *tdh* and/or *trh* genes. Only small percentage of *V. parahaemolyticus* isolates from food and environmental samples carrying *tdh* and/or *trh* genes. This statement was in agreement with our findings as *V. parahaemolyticus* strains with *tdh* gene and strains with both *tdh* and *trh* genes were detected in low level, 8.5 and 1.0%, respectively, in short mackerels. No *trh*+ *V. parahaemolyticus* strains was found in all samples can be associated with the warmer climate in Malaysia as Rodriguez-Castro et al. (2010) reported that *trh*+ strains more prevailing in the coldest water and *tdh*+ *V. parahaemolyticus* tends to disseminate in the warmer water. Even in clinical *V. parahaemolyticus* strains only minority of the isolates carried the *trh* genes. Suthienkul et al. (1995) reported merely 10 out of 489 (2%) *V. parahaemolyticus* isolates isolated from patients with acute gastroenteritis possessed the *trh* genes and 27 out

of 489 (6%) isolates harbored both the *trh* and *tdh* genes. Bhoopong et al. (2007) revealed only 0.5% (3/629) of the clinical *V. parahaemolyticus* isolates from the 63 patients in Thailand carried the *trh* gene alone, compared to 87.4% (550/629) and 7% (44/629) of the isolates possessed the *tdh* gene and both genes, respectively. Chen et al. (2016) reported 93% and 1% of the 501 clinical *V. parahaemolyticus* isolates from southeastern China were carried *tdh* gene and *trh* gene, respectively. However, distributions of *tdh*+ and/or *trh*+ strains may vary depend on the geographical region, sample source, and detection method (Raghunath, 2015).

V. parahaemolyticus isolates obtained from short mackerels displayed a high level of resistance to penicillin and ampicillin. This finding was consistent with the results reported by Letchumanan et al. (2015a) where 82% of the isolates from shrimp samples were resistant to ampicillin. Besides, Elexson et al. (2014) reported all of the *V. parahaemolyticus* isolates from cultured seafood products were resistant to both penicillin and ampicillin. Based on these findings, resistant to penicillin and ampicillin could be probably due to extensively used of antibiotics in aquaculture and the impact of antimicrobial residues in aquatic systems. It is also likely due to the complexity of the Gram-negative bacteria outer membrane which inhibits antibiotic compounds to pass through the outer membrane (IFT, 2006; Blair et al., 2014). Penicillin and ampicillin are therefore ineffective for the treatment of *V. parahaemolyticus* infections. Nevertheless, the majority of the isolates were susceptible to most antibiotics tested in this study. Susceptibility profiles of *V. parahaemolyticus* isolates to antibiotic classes such as tetracycline, phenicols, quinolone, and cephalosporin were comparable with many other studies reported in several countries and sample sources (Lesmana et al., 2001; Han et al., 2007; Yano et al., 2011; Ottaviani et al., 2013). In this study, 37.31% of isolates with MAR value more than 0.2 indicating samples originated from a high risk source of contamination where several antibiotics are used (Krumperman, 1983). Widespread usage of antibiotics in clinical, agriculture, aquaculture, and livestock production can result in antimicrobial residues present in the environment and dispersion of antimicrobial residues that reach the marine environment could lead to selective pressure on the marine bacteria and the emergence of MDR bacterial strains in marine life.

Misuse and overuse of antibiotics are recognized as two of the major factors contribute to the development of resistance genes in bacteria and widespread dissemination of MDR bacterial strains. An alternative to antibiotics is urgently needed in order to overcome the continuous emergence of MDR bacterial strains in the environment (Tan et al., 2016). Research on alternative approaches such as the use of bacteriophage and probiotics has become possible solution to replace or reduce the use of antibiotics. Bacteriophages are bacteria viruses with the ability to attack and destroy bacteria cells. Jun et al. (2014) demonstrated bacteriophage-based therapies displays effectual protection against multiple antibiotics resistant *V. parahaemolyticus* strains infection in mice. Lomelí-Ortega and Martínez-Díaz (2014) reported two isolated lytic bacteriophages can be used to control vibriosis in whiteleg shrimp larvae (*Litopenaeus vannamei*). Probiotics are organisms or substances that bring beneficial effect to the host (Hai, 2015). Tan et al. (2016) proposed the feed supplemented with the genus of *Streptomyces* bacteria as probiotics could protect aquaculture livestock from pathogens and enhance the growth performance of the aquatic cultured organisms. Augustine et al. (2016) demonstrated *Streptomyces rubrolavendulae* M56 biogranules showed a competitive exclusion effect on *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis,* and *V. harveyi* can be used as a promising alternative to the use of antibiotics in the prawn larval production systems. Although the application of bacteriophages and probiotics offer promising alternative to antibiotics, efforts for continuous monitoring of *V. parahaemolyticus* antibiotic resistance patterns and risk assessment on the use of antibiotics in therapeutic and non-therapeutic purposes is still needed to overcome the development of MDR bacterial strains.

CONCLUSION

V. parahaemolyticus not only accumulates in shellfish and crustaceans but also can be found in the short mackerels at high concentrations. *V. parahaemolyticus* multiply more rapidly and reach the highest concentrations during warmer months. In Malaysia, warm temperatures remain fairly constant all year round thus it is expected high initial concentrations of *V. parahaemolyticus* can be found in the seafood. Preserving seafood at low temperature conditions can limit and control the growth of *V. parahaemolyticus*. Unbroken cold chain and cold storage facilities available in the hypermarkets maintained seafood products freshness as well as minimized the growth of foodborne pathogen such as *V. parahaemolyticus*. The low detection rate of pathogenic *V. parahaemolyticus* strains in the short mackerels may still at risk for food poisoning if storage conditions and preparation procedures are not receiving proper attention.

AUTHOR CONTRIBUTIONS

CT is the corresponding author for this work. TM, CK, TT, WC, YL, JP, OR, MN, and NY provided assistance and guidance in throughout the research. YR, YN, MN, and SR are the mentor in the research study and assist in manuscript checking.

ACKNOWLEDGMENTS

This research was funded by a Research University Grant Scheme Initiative Six (RUGS 6) of Universiti Putra Malaysia (GP-IPS 9438703) and Fundamental Research Grant Scheme (FRGS) of Ministry of Higher Education (MOHE), Malaysia (02-01-14-1475FR) and, in part, by the Kakenhi Grant-in-Aid for Scientific Research (KAKENHI 24249038), Japan Society for the Promotion of Sciences and grant-in-aid of Ministry of Health, Labor and Welfare, Japan.

REFERENCES

ASEAN (2013). *Appendix II: List of Chemicals Used in Aquaculture by ASEAN Member States*. Available online at: http://www.asean.org/wp-content/uploads/images/Community/AEC/AMAF/UpdateApr2014/ASEAN%20Guideliness%20for%20Chemicals%20Final%20Draft%20Malaysia%20OK.pdf

Augustine, D., Jacob, J. C., and Philip, R. (2016). Exclusion of *Vibrio* spp. by an antagonistic marine actinomycete *Streptomyces rubrolavendulae* M56. *Aquacult. Res.* 47, 2951–2960. doi: 10.1111/are.12746

Baba, K., Yamasaki, S., Nishibuchi, M., and Takeda, Y. (1992). Examination by site-directed mutagenesis of the amino acid residues of the thermostable direct hemolysin of *Vibrio parahaemolyticus* required for its hemolytic activity. *Microb. Pathog.* 12, 279–287. doi: 10.1016/0882-4010(92)90046-Q

Baffone, W., Pianetti, A., Bruscolini, F., Barbieri, E., and Citterio, B. (2000). Occurrence and expression of virulence-related properties of Vibrio species isolated from widely consumed seafood products. *Int. J. Food Microbiol.* 54, 9–18. doi: 10.1016/S0168-1605(99)00189-0

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C. L., Jones, D. D., and Kaysner, C. A. (1999). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh, tdh and trh. *J. Microbiol. Methods* 36, 215–225. doi: 10.1016/S0167-7012(99)00037-8

Bhoopong, P., Palittapongarnpim, P., Pomwised, R., Kiatkittipong, A., Kamruzzaman, M., Nakaguchi, Y., et al. (2007). Variability of properties of *Vibrio parahaemolyticus* strains isolated from individual patients. *J. Clin. Microbiol.* 45, 1544–1550. doi: 10.1128/JCM.02371-06

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. V. (2014). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Chen, Y., Chen, X., Yu, F., Wu, M., Wang, R., Zheng, S., et al. (2016). Serology, virulence, antimicrobial susceptibility and molecular characteristics of clinical *Vibrio parahaemolyticus* strains circulating in southeastern China from 2009 to 2013. *Clin. Microbiol. Infect.* 22, 258.e9–258.e16. doi: 10.1016/j.cmi.2015.11.003

CLSI (2010). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline, 2nd Edn*. CLSI document M45-A2. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2016). *Performance Standards for Antimicrobial Susceptibility Testing, 26th Edn*. CLSI supplement M100S. Wayne, PA: Clinical and Laboratory Standards Institute.

Elexson, N., Afsah-Hejri, L., Rukayadi, Y., Soopna, P., Lee, H. Y., Tuan Zainazor, T. C., et al. (2014). Effect of detergents as antibacterial agents on biofilm of antibiotics-resistant *Vibrio parahaemolyticus* isolates. *Food Control.* 35, 378–385. doi: 10.1016/j.foodcont.2013.07.020

Fabbri, A., Falzano, L., Frank, C., Donelli, G., Matarrese, P., Raimondi, F., et al. (1998). *Vibrio parahaemolyticus* thermostable direct hemolysin modulates cytoskeletal organization and calcium homeostasis in intestinal cultured cells. *Infect. Immun.* 67, 1139–1148.

FAO (2016). *Drivers, Dynamics and Epidemiology of Antimicrobial Resistance in Animal Production.* Available online at: http://www.fao.org/3/a-i6209e.pdf

FDA (2011). *Appendix 5: FDA and EPA Safety Levels in Regulations and Guidance.* Available online at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM251970.pdf

Hai, N. V. (2015). The use of probiotics in aquaculture. *J. Appl. Microbiol.* 119, 917–935. doi: 10.1111/jam.12886

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W., and Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Appl. Environ. Microbiol.* 73, 7096–7098. doi: 10.1128/AEM.01116-07

Hara-kudo, Y., Sugiyama, K., Nishibuchi, M., Chowdhury, A., Yatsuyanagi, J., Saito, A., et al. (2003). Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. *Appl. Environ. Microbiol.* 69, 3883–3891. doi: 10.1128/AEM.69.7.3883-3891.2003

Honda, T., and Iida, T. (1993). The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. *Rev Med Microbiol.* 4, 106–113. doi: 10.1097/00013542-199304000-00006

Honda, T., Ni, Y. X., and Miwatani, T. (1988). Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect. Immun.* 56, 961–965.

Honda, T., Ni, Y., Miwatani, T., Adachi, T., and Kim, J. (1992). The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. *Can. J. Microbiol.* 38, 1175–1180. doi: 10.1139/m92-192

Huang, J. Y., Henao, O. L., Griffin, P. M., Vugia, D. J., Cronquist, A. B., Hurd, S., et al. (2016). Infection with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance – foodborne diseases active surveillance network, 10 U.S. sites, 2012-2015. *Morb. Mortal. Wkly. Rep.* 65, 368–371. doi: 10.15585/mmwr.mm6514a2

IFT (2006). Antimicrobial resistance: implications for the food system. *Compr. Rev. Food Sci. Food Saf.* 5, 71-137. doi: 10.1111/j.1541-4337.2006.00004.x

Jun, J. W., Shin, T. H., Kim, J. H., Shin, S. P., Han, J. E., Heo, G. J., et al. (2014). Bacteriophage therapy of a *Vibrio parahaemolyticus* infection caused by a multiple-antibiotic-resistant O3:K6 pandemic clinical strain. *J. Infect. Dis.* 210, 72–78. doi: 10.1093/infdis/jiu059

Kaysner, C. A., and DePaola, A. J. (2004). *BAM: Vibrio.* Available online at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm

Kim, Y. B. U., Okuda, J. U. N., Matsumoto, C., Takahashi, N., Hashimoto, S., and Nishibuchi, M. (1999). Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. *J. Clin. Microbiol.* 37, 1173–1177.

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* 46, 165–170.

Lesmana, M., Subekti, D., Simanjuntak, C. H., Tjaniadi, P., Campbell, J. R., and Oyofo, B. A. (2001). *Vibrio parahaemolyticus* associated with cholera-like diarrhea among patients in North Jakarta, Indonesia. *Diagn. Microbiol. Infect. Dis.* 39, 71–75. doi: 10.1016/S0732-8893(00)00232-7

Letchumanan, V., Yin, W. F., Lee, L. H., and Chan, K. G. (2015a). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Front. Microbiol.* 6:33. doi: 10.3389/fmicb.2015.00033

Letchumanan, V., Yin, W. F., Lee, L. H., and Chan, K. G. (2015b). Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* from shellfish in Selangor, Malaysia. *Front. Microbiol.* 6:1417. doi: 10.3389/fmicb.2015.01417

Lomelí-Ortega, C. O., and Martínez-Díaz, S. F. (2014). Phage therapy against *Vibrio parahaemolyticus* infection in the whiteleg shrimp (*Litopenaeus vannamei*) larvae. *Aquaculture* 434, 208–211. doi: 10.1016/j.aquaculture.2014.08.018

Malcolm, T. T. H., Cheah, Y. K., Radzi, C. W. J. W. M., Kasim, F. A., Kantilal, H. K., John, T. Y. H., et al. (2015). Detection and quantification of pathogenic *Vibrio parahaemolyticus* in shellfish by using multiplex PCR and loop-mediated isothermal amplification assay. *Food Control.* 47, 664–671. doi: 10.1016/j.foodcont.2014.08.010

Nakaguchi, Y. (2013). Contamination by *Vibrio parahaemolyticus* and its virulent strains in seafood marketed in Thailand, Vietnam, Malaysia, and Indonesia. *Trop. Med. Health* 41, 95–102. doi: 10.2149/tmh.2011-06

Newton, A. E., Garrett, N., Stroika, S. G., Halpin, J. L., Turnsek, M., and Mody, R. K. (2014). Increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic Coast shellfish - 2013. *Morb. Mortal. Wkly. Rep.* 63, 335–336.

Nishibuchi, M., Taniguchi, T., Misawa, T., Khaeomanee-Iam, V., Honda, T., and Miwatani, T. (1989). Cloning and nucleotide sequence of the gene (trh) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infect. Immun.* 57, 2691–2697.

Ohno, S., Tazawa, T., Kon, M., Uno, Y., Terao, M., and Goto, K. (1993). *Contamination by Vibrio parahaemolyticus of Fish Landed at Fishery Markets in Niigata Prefecture in Japanese.* Niigataken Eisei Kogai Kenkyusyo Nenpo [Annual Report of Niigata Prefectural Research Laboratory for Health and Environment] 9, 77–82.

Ottaviani, D., Leoni, F., Talevi, G., Masini, L., Santarelli, S., Rocchegiani, E., et al. (2013). Extensive investigation of antimicrobial resistance in *Vibrio parahaemolyticus* from shellfish and clinical sources, Italy. *Int. J. Antimicrob. Agents* 42, 191–193. doi: 10.1016/j.ijantimicag.2013.05.003

Raghunath, P. (2015). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Front. Microbiol.* 5:805. doi: 10.3389/fmicb.2014.00805

Raimondi, F., Kao, J. P. Y., Fiorentini, C., Fabbri, A., Donelli, G., Gasparini, N., et al. (2000). Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in *in vitro* systems. *Infect. Immun.* 68, 3180–3185. doi: 10.1128/IAI.68.6.3180-3185.2000

Rodgers, C., Parveen, S., Chigbu, P., Jacobs, J., Rhodes, M., and Harter-Dennis, J. (2014). Prevalence of *Vibrio parahaemolyticus*, and *Vibrio vulnificus* in blue crabs (*Callinectes sapidus*), seawater and sediments of the Maryland Coastal Bays. *J. Appl. Microbiol.* 117, 1198–1209. doi: 10.1111/jam.12608

Rodriguez-Castro, A., Ansede-Bermejo, J., Blanco-Abad, V., Varela-Pet, J., Garcia-Martin, O., and Martinez-Urtaza, J. (2010). Prevalence and genetic diversity of pathogenic populations of *Vibrio parahaemolyticus* in coastal waters of Galicia, Spain. *Environ Microbiol Rep.* 2, 58–66. doi: 10.1111/j.1758-2229.2009.00064.x

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States - major pathogens. *Emerging Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.P11101

Shariff, M., Nagaraj, G., Chua, F. H. C., and Wang, Y. G. (2000). "The use of chemicals in aquaculture in Malaysia and Singapore," in *Proceedings of the Meeting on the Use of Chemicals in Aquaculture in Asia* (Iloilo), 127–140.

Su, Y. C., and Liu, C. (2007). *Vibrio parahaemolyticus*: a concern of seafood safety. *Food Microbiol.* 24, 549–558. doi: 10.1016/j.fm.2007.01.005

Suthienkul, O., Ishibashi, M., Iida, T., Nettip, N., Supavej, S., Eampokalap, B., et al. (1995). Urease production correlates with possession of the trh gene in *Vibrio parahaemolyticus* strains isolated in Thailand. *J. Infect. Dis.* 172, 1405–1408. doi: 10.1093/infdis/172.5.1405

Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, H., Fukushima, S., et al. (1992). Detection of the thermostable direct hemolysin gene (tdh) and the thermostable direct hemolysin-related hemolysin gene (trh) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol. Cell. Probes* 6, 477–487. doi: 10.1016/0890-8508(92)90044-X

Takahashi, A., Kenjyo, N., Imura, K., Myonsun, Y., and Honda, T. (2000). Cl-secretion in colonic epithelial cells induced by the *Vibrio parahaemolyticus* hemolytic toxin related to thermostable direct hemolysin. *Infect. Immun.* 68, 5435–5438. doi: 10.1128/IAI.68.9.5435-5438.2000

Tan, L. T. H., Chan, K. G., Lee, L. H., and Goh, B. H. (2016). Streptomyces bacteria as potential probiotics in aquaculture. *Front. Microbiol.* 7:79. doi: 10.3389/fmicb.2016.00079

Van Hoek, A. H. A. M., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., and Aarts, H. J. M. (2011). Acquired antibiotic resistance genes: an overview. *Front. Microbiol.* 2:203. doi: 10.3389/fmicb.2011.00203

Watanabe, T., Tazawa, T., Kon, M., Ohno, S., Uno, Y., Terao, M., et al. (1994). "Growth of *Vibrio parahaemolyticus* in different methods of cooking fish," in *Proceeding of the National Conference of Food Safety Inspection*, 113–116.

Weese, J. S., Giguère, S., Guardabassi, L., Morley, P. S., Papich, M., Ricciuto, D. R., et al. (2015). ACVIM consensus statement on therapeutic antimicrobial use in animals and antimicrobial resistance. *J. Vet. Intern. Med.* 29, 487–498. doi: 10.1111/jvim.12562

Wong, H. C., Chen, M. C., Liu, S. H., and Liu, D. P. (1999). Incidence of highly genetically diversified *Vibrio parahaemolyticus* in seafood imported from Asian countries. *Int. J. Food Microbiol.* 52, 181–188. doi: 10.1016/S0168-1605(99)00143-9

Wong, K. C., Brown, A. M., Luscombe, G. M., Wong, S. J., and Mendis, K. (2015). Antibiotic use for Vibrio infections: important insights from surveillance data. *BMC Infect. Dis.* 15:226. doi: 10.1186/s12879-015-0959-z

Xu, X., Wu, Q., Zhang, J., Cheng, J., Zhang, S., and Wu, K. (2014). Prevalence, pathogenicity, and serotypes of *Vibrio parahaemolyticus* in shrimp from Chinese retail markets. *Food Control* 46, 81–85. doi: 10.1016/j.foodcont.2014.04.042

Yanagihara, I., Nakahira, K., Yamane, T., Kaieda, S., Mayanagi, K., Hamada, D., et al. (2010). Structure and functional characterization of *Vibrio parahaemolyticus* thermostable direct hemolysin. *J. Biol. Chem.* 285, 16267–16274. doi: 10.1074/jbc.M109.074526

Yano, Y., Hamano, K., Satomi, M., and Tsutsui, I. (2011). Diversity and characterization of oxytetracycline-resistant bacteria associated with non-native species, white-leg shrimp (*Litopenaeus vannamei*), and native species, black tiger shrimp (Penaeus monodon), intensively cultured in Thailand. *J. Appl. Microbiol.* 110, 713–722. doi: 10.1111/j.1365-2672.2010.04926.x

Ye, M., Huang, Y., and Chen, H. (2012). Inactivation of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oysters by high-hydrostatic pressure and mild heat. *Food Microbiol.* 32, 179–184. doi: 10.1016/j.fm.2012.05.009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Tan, Malcolm, Kuan, Thung, Chang, Loo, Premarathne, Ramzi, Norshafawatie, Yusralimuna, Rukayadi, Nakaguchi, Nishibuchi and Radu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 June 2017
doi: 10.3389/fmicb.2017.01140

A Novel Approach to Predict the Growth of *Staphylococcus aureus* on Rice Cake

Jun Wang[1], *Shige Koseki*[2], *Mi-Ja Chung*[3] *and Deog-Hwan Oh*[4]*

[1] College of Food Science and Engineering, Qingdao Agricultural University, Qingdao, China, [2] Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, [3] Department of Food Science and Nutrition, College of Health, Welfare and Education, Gwangju University, Gwangju, South Korea, [4] Department of Food Science and Biotechnology, Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, South Korea

This study aimed to investigate the growth kinetics of *Staphylococcus aureus* on rice cake and to determine the shelf life based on the probability model of the increase in *S. aureus* contamination on rice cake. Secondary models were developed based on the growth parameters derived from the Baranyi model at constant temperatures (15, 25, 35, and 45°C). External validation was then conducted using additional data under experimental conditions not used in development of the models to verify the performance and reliability of the developed model through different goodness-of-fit indices. Furthermore, the growth of *S. aureus* on rice cake under dynamic temperature was obtained with the root mean square error (RMSE) of 0.218 and the 90.9% acceptable prediction rate. In addition, probability models of the 1-, 2-, 3-, and 4-log increases of *S. aureus* on rice cake were also developed from the data, which could provide the probability and the time to a certain log increase. The results of validation demonstrated that the developed predictive model and the obtained growth parameters could be used for evaluating the growth behavior of *S. aureus* on rice cake under different conditions, and qualified to supply sufficient information for microbiological risk assessment studies of *S. aureus* on rice cake in Korea.

Keywords: *Staphylococcus aureus*, predictive microbiology, rice cake, growth model, probability model

OPEN ACCESS

Edited by:
Pierina Visciano,
University of Teramo, Italy

Reviewed by:
Antonio Valero,
Universidad de Córdoba, Spain
Miriam R. Garcia,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

***Correspondence:**
Deog-Hwan Oh
deoghwa@kangwon.ac.kr

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *03 March 2017*
Accepted: *06 June 2017*
Published: *22 June 2017*

Citation:
Wang J, Koseki S, Chung M-J and Oh D-H (2017) A Novel Approach to Predict the Growth of Staphylococcus aureus on Rice Cake. Front. Microbiol. 8:1140. doi: 10.3389/fmicb.2017.01140

INTRODUCTION

Rice cake is one of the most popular Korean traditional foods. Many types of rice cake can be prepared via the use of different ingredients and different manufacturing processes. *Gyungdan,* a ball-shaped rice cake filled with delicious, sweet red bean paste and rolled in sesame seeds, is the most common variety of "tteok." The major ingredients of rice cakes are rice flour and water, which causes rapid starch retrogradation during storage (Riva et al., 2000; Wu et al., 2009). Korean traditional rice cakes are generally packaged with linear low-density polyethylene after steaming and cooling at room temperature, and then distributed throughout markets (Lee et al., 2011). Oh et al. (2007) reported that 19.3% of the rice cakes with filling in Korea were contaminated with *Staphylococcus aureus.* The most important bacteria causing food poisoning in Korea are *Salmonella* spp., *Vibrio* spp., and *S. aureus.* These bacteria account for 85–90% of the outbreaks and cases of illness in Korea (Park et al., 2001). In addition, *Salmonella* spp., *S. aureus*, and *Bacillus cereus* are the major microbiological hazards of cereal grains and related products (FDA, 2003).

In the past decades, foodborne illness has been a serious public health issue in both developed and developing countries. *S. aureus* is able to produce a variety of toxins and many disease syndromes, and many detection method of *S. aureus* were developed and reported (Dabrowski and Medrala, 2004; Zhao et al., 2014; Li et al., 2016). In the United States, total 17 foodborne outbreaks caused by *Staphylococcus* enterotoxin and 566 outbreak-associated illnesses were reported to the CDC in 2014 (CDC, 2016). In Korea, 22.76% of the 10,676 foodborne illnesses from 1981 to 1995 caused by bacteria were associated with *S. aureus* strains, while 10.8% of the 33,353 patients suffering from food poisoning from 2001 to 2005 had illness related to enterotoxins produced by *S. aureus* (Lee et al., 2001; Yoon et al., 2011). Risk related to *S. aureus* and staphylococcal enterotoxins in fluid milk were estimated and demonstrated from the consumption of milk (Ding et al., 2016). It is necessary to estimate the effects of environmental factors on the growth and survival of *S. aureus* on rice cake, as it is the most popular traditional food in Korea.

The methodologies of primary and secondary modeling approaches and validation indices are based on well-established in many publications. But the ways to develop the growth probability model and the kinetic models under fluctuating temperature indicating the different log-increase probability at different temperatures and including as a dependent variable in the logistic models, respectively, are rarely reported. The objective of the present study was to develop a novel approach to predict the growth kinetics of *S. aureus* on rice cake under various environmental conditions. The Baranyi and Roberts model and Ratkowsky square root equation were selected to develop the primary and secondary models to evaluate the growth of *S. aureus* on rice cake under 15, 25, 35, and 45°C. In addition, the kinetic models under fluctuating temperature for the growth of *S. aureus* on rice cake was developed along with a probability model of 1-, 2-, 3-, and 4-log increase were also developed based on the *S. aureus* growth data on rice cakes to identify the shelf life.

MATERIALS AND METHODS

Preparation of Strains

Staphylococcus aureus strains ATCC 12598, ATCC 25923, and ATCC 12600, obtained from the department of Food Science, University of Georgia, were used in this study. All strains were maintained at −70°C in tryptic soy broth (TSB, Difco, Sparks, MD, USA) containing 0.6% yeast extract (YE, Difco, USA) and 20% glycerol. For experimental purposes, the stock cultures of the strains were activated in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 35°C for 24 h in order to reach to the stationary phase. The bacteria were harvested by centrifugation (3,000 × g) at 4°C for 10 min, and then washed twice using 0.1% (w/v) sterile peptone water (PW; Difco, USA). The bacterial suspensions were mixed at equal concentrations to obtain a mixture with the final population level of approximately 5 log cfu/mL.

Preparation and Inoculation of Samples

Gyungdan, a ball-shaped rice cake filled with sweet red bean paste and rolled in sesame seeds, were purchased from a local supermarket in Chuncheon, Korea. The pH and water activity of rice cake was approximately 6.14 and 0.975, respectively. The samples were cut in half using a sterile knife and placed on sterile aluminum foil in a lamella flow hood. The rice cakes were divided into 10 g samples with a balance to prepare for inoculation. For inoculation, 0.1 mL of the mixed culture (5 log cfu/mL) was applied to the samples by depositing droplets with a micropipettor. This procedure resulted in an initial pathogen inocula level of approximately 3 log cfu/g.

Growth Experiments of *S. aureus*

The inoculated samples were packaged with polyethylene and transferred into the growth chamber (BF-600GC, BioFree, Seoul, Korea). Uninoculated samples were used as a control. For the purpose of model development, the inoculated samples were stored at 5, 15, 25, 35, and 45°C at two different levels (50 and 80%) of relative humidity until they reached the stationary phase. As for model validation, the experiments were conducted at 10, 20, 30, and 40°C within the region of interpolation of the model. Sampling was generally carried out for enumeration based on the designed intervals, depending on the different incubation temperatures. Two samples were tested at each time interval. The growth studies were conducted in triplicate for each combination of conditions. Experimental period was set approximately 25 h at 35 and 45°C, 30 h at 30 and 40°C, and approximately 50 h at 20 and 25°C. However, longer periods were selected for the experiment at 5, 10, and 15°C.

Enumeration of *S. aureus*

At each sampling time, the 10 g rice cake samples to be tested were taken from the growth chamber and put into a sterile 400-mL stomaching bag with a filter (Nasco Whirl-Pak, Janesville, WI, USA) then mixed with 90 mL of 0.1% sterilized PW. Samples were then pummeled in a Seward Stomacher (400 Circulator, Seward, London, UK) at 200 rpm for 2 min. After homogenization, 1 mL of the sample suspension containing *S. aureus* was added into 9 mL 0.1% sterilized PW, and serial 10-fold dilutions were performed using 0.1% sterilized PW. Subsequently, 0.1 mL of the obtained samples or dilutions was plated in duplicate on Baird-Parker Agar (Difco Co., USA). After incubation of the plates at 35°C for 24 h, colonies were counted and the data were expressed as a logarithm of the population (log cfu/g). Means of cell populations from each treatment were calculated from three replications.

Kinetic Growth Model Development

Primary Modeling

Experimental data of *S. aureus* growth on the rice cake samples were collected for each designed trial with the different combinations of temperature and relative humidity. The specific growth rate (μ, 1/h) and lag time (LT, h) for each growth curve were generated by fitting the raw data into the Baranyi and Roberts model (Baranyi and Roberts, 1994). Analysis was carried out with R software (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/).

An R-software package *nlstools* was used to fit the Baranyi and Roberts model.

Secondary Modeling

The Ratkowsky's square root model (Equation 1; Ratkowsky et al., 1982; Oscar, 2002) was used to develop secondary models for the μ, obtained from different primary models by non-linear regression using the Origin 8.5 software package (OriginLab corporation, Northampton, MA 01060, USA) based upon the generated growth parameters.

$$\sqrt{\mu} = a(T - T_{min}) \quad (1)$$

where μ is specific growth rate (1/h), T is the temperature (°C), T_{min} is the minimum temperature (°C) required for growth, and a is the regression coefficient.

Differential Secondary Modeling

Staphylococcus aureus growth under fluctuating temperature.

In order to simulate the growth of *S. aureus* on rice cake, the Baranyi and Roberts (1994) was implemented as follows:

$$\frac{dq}{dt} = \mu q, q(0) = q_0 \quad (2)$$

$$\frac{dN}{dt} = \frac{q}{1+q}\mu\left(1 - \frac{N}{N_{max}}\right)N, N(0) = N_0 \quad (3)$$

where N denotes the bacterial cell concentration (CFU/g) at time t, q is a dimensionless quantity related to the physiological state of the cells, μ is the maximum specific growth rate (1/h), and $N_{\max}$ represents the maximum population density of the bacteria (CFU/g). The q_0 and N_0 represents the initial value of the q and N, respectively. For the initial value of q_0 which is a measure of the initial physiological state of the cells, a geometric mean value for the physiological state parameter α_0 was estimated from the constant temperature experimental data. It should be noted that the relationship between lag time (λ) and α_0 could be shown as follows:

$$\mu \times \mathrm{LT} = \ln\left(1 + \frac{1}{q_0}\right) = -\ln(\alpha_0) \quad (4)$$

The models for μ (Equation 1) along with $N_{\max}$ and q_0 were substituted into Equations (2, 3), and the temperature allowed to be dependent on time. The system was solved numerically by the fourth-order Runge-Kutta method as a means of obtaining predictions of bacterial concentration using statistical software R (deSolve package). The temperature profile was recorded continuously using a data logger with an interval of 10 s and set to stimulate real life conditions during storage and transportation.

Growth Probability Model Development

For each growth probability model development, each sampling point was scored with values of 0 and 1 to indicate whether or not to obtain 1-, 2-, 3-, and 4-log increases of *S. aureus* growth, respectively. The probability of a 1-, 2-, 3-, and 4-log increases of *S. aureus* from the growth data on rice cakes was collected and fitted to a logistic regression model using R software (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/). The analyses were made with the glm() function that fits generalized linear models in R-software package STATS. The initial model for fitting had the following form after a minor modification according to the approach described by Ratkowsky and Ross (1995), Koseki et al. (2009), and Agresti (2007):

$$\mathrm{Logit}\,(P) = \alpha_0 + \alpha_1\mathrm{Temp} + \alpha_2 \cdot \ln(\mathrm{Time}) \quad (5)$$

where P is the probability of an arbitrary log-increase based on *S. aureus* growth, $\mathrm{Logit}\,(P) = \ln(\frac{P}{1-P})$, $\alpha_0 - \alpha_2$ are the coefficients to be estimated, Temp is the storage temperature, and Time is the time at which the increase of *S. aureus* growth reached the set values.

To evaluate the goodness-of-fit the developed model, the maximum rescaled R-square statistic, the Hosmer-Lemeshow goodness-of-fit statistic, and the receiver operating characteristic (ROC) curve were used (Agresti, 2007). The maximum rescaled R^2 for use with binomial error was proposed as a generalization of the coefficient of determination R that is commonly used in regression applications involving normally distributed error (Nagelkerke, 1991; Tienungoon et al., 2000). The Hosmer-Lemeshow goodness-of-fit statistic, which involves grouping objects into a contingency table and calculating a Pearson chi-square statistic, was proposed as a means of estimating goodness of fit (Tienungoon et al., 2000). Small values of the statistic (large P-values) indicate a good fit of the model to the data. The area under the ROC curve, c, is a measure of discrimination, obtained from a plot of sensitivity, i.e., the proportion of observed events that were correctly predicted to be events, against the complement of specificity, i.e., the proportion of nonevents that were correctly predicted to be nonevents. The closer the value of c is to 1, the greater is the discrimination. In epidemiological studies, a c value of >0.7 is considered acceptable discrimination, a c value of >0.8 as good discrimination, and a c value of >0.9 as excellent discrimination (Lemeshow and Jean-Roger, 1994).

Validation of the Predictive Models

The performance and reliability of the developed models should be validated before actual application. The validation step was conducted using experimental data which was not used for model development. In the validation step, several statistical indices, such as bias factor (B_f, Equation 6), accuracy factor (A_f, Equation 7), the root mean square error (RMSE, Equation 8; McKellar and Lu, 2004) and %standard error of prediction (%SEP, Equation 9; Garcia-Gimeno et al., 2005) were employed as follows:

$$B_f = 10^{\left(\sum_{i=1}^{n}\log(\mu_{observed}/\mu_{predicted})/n\right)} \quad (6)$$

$$A_f = 10^{\left(\sum_{i=1}^{n}\left|\log(\mu_{predicted}/\mu_{observed})\right|/n\right)} \quad (7)$$

$$RMSE = \sqrt{\frac{\sum(\mu_{observed} - \mu_{predicted})^2}{n}} \quad (8)$$

$$\%SEP = \frac{100}{\mathrm{Average}\,(\mu_{observed})}\sqrt{\frac{\sum\left(\mu_{observed} - \mu_{predicted}\right)^2}{n}} \quad (9)$$

where n is the number of observations, $\mu_{observed}$ is the observed value, and $\mu_{predicted}$ is the predicted value.

Differential secondary model performance was evaluated using the RMSE. In addition, the acceptable prediction zone method was used for evaluation of the model performance (Oscar, 2005). The prediction errors or relative errors (REs) for the individual fitted cases were calculated according to the following equation:

$$\mathrm{RE} = \frac{\text{Predicted} - \text{Observed}}{\text{Predicted}} \quad (10)$$

The model gives fail-safe predictions when the RE is <0, while fail-dangerous predictions at RE values >0. The proportion of RE (pRE) in the acceptable prediction zone of RE −0.6 to 0.3, wherein the proportion of ≥0.70 indicated an acceptable model, was used to evaluate the performance of the obtained model.

Statistical Analysis

All the data from the three replicates were expressed as log colony forming units per gram (log CFU/g) and used together for model development. Statistical analysis was performed using IBM SPSS statistics 20 (IBM Corporation, New York, USA). Data were expressed as means ± standard deviation (SD). All data were analyzed using Analysis of variance (ANOVA). Statistical significance of growth parameters was analyzed by *t*-test at a significance level of 0.05. Values of validation indices were calculated in a Microsoft Excel spreadsheet.

RESULTS AND DISCUSSION

Growth of *S. aureus* on Rice Cake at Different Temperatures

The experimental data of *S. aureus* on rice at all the designed temperatures for model development and validation were collected and presented in **Figures 1**, **2**, respectively. Experimental observations revealed *S. aureus* to be undetectable on the uninoculated rice cake samples (control). *S. aureus* did not grow well on the rice cake stored at 5 and 10°C (**Figure 3**), and a decline in the *S. aureus* cells was observed at 5°C. This was similar with previous studies which indicated *S. aureus* could grow at temperatures as low as 7°C (ICMSF, 1980). In contrast, no growth was observed at 10°C for the storage period of up to 7 days. The reason may be because the target samples were different food matrices, and Staphylococci are particularly sensitive to nutrient depletion. The data at these lower incubation temperatures (5 and 10°C) were not included in the development and validation of the model. The final cell populations of *S. aureus* at 15, 25, 35, and 45°C were 7.45, 7.55, 7.44, and 7.65 log CFU/mL, respectively, indicating that higher temperatures led to higher final cell populations. Among them, the higher values at 15 and 25°C are due to the higher initial population levels. Similar results were demonstrated in our previous research, in which the temperature was more influential on pathogen growth on the food matrix than humidity (Wang and Oh, 2012; Wang et al., 2012).

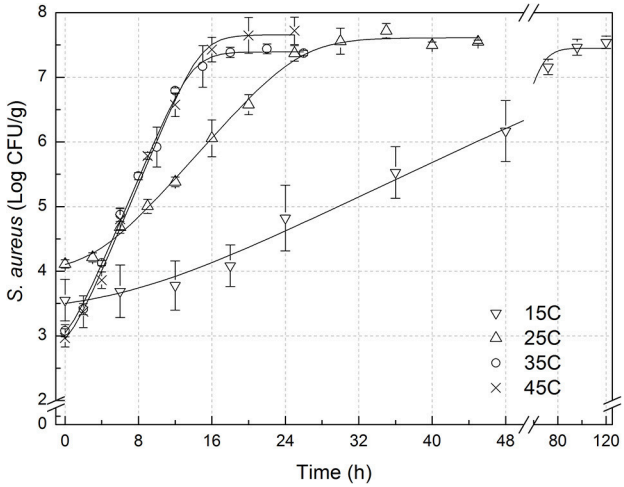


FIGURE 1 | Observed growth data and fitted primary models of *Staphylococcus aureus* on rice cake at 15, 25, 35, and 45°C.

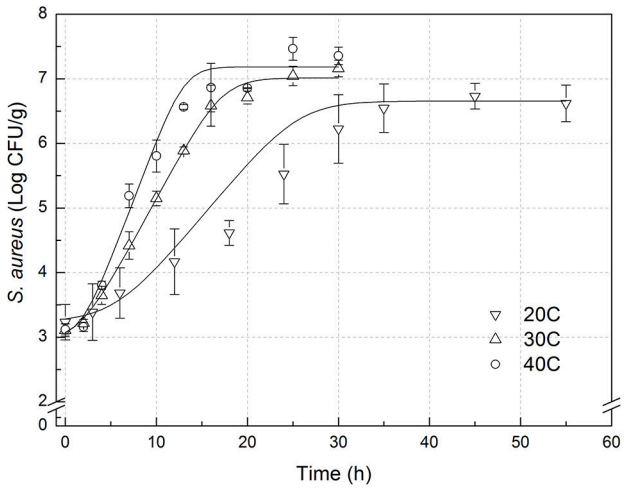


FIGURE 2 | Observed growth data and fitted primary models of *Staphylococcus aureus* on rice cake at 20, 30, and 40°C.

Development of *S. aureus* Growth Models on Rice Cake

Development of Primary Models

In order to develop suitable primary models, which fit the growth data of *S. aureus* on rice cake, the growth curves of *S. aureus* at different environmental conditions were fitted into the Baranyi and Roberts model. The fitted curves at different temperatures were shown in **Figures 1**, **2**. The results of analysis of the derived growth parameters including μ and LT indicated no significant differences between μ and LT values among different relative humidity ($p < 0.05$), regardless of the storage temperatures. **Figures 1**, **2** showed that lag phase could be only relevant for low temperatures. Therefore, the experimental data collected at the different relative humidity levels were combined to estimate

specific growth rate of *S. aureus* on rice cake. The results of fitted parameters are summarized in **Table 1**.

Development of Secondary Models

Following the test of statistical significance of μ obtained from the primary models, only temperature was considered as a factor influencing the growth of *S. aureus* on rice cakes. The square root model, a special case of Belehradék's temperature function with the exponent of two, was suitable to describe the relationship between temperature and microbial growth rates (McMeekin et al., 2013). All μ-values estimated from the primary models were employed to develop the secondary models using a square root model as follows:

$$\sqrt{\mu} = 0.0173^{*}(\mathrm{T} + 10.03) \quad (11)$$

The fitted parameters of the Equation (11) are summarized in **Table 2**.

Validation of Predictive Models

In the present study, external validation was conducted using additional data under experimental conditions (**Figure 2**) which were not used for development of the models. Bias and accuracy factors are usually calculated for model validation. The B_f value of the predictive model for μ was 1.131. Based on the criterion proposed by Ross (1999), the results indicated that the performance of developed models could be considered as acceptable. The acceptable range of an accuracy factor determined the number of environmental parameters in a kinetic model with 0.10–0.15 units increased for each predictive variable (Ross et al., 2000). In this study, the acceptable range for A_f should be <1.15 since just temperature was employed. The A_f value of the predictive model for μ was 1.131. The results indicated that the performance of developed model for μ could be considered as acceptable. RMSE was used to evaluate the performance of the predictive model. The lower the RMSE, the better goodness-of-fit the model shows (Wang et al., 2012). The RMSE value of the predictive model for μ was 0.086. Previous works reported errors of the prediction of growth rate ranging from 0.27 to 0.30 (Sutherland et al., 1994; Olmez and Aran, 2005), which was greater than that for μ in the present study. The results indicated that the developed model showed a good quality of fit for the experimental data. The %SEP has the advantage of being dimensionless, and calculation of the %SEP for REs has the advantage of not being dependent on the magnitude of the measurements. The %SEP value of the predictive model for μ on rice cake was 11.1%. In several previous studies, it was reported that the %SEP value for growth rate of *Leuconostoc mesenteroides* for external validation in aerobic conditions ranged from 14.37 to 22.88% (Garcia-Gimeno et al., 2005; Hervas-Martinez et al., 2006). Compared with the previously reported results, both values obtained herein proved the acceptable goodness of the proposed models.

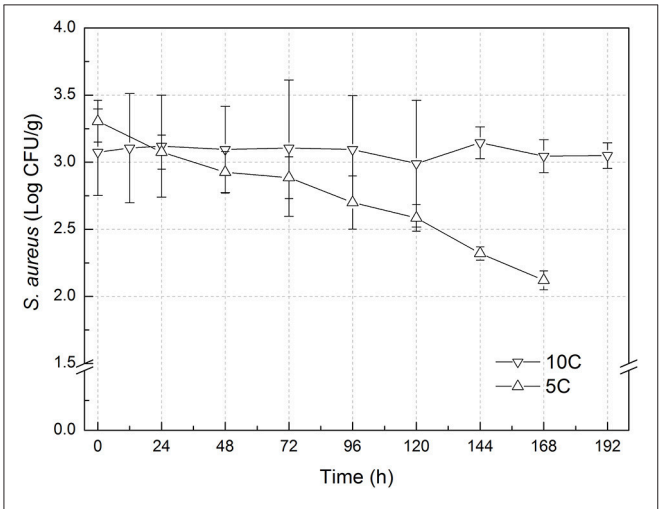


FIGURE 3 | Observed growth data of *Staphylococcus aureus* on rice cake at 5 and 10°C.

TABLE 2 | Estimated Specific growth rate (μ_{max}) of the secondary model for growth of *Staphylococcus aureus* on rice cake.

	parameter	Value	L95CI[a]	U95CI
GR	a	0.0173	0	0.034
	Tmin	−10.03	−51.5	31.4

[a] *Confidence intervals (95%) of the estimates.*

TABLE 1 | Growth parameters (μ_{max}, specific growth rate and lag time) of *Staphylococcus aureus* on rice cake at different experimental conditions.

Temperature (°C)	μ_{max} (1/h)	Std. Error	95% CI[a]		Lag time (h)	Std. Error	95% CI		R^2
			Lower	Upper			Lower	Upper	
15°C	0.157	0.014	0.130	0.184	7.860	3.520	0.961	14.759	0.948
25°C	0.372	0.013	0.346	0.398	3.850	0.624	2.627	5.073	0.989
35°C	0.771	0.024	0.724	0.818	0.865	0.330	0.218	1.512	0.989
45°C	0.779	0.029	0.722	0.836	0.923	0.398	0.143	1.703	0.987
20°C	0.394	0.064	0.269	0.520	5.770	2.452	0.964	10.575	0.983
30°C	0.600	0.037	0.528	0.673	2.018	0.768	0.512	3.524	0.974
40°C	0.858	0.084	0.694	1.023	1.993	0.866	0.296	3.690	0.959

[a] *Confidence intervals of the parameters.*

S. aureus Growth on Rice Cake at Dynamic Temperatures

To simulate growth of *S. aureus* on rice cake under real temperature profile, we calculated the number of *S. aureus* by using simultaneous differential equations (Equations 2, 3). The physiological state parameter q_0 was determined by average of the estimated fitted parameters of four iso-thermal growth curves. The q_0 of the tested growth curves of *S. aureus* was determined as 0.20 ± 0.14. **Figure 4** demonstrated one of the representing results under fluctuating temperature condition. The RMSE for *S. aureus* growth on rice cake was 0.218, while the acceptable prediction rate was 90.9%. These indices exhibited acceptable performance of the developed model for *S. aureus* growth on rice cake under the tested temperatures. In the present study, just one of the representing fluctuating temperature condition was monitored. The model was not tested for significant changes in temperature. Although the predictive capabilities of the model under dynamic temperature were not conducted, the calculation procedure would enable to correspond to real temperature history during distribution.

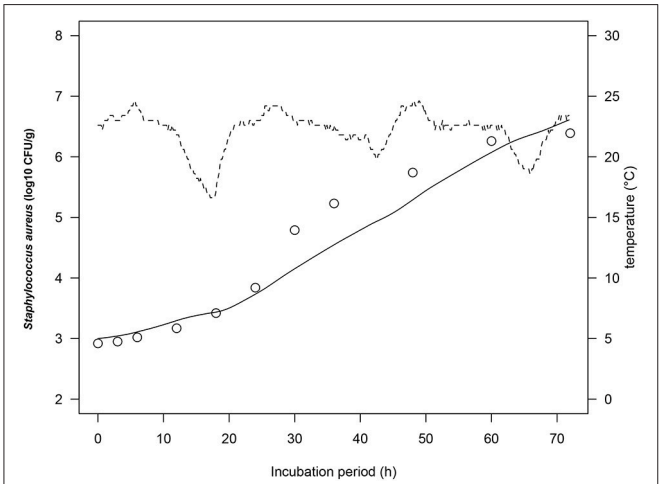


FIGURE 4 | Growth curves of *Staphylococcus aureus* on rice cake at dynamic temperatures (dashed lines) during the stimulated storage period. The solid line represents the prediction made from the developed model for *S. aureus*. ◯ represents the observed values of *S. aureus* growth on rice cake.

Fujikawa et al. (2004) developed a new logistic model for bacterial growth at dynamic temperatures using a numerical solution with the fourth-order Runge–Kutta method and demonstrated that the newly developed model could successfully predict *Escherichia coli* and *Salmonellae* growth curves for various patterns of the temperature history. Subsequently, *S. aureus* growth in sterilized milk were successfully predicted at various patterns of varying temperature using the newly developed logistic model, while its enterotoxin amounts predicted with the developed regression line for Staphylococcal enterotoxins were higher than the observed values (Fujikawa and Morozumi, 2006). In addition, comparison of the dynamic models based on the Baranyi model and modified logistic model could produce almost the same growth curves for varying temperature histories (Fujikawa and Morozumi, 2005).

Development of Probabilistic Model

In order to determine the effect of temperature on the growth of *S. aureus* on rice cake during the storage period, the probability of the time required to reach a certain level was determined using the logistic regression model described in Equation (4). The estimated parameters of the logistic regression models for 1-, 2- 3-, and 4-log increases of *S. aureus* expansion on rice cake are presented in **Table 3**. The information of goodness-of-fit of the model, such as 95% confidence intervals of the

TABLE 3 | Estimated parameters of the logistic regression for arbitrary growth increases of *Staphylococcus aureus* on rice cake.

Increases	Parameters	Estimates	Std. Error	95% CI[a]		Maximum rescaled R^2	*c* (area under ROC[b] curve)	Hosmer-lemeshow (goodness of fit)
				Lower	Upper			
1-log	α0	−22.317	3.950	−30.058	−14.576	0.806	0.974	2.938 with 8 df[c] ($P = 0.938$)
	α1	0.355	0.067	0.225	0.486			
	α2	5.835	1.002	3.872	7.799			
2-log	α0	−32.48	5.542	−43.342	−21.618	0.831	0.981	4.492 with 8 df ($P = 0.810$)
	α1	0.441	0.079	0.286	0.596			
	α2	7.667	1.288	5.143	10.191			
3-log	α0	−33.43	5.236	−43.693	−23.167	0.785	0.970	2.688 with 8 df ($P = 0.952$)
	α1	0.435	0.072	0.294	0.576			
	α2	7.077	1.092	4.937	9.217			
4-log	α0	−29.500	5.064	−39.425	−19.575	0.567	0.942	8.931 with 8 df ($P = 0.348$)
	α1	0.400	0.070	0.263	0.537			
	α2	4.998	0.898	3.238	6.758			

[a] *Confidence intervals (95%) of the estimates.*
[b] *Receiver operating curve.*
[c] *Degree of freedom.*

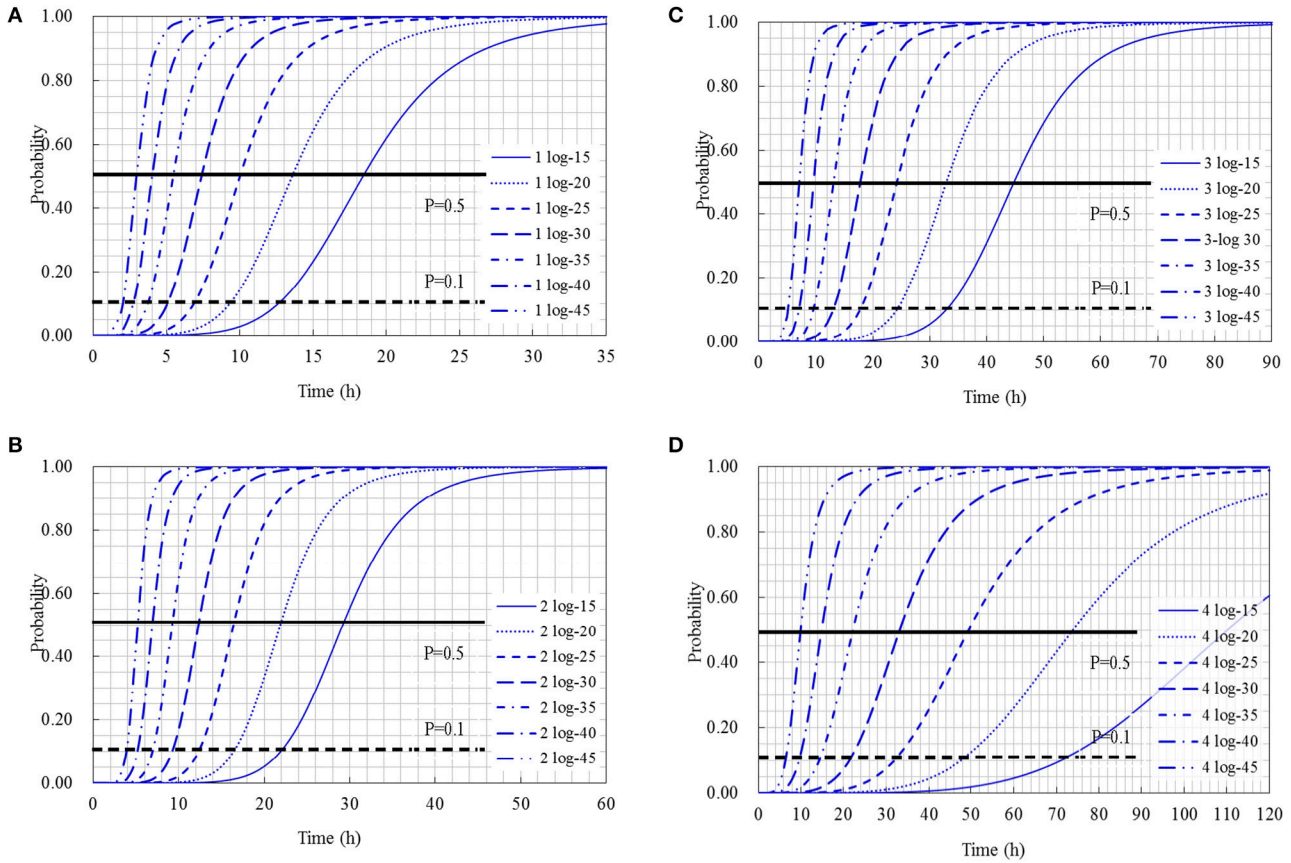


FIGURE 5 | Probability of a certain log increases of *Staphylococcus aureus* occurring at different temperatures: **(A)** 1-log increases; **(B)** 2-log increases; **(C)** 3-log increases; **(D)** 4-log increases. Solid and dashed lines represent the probability ($p = 0.5$ and $p = 0.1$) reaching to a certain log increases of *S. aureus* growth.

parameters, maximum rescaled R^2, area under ROC curve, and Hosmer-Lemeshow goodness of fit are also listed in **Table 3**. According to the performance statistics, the derived parameters for the developed logistic regression models showed excellent discrimination for all the log increases.

The cumulative probability distributions predicted by the developed models listed in **Table 3** are shown in **Figure 5** for the 1-, 2-, 3-, and 4-log increases of *S. aureus* on rice cake, respectively. From the figure, the probability of the time required to reach a certain level of *S. aureus* contamination on the rice cakes at different temperatures could be determined. Meanwhile, the probability of the time required to reach different levels of *S. aureus* on rice cake at a certain temperature is presented in **Figure 6**.

Using the obtained model, the Logit (P) values can be obtained, and the probabilities of *S. aureus* growth on rice cake can be estimated. Referred the reported limits on the growth of bacteria (Presser et al., 1998; Tienungoon et al., 2000; Le Marc et al., 2005; Hwang and Juneja, 2011) that $p \leq 0.1$ indicates an "unlikely to grow" or "no-growth" region, $p > 0.5$ indicates a "likely to grow" or "growth" region, and p values between 0.1 and 0.5 indicate an "uncertainty" region, in the present study, the criterion of a certain log increases of *S. aureus* was set to be $P = 0.5$ and $P = 0.1$. That is to say, as for a certain level of logistic regression model, $P \leq 0.1$ indicates an "unlikely reach" region of a certain log increase, $P > 0.5$ indicates a "reach" region, and *P*-values between 0.1 and 0.5 indicate a "likely reach" region (**Figure 5**). According to this criterion, we could derive an equation through transformation from the obtained logistic regression models shown in **Table 3** to calculate the time to reach a certain log increase of *S. aureus* at different temperatures within the limits of the experimental design. The equations to calculate how long time to reach 1-, 2-, 3-, and 4-log increase of *S. aureus* were listed as following:

$$t = \exp\left((22.317 - 0.355 * \text{Temp})/5.835\right) \quad (12)$$

$$t = \exp\left((32.48 - 0.441 * \text{Temp})/7.667\right) \quad (13)$$

$$t = \exp\left((33.43 - 0.435 * \text{Temp})/7.077\right) \quad (14)$$

$$t = \exp\left((29.50 - 0.400 * \text{Temp})/4.998\right) \quad (15)$$

where Temp is the storage temperature, and t is the time at which 1-, 2-, 3-, and 4-log increases of *S. aureus* reached, respectively.

Time and temperature abuse would lead to results of pathogenic bacteria growth and toxin formation. *S. aureus* concentrations of more than 10^5 CFU/mL were unacceptable, since numbers of *S. aureus* higher than 10^5 most often lead

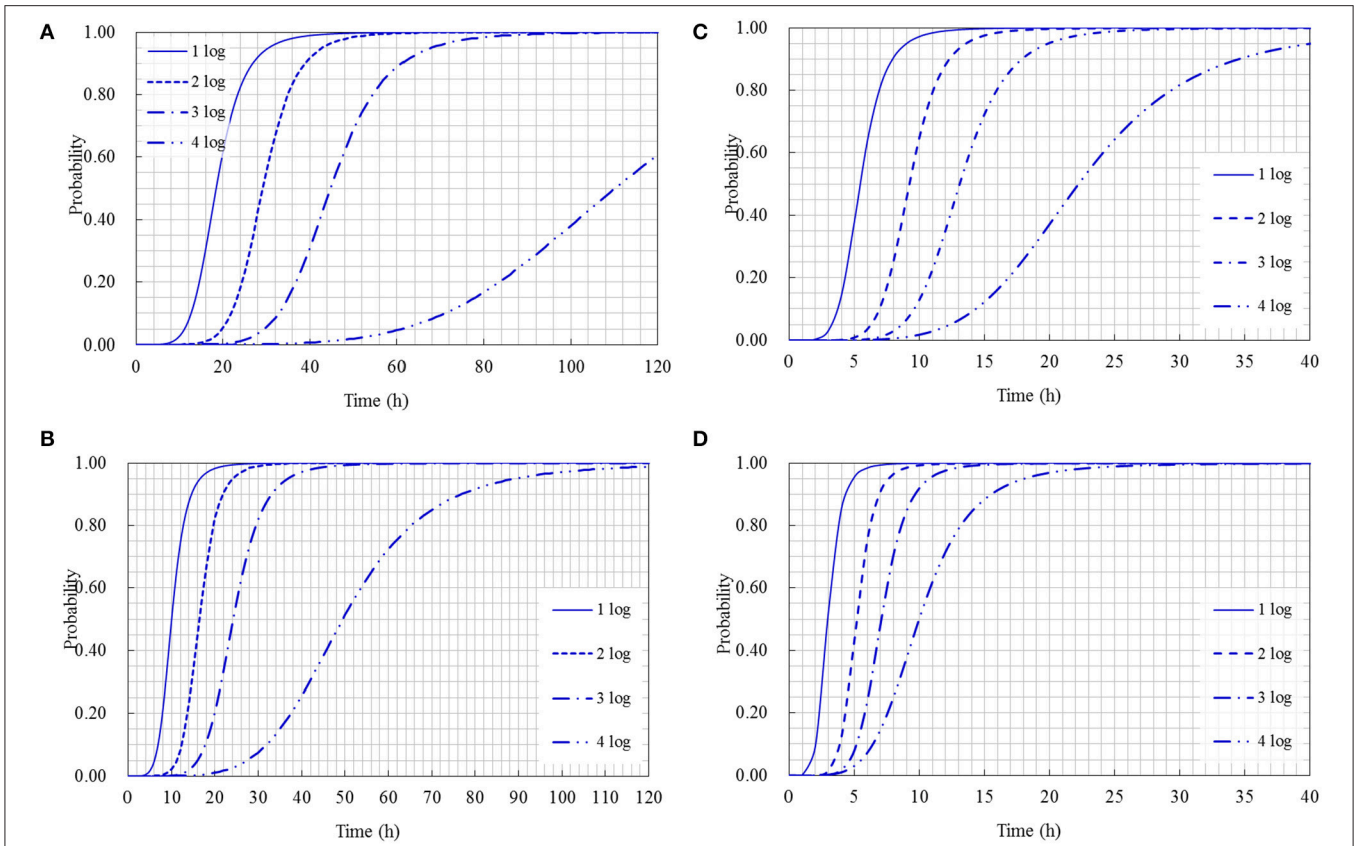


FIGURE 6 | Probability of different log increases of *Staphylococcus aureus* at a certain time at the following temperature: **(A)** 15°C; **(B)** 25°C; **(C)** 35°C; **(D)** 45°C.

to human illness (Food and Drug Administration, 2012). In addition, it is extremely likely to produce Staphylococcal enterotoxins under specific environmental conditions when the *S. aureus* level reaches 10^5 CFU/mL (Heidinger et al., 2009). Fujikawa and Morozumi (2006) reported that temperature dependency of the rate constant of staphylococcal enterotoxin A (ng/mL/h) could be calculated as follows:

$$p_{toxin} = 0.0376 * T - 0.559 \quad (16)$$

where p_{toxin} is the production rate of staphylococcal enterotoxin A, T is temperature. Based on Equation (16), enterotoxin production rate at each temperature would be estimated. According to Equation (14), it is easy to know the time to reach 3-log increase of *S. aureus* growth at different temperatures within the limits of the experimental design. As shown in **Figure 5c**, the area above the solid line was "reach" region, and the time to reach 3-log increase of *S. aureus* growth at 15, 25, 35, and 45°C were 44.8, 24.2, 13.1, and 7.1 h, respectively. At the same time, the probability to reach 3-log increase could be obtained. Finally, the hazardous levels at each temperature could be evaluated considering the probability models and the enterotoxin production rate. If we could check the initial contamination level of rice cake, it is convenient to monitor the status of rice cake products. These logistic regression models could identify the shelf life of rice cake based on the initial levels of *S. aureus* contamination. From a food industry standpoint, to control the growth conditions of pathogens contributing to levels exceeding 10^5 will be of key importance. Therefore, the developed probability models will be very useful for food safety management and microbiological risk assessment of *S. aureus* on rice cake in Korea.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by JW, MC, and DO. Microbiological and data analyses were performed by JW and SK. The paper was written by JW, SK, and DO.

ACKNOWLEDGMENTS

This research was supported by a grant (10162KFDA995) from the Korea Food & Drug Administration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01140/full#supplementary-material

REFERENCES

Agresti, A. (2007). *An Introduction to Categorical Data Analysis*, *2nd Edn*. Hoboken, NJ: John Wiley and Sons, Inc.

Baranyi, J., and Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol.* 23, 277–294. doi: 10.1016/0168-1605(94)90157-0

CDC (2016). *Surveillance for Foodborne Disease Outbreaks, United States, 2014, Annual Report*. Atlanta: US Department of Health and Human Services.

Dabrowski, W. M., and Medrala, D. (2004). "Bacterial toxins," in *Toxins in Food*, eds W. M. Dabrowski and Z. E. Sikorski (Boca Raton, FL: CRC Press, LLC, US), 187–210.

Ding, T. Yu, Y. Y., Schaffner, D. W., Chen, S. G., Ye, Q. U., and Liu, D.-H. (2016). Farm to consumption risk assessment for *Staphylococcus aureus* and staphylococcal enterotoxins in fluid milk in China. *Food Control* 59, 636–643. doi: 10.1016/j.foodcont.2015.06.049

FDA. (2003). Analysis of microbial hazards related to time/temperature control of foods for safety. *Compr. Rev. Food Sci. Food Safety* 2, 33–41. doi: 10.1111/j.1541-4337.2003.tb00049.x

Food and Drug Administration (2012). "*Staphylococcus aureus,*" *Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins. 2nd Edn.* (FDA), 80–84. Available online at: https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf

Fujikawa, H., Kai, A., and Morozumi, S. (2004). A new logistic model for *Escherichia coli* growth at constant and dynamic temperatures. *Food Microbiol.* 21, 501–509. doi: 10.1016/j.fm.2004.01.007

Fujikawa, H., and Morozumi, S. (2005). Modeling surface growth of *Escherichia coli* on agar plates. *Appl. Environ. Microbiol.* 71, 7920–7926. doi: 10.1128/AEM.71.12.7920-7926.2005

Fujikawa, H., and Morozumi, S. (2006). Modeling *Staphylococcus aureus* growth and enterotoxin production in milk. *Food Microbiol.* 23, 260–267. doi: 10.1016/j.fm.2005.04.005

Garcia-Gimeno, R. M., Hervas-Martinez, C., Rodriguez-Perez, R., and Zurera-Cosano, G. (2005). Modelling the growth of *Leuconostoc mesenteroides* by artificial neural networks. *Int. J. Food Microbiol.* 105, 317–332. doi: 10.1016/j.ijfoodmicro.2005.04.013

Heidinger, J. C., Winter, C., K., and Cullor, J., S. (2009). Quantitative microbial risk assessment for *Staphylococcus aureus* and *Staphylococcus enterotoxin* A in raw milk. *J. Food Prot.* 72, 1641–1653. doi: 10.4315/0362-028X-72.8.1641

Hervas-Martinez, C., Garcia-Gimeno, R. M., Martinez-Estudillo, A. C., Martinez-Estudillo, F. J., and Zurera-Cosano, G. (2006). Improving microbial growth prediction by product unit neural networks. *J. Food Sci.* 71, M31–M38. doi: 10.1111/j.1365-2621.2006.tb08904.x

Hwang, C. A., and Juneja, V. (2011). Effects of salt, sodium pyrophosphate, and sodium lactate on the probability of growth of *Escherichia coli* O157:H7 in ground beef. *J. Food Prot.* 74, 622–626. doi: 10.4315/0362-028X.JFP-10-325

ICMSF (1980). *Microbial Ecology of Foods. Vol. 1, Factors Affecting Life and Death of Microorganisms*. Orlando, FL: International Commission on Microbiological Specification for Foods; Academic Press.

Koseki, S., Matsubara, M., and Yamamoto, K. (2009). Prediction of a required log reduction with probability for enterobacter sakazakii during high-pressure processing, using a survival/death interface model. *Appl. Environ. Microb.* 75, 1885–1891. doi: 10.1128/AEM.02283-08

Lee, K. A., Kim, K. T., and Paik, H. D. (2011). Physicochemical, microbial, and sensory evaluation of cook-chilled Korean traditional rice cake (Backseolgi) during storage via various packaging methods. *Food Sci. Biotechnol.* 20, 1069–1074. doi: 10.1007/s10068-011-0145-z

Lee, W. C., Lee, M. J., Kim, J. S., and Park, S. Y. (2001). Foodborne illness outbreaks in Korea and Japan studied retrospectively. *J. Food Prot.* 64, 899–902. doi: 10.4315/0362-028X-64.6.899

Le Marc, Y., Pin, C., and Baranyi, J. (2005). Methods to determine the growth domain in a multidimensional environmental space. *Int. J. Food Microbiol.* 100, 3–12. doi: 10.1016/j.ijfoodmicro.2004.10.003

Lemeshow, S., and Jean-Roger, L. (1994). Modeling the severity of illness of icu patients: a systems update. *JAMA* 272, 1049–1055. doi: 10.1001/jama.1994.03520130087038

Li, J., Ahn, J., Liu, D., Chen, S., Ye, X., and Ding, T. (2016). Evaluation of ultrasound-induced damage to *Escherichia coli* and *Staphylococcus aureus* by flow cytometry and transmission electron microscopy. *Appl. Environ. Microbiol.* 82, 1828–1837. doi: 10.1128/AEM.03080-15

McKellar, R. C., and Lu, X. (2004). *Modeling Microbial Responses in Food*. Boca Raton, FL: CRC Press.

McMeekin, T., Olley, J., Ratkowsky, D., Corkrey, R., and Ross, T. (2013). Predictive microbiology theory and application: Is it all about rates? *Food Control* 29, 290–299. doi: 10.1016/j.foodcont.2012.06.001

Nagelkerke, N. (1991). A note on a general definition of the coefficient of determination. *Biometrika* 78, 691–692. doi: 10.1093/biomet/78.3.691

Oh, S. K., Lee, N., Cho, Y. S., Shin, D. B., Choi, S. Y., and Koo, M. (2007). Occurrence of toxigenic *Staphylococcus aureus* in ready-to-eat food in Korea. *J Food Protect* 70, 1153–1158. doi: 10.4315/0362-028X-70.5.1153

Olmez, H. K., and Aran, N. (2005). Modeling the growth kinetics of *Bacillus cereus* as a function of temperature, pH, sodium lactate and sodium chloride concentrations. *Int. J. Food Microbiol.* 98, 135–143. doi: 10.1016/j.ijfoodmicro.2004.05.018

Oscar, T. P. (2005). Validation of lag time and growth rate models for *Salmonella typhimurium*: acceptable prediction zone method. *J. Food Sci.* 70, M129–M137. doi: 10.1111/j.1365-2621.2005.tb07103.x

Oscar, T. P. (2002). Development and validation of a tertiary simulation model for predicting the potential growth of *Salmonella typhimurium* on cooked chicken. *Int. J. Food Microbiol.* 76, 177–190. doi: 10.1016/S0168-1605(02)00025-9

Park, H. O., Kim, C. H., Woo, G. J., Park, S. H., Lee, D. H., and Park, K. H. (2001). Monitoring and trends analysis of food poisoning outbreaks occurred in recent years in Korea. *J. Food Hyg. Saf.* 16, 280–294.

Presser, K. A., Ross, T., and Ratkowsky, D. A. (1998). Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. *Appl. Environ. Microbiol.* 64, 1773–1779.

Ratkowsky, D. A., Olley, J., McMeekin, T. A., and Ball, A. (1982). Relationship between temperature and growth rate of bacterial cultures. *J. Bacteriol.* 149, 1–5.

Ratkowsky, D. A., and Ross, T. (1995). Modeling the bacterial-growth go growth interface. *Lett. Appl. Microbiol.* 20, 29–33. doi: 10.1111/j.1472-765X.1995.tb00400.x

Riva, M., Fessas, D., and Schiraldi, A. (2000). Starch Retrogradation in Cooked Pasta and Rice. *Cereal Chem. J.* 77, 433–438. doi: 10.1094/CCHEM.2000.77.4.433

Ross, T. (1999). *Predictive Food Microbiology Models in the Meat Industry (MSRC.003)*. Sydney, NSW: Meat and Livestock.

Ross, T., Dalgaard, P., and Tienungoon, S. (2000). Predictive modelling of the growth and survival of Listeria in fishery products. *Int. J. Food Microbiol.* 62, 231–245. doi: 10.1016/S0168-1605(00)00340-8

Sutherland, J. P., Bayliss, A. J., and Roberts, T. A. (1994). Predictive modelling of growth of *Staphylococcus aureus*: the effects of temperature, pH and sodium chloride. *Int. J. Food Microbiol.* 21, 217–236. doi: 10.1016/0168-1605(94)90029-9

Tienungoon, S., Ratkowsky, D. A., McMeekin, T. A., and Ross, T. (2000). Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Appl. Environ. Microbiol.* 66, 4979–4987. doi: 10.1128/AEM.66.11.4979-4987.2000

Wang, J., Membre, J. M., Ha, S. D., Bahk, G. J., Chung, M. S., and Oh, D. H. (2012). Modeling the combined effect of temperature and relative humidity on *Escherichia coli* O157:H7 on lettuce. *Food Sci. Biotechnol.* 21, 859–865. doi: 10.1007/s10068-012-0111-4

Wang, J., and Oh, D. H. (2012). Effect of temperature and relative humidity on growth behavior of *Escherichia Coli* O157:H7 ON spinach using response surface methodology. *J. Food Saf.* 32, 296–304. doi: 10.1111/j.1745-4565.2012.00380.x

Wu, Y., Chen, Z., Li, X., and Li, M. (2009). Effect of tea polyphenols on the retrogradation of rice starch. *Food Res. Int.* 42, 221–225. doi: 10.1016/j.foodres.2008.11.001

Yoon, S. K., Ghafoor, K., Kang, Y. S., Sohn, M. G., Hwang, I. G., and Park, J. (2011). Exposure assessment of *Staphylococcus aureus* in Kimbab, a ready-to-eat Korean food. *Food Sci. Biotechnol.* 20, 23–28. doi: 10.1007/s10068-011-0004-y

Zhao, X., Lin, C. W., Wang, J., and Oh, D. H. (2014). Advances in rapid detection methods for foodborne pathogens. *J. Microbiol. Biotechnol.* 24, 297–312. doi: 10.4014/jmb.1310.10013

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Wang, Koseki, Chung and Oh. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 June 2017
doi: 10.3389/fmicb.2017.01136

Comparative Genomic Characterization of the Highly Persistent and Potentially Virulent *Cronobacter sakazakii* ST83, CC65 Strain H322 and Other ST83 Strains

*Hannah R. Chase[1], Gopal R. Gopinath[1], Athmanya K. Eshwar[2], Andrea Stoller[2], Claudia Fricker-Feer[3], Jayanthi Gangiredla[1], Isha R. Patel[1], Hediye N. Cinar[1], HyeJin Jeong[1], ChaeYoon Lee[1], Flavia Negrete[1], Samantha Finkelstein[1], Roger Stephan[2], Ben D. Tall[1] and Angelika Lehner[2]**

[1] Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, Laurel, MD, United States, [2] Institute for Food Safety and Hygiene, University of Zurich, Zurich, Switzerland, [3] Quality Assurance and Food Safety Department, Hochdorf Swiss Nutrition Ltd, Hochdorf, Switzerland

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Ondřej Holý, Palacký University, Olomouc, Czechia
Séamus Fanning, University College Dublin, Ireland

***Correspondence:**
Angelika Lehner
lehnera@fsafety.uzh.ch

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *03 May 2017*
Accepted: *06 June 2017*
Published: *26 June 2017*

Citation:
Chase HR, Gopinath GR, Eshwar AK, Stoller A, Fricker-Feer C, Gangiredla J, Patel IR, Cinar HN, Jeong H, Lee C, Negrete F, Finkelstein S, Stephan R, Tall BD and Lehner A (2017) Comparative Genomic Characterization of the Highly Persistent and Potentially Virulent Cronobacter sakazakii ST83, CC65 Strain H322 and Other ST83 Strains. Front. Microbiol. 8:1136. doi: 10.3389/fmicb.2017.01136

Cronobacter (*C.*) *sakazakii* is an opportunistic pathogen and has been associated with serious infections with high mortality rates predominantly in pre-term, low-birth weight and/or immune compromised neonates and infants. Infections have been epidemiologically linked to consumption of intrinsically and extrinsically contaminated lots of reconstituted powdered infant formula (PIF), thus contamination of such products is a challenging task for the PIF producing industry. We present the draft genome of *C. sakazakii* H322, a highly persistent sequence type (ST) 83, clonal complex (CC) 65, serotype O:7 strain obtained from a batch of non-released contaminated PIF product. The presence of this strain in the production environment was traced back more than 4 years. Whole genome sequencing (WGS) of this strain together with four more ST83 strains (PIF production environment-associated) confirmed a high degree of sequence homology among four of the five strains. Phylogenetic analysis using microarray (MA) and WGS data showed that the ST83 strains were highly phylogenetically related and MA showed that between 5 and 38 genes differed from one another in these strains. All strains possessed the pESA3-like virulence plasmid and one strain possessed a pESA2-like plasmid. In addition, a pCS1-like plasmid was also found. In order to assess the potential *in vivo* pathogenicity of the ST83 strains, each strain was subjected to infection studies using the recently developed zebrafish embryo model. Our results showed a high (90–100%) zebrafish mortality rate for all of these strains, suggesting a high risk for infections and illness in neonates potentially exposed to PIF contaminated with ST83 *C. sakazakii* strains. In summary, virulent ST83, CC65, serotype CsakO:7 strains, though rarely found intrinsically in PIF, can persist within a PIF manufacturing facility for years and potentially pose significant quality assurance challenges to the PIF manufacturing industry.

Keywords: *Cronobacter*, persister, environmental microarray, whole genome sequencing

INTRODUCTION

Cronobacter is a genus of Gram-negative bacteria of the family *Enterobacteriaceae*. These bacteria have been associated with cases of illnesses in neonates for which fatality rates ranging between 40 and 80% have been reported (Tall et al., 2014). Clinical manifestations of infection include necrotizing enterocolitis, septicemia and neonatal meningitis. Infections in infants have been epidemiologically linked to consumption of contaminated batches of reconstituted powdered infant formula (PIF) (Hunter et al., 2008).

Cronobacter is capable of surviving under extreme desiccation (high osmotic stress) conditions and it is assumed that this characteristic contributes to its survival in PIF (and dried milk) factories, dried food products, and dry environments (Osaili and Forsythe, 2009; Osaili et al., 2009). Currently there are seven species described: *C. sakazakii*, *C. malonaticus, C. turicensis, C. universalis, C. condimenti, C. muytjensii,* and *C. dublinensis* all of which, except *C. condimenti* have been noted to cause infections in humans (Iversen et al., 2008; Joseph et al., 2012a). Of these species, *C. sakazakii* has been the most commonly isolated species from ill patients as well as from PIF products and production environments (Jolley and Maiden, 2010; Joseph et al., 2012b).

In April 2016 during routine testing of PIF packed products that were ready for distribution, a *C. sakazakii* isolate, H322, was recovered. A follow-up epidemiological investigation of this isolate and other isolates obtained from the facility's production environment using macro restriction typing (Pulsed Field Gel Electrophoresis, PFGE) identified two other isolates which possessed indistinguishable PFGE patterns with H322, the oldest isolate dating back to 2012. This result suggested that these isolates were phylogenetically related and that the strain may have been persisting within the facility for more than 4 years (Stoller et al., 2016). Further characterization revealed that the strains possessed the serotype CsakO:7 LPS and sequence type (ST) ST83/CC65. In order to obtain further insights into the genes and features possibly responsible for the long-term persistence of these strains, whole genome sequencing (WGS), as well as microarray analysis, was applied to these strains as well as to one more ST83 isolate with a pulsotype distinct from this persistent clone. In addition, since no data on the putative pathogenicity of ST83 strains are available to date, we performed infection experiments in zebrafish embryos in order to assess information on the potential *in vivo* pathogenicity of the strains (Fehr et al., 2015; Eshwar et al., 2016).

MATERIALS AND METHODS

Bacterial Strains

The five ST83 strains investigated in this study were isolated from PIF product (H322) and production environment (A31, H2399, H1191, H2397) in Switzerland between 2011 and 2016 during the routine hygiene monitoring performed by the PIF production company. The metadata associated with these strains is shown in **Table 1**. Putative *Cronobacter* strains were isolated according to the ISO/TS22964:2006 method. Isolates were identified using the genus- (alpha glucosidase) and species- (*rpoB*) specific PCR assays described by Lehner et al. (2006, 2012) and Stoop et al. (2009). Strains *C. sakazakii* ATCC 29544^T (C. sakO:1, ST8, clinical, child's throat (United States), and NM1242 (C. sakO:4, ST4, clinical, brain exudate, United States) together with the non-pathogenic *E. coli* strain Xl1blue were included in zebrafish embryo infection studies (Eshwar et al., 2016).

TABLE 1 | Details of *C. sakazakii* ST83 strains sequenced in this study.

Strain ID	Source	Date of isolation	O-type	ST/CC
H322	PIF product	21.04.2016	O:7	83/65
A31	PIF environment, floor drain	14.06.2012	O:7	83/65
H1191	PIF environment, vacuum cleaner	06.02.2012	O:7	83/65
H2399	PIF environment, vacuum cleaner	07.11.2011	O:7	83/65
H2397	PIF environment, vacuum cleaner	07.11.2011	O:7	83/65

Preparation of Genomic DNA for PCR

Strains were grown in LB (Thermo Fisher Scientific AG, Zürich) at 37°C overnight and genomic DNA was isolated from 2 ml of culture using the Qiagen Blood and Tissue Kit according to the manufacturer's instructions. 10–20 ng DNA was then used as the template in 50 μl PCR reactions.

Serotyping PCR

O-antigen serotypes were determined by applying the scheme proposed by Sun et al. (2011).

Multi-Locus Sequence Typing (MLST)

Multi-locus sequence typing was performed following the protocol described by Joseph et al. (2012b) or by submitting genome sequences using the *Cronobacter* MLST website[1] (Jolley and Maiden, 2010). Sequencing of PCR amplicons was outsourced (Microsynth, Balgach, Switzerland).

Genomic DNA Isolation for WGS and Microarray Analyses

All strains were grown overnight in a shaker incubator (160 rpm) at 37°C in 5 ml of Trypticase soy broth (BBL, Becton Dickinson, Franklin Lakes, NJ, United States) supplemented with 1% NaCl (final conc.). Isolation of genomic DNA was performed on 2 ml cultures using the robotic QIACube workstation and the automated Qiagen DNeasy technology (Qiagen, Inc.) following the manufacturer's recommendations. Characteristically, 5–50 μg of purified genomic DNA was obtained in a final elution volume of 200 μl and used for plasmid typing and WGS analyses. For microarray analysis, the purified DNA was further concentrated using an Amicron Ultracel-30 membrane filter (30,000 molecular weight cut-off, 0.5 ml Millipore Corp.; Billerica, MA, United States) to a final volume of 10–25 μl as described by Tall et al. (2015).

[1] http://pubmlst.org/cronobacter/

Microarray Analysis

The microarray used in this study is an Affymetrix MyGeneChip Custom Array (Affymetrix design number: FDACRONOa520845F) which utilizes the whole genome sequences of 15 *Cronobacter* strains, as well as 18 plasmids. These 15 strains encompassed all proposed species of *Cronobacter*. A $\geq$ 97% identity threshold level between gene homologs to positively predict allelic coverage as described by Tall et al. (2015) was used to design the array. Each gene is represented on the array by 22 unique 25-mer oligonucleotide probes, as described by Jackson et al. (2011) and Tall et al. (2015). Genomic DNA was hybridized, washed in the Affymetrix FS-450 fluidics station, and evaluated on the Affymetrix GeneChip® Scanner 3000 (AGCC software) as described by Jackson et al. (2011) and as modified by Tall et al. (2015). All reagents for hybridizing, staining and washing were made in conjunction with the Affymetrix GeneChip® Expression Analysis Technical Manual (Affymetrix, 2014). For each genetic locus represented on the microarray, probe set intensities were assessed using the Robust MultiArray Averaging (RMA) function in the Affymetrix package of R-Bioconductor as described by Bolstad et al. (2003). RMA summarization, normalization, and polishing was done on the data received and final probe set values were determined as explained by Jackson et al. (2011) and as modified by Tall et al. (2015). Gene differences were determined and phylogenetic trees were created using the SplitsTree 4 neighbor net joining method, and scatter plots were used for verification of the RMA-summarized probe set intensities as described by Jackson et al. (2011) and as modified by Tall et al. (2015).

WGS Assembly and Comparative Genomic Analysis

Whole genome sequencing was carried out using the MiSeq platform (Illumina, San Diego, CA, United States), and a Nextera XT library kit. Trimmed Fastq data sets were *de novo* assembled through the CLC Genomics Workbench software version 7.0 (CLC bio, Aarhus, Denmark). WGS assemblies in FASTA format were submitted to NCBI under *Cronobacter* GenomeTrakr project as described by Grim et al. (2015) and Chase et al. (2016). Genome annotation was conducted through the RAST annotation server[2] (Aziz et al., 2008; Overbeek et al., 2014). Genomes were submitted to NCBI under the *Cronobacter* GenomeTrakr Project: FDA-CFSAN bioproject number PRJNA258403 and accession numbers are given in **Table 2**. A local BLAST database was built to query and use with in-house perl scripts (will provide to users upon request). Homologs of conserved genes in these *Cronobacter* isolates were first identified using 4255 known CDS present in the NCBI GenBank annotation of *C. sakazakii* BAA-894 (GCA_000017665.1). Multiple sequence alignments to detect single nucleotide polymorphisms (SNPs) and Neighbor-Joining (NJ) algorithm based cladistic analysis of the resulting SNP matrix was carried out using MEGA7 suite (Kumar et al., 2016). To create a pseudomolecule of the pCS1-like native plasmid in H322, the WGS assembly was first mapped to pCS1 sequence (CP012254). The mapping of contigs was ordered in sequence and combined together to form an ungapped scaffold by manual curation. The resulting pseudomolecule represented the pCS1-like (pH322) sequences found in H322. Progressive Mauve implementation using Geneious 9 suite (Kearse et al., 2012) was used for global alignment.

[2]http://www.nmpdr.org/FIG/wiki/view.cgi/FIG/RapidAnnotationServer

Zebrafish Embryo Infection Model

Husbandry, breeding, bacterial inoculum preparation, and infection of the embryos was carried out by microinjection of approx. 50 CFU of bacteria into the yolk sac of 2 dpf embryos according to the procedure described in the study by Eshwar et al. (2016). Virulence was assessed by determination of lethality (30 embryos per experiment/strain) over 72 h post-infection. The following controls were included: infection with (apathogenic) *E. coli* Xl1blue, injections with Delbecco's Phosphate Buffered Saline (DPBS), and non-injected embryos. The maximum age reached by the embryos during experimentation was 120 hpf (72 hpi) for which no license is required from the cantonal veterinary office since embryos had not yet reached free feeding stage.

RESULTS AND DISCUSSION

Strain H322 was isolated from a contaminated batch of non-released PIF in April 2016. Subsequent PFGE analysis of *C. sakazakii* isolates obtained during the regular monitoring program performed by the production facility over the last 5 years revealed two more isolates (A31, H1191) exhibiting indistinguishable PFGE profiles, the oldest one dating back to February 2012 (data not shown). These results suggest that these strains have been persisting in the production environment for more than 4 years. Further analysis identified these strains as being of serotype O:7 and MLST ST83, CC65. Details on the strains are given in **Table 1**.

TABLE 2 | Overview of whole genomic characteristics of the *C. sakazakii* ST83 strains used in this study.

Strain ID	Genome size (mbp)	No. of contigs	%GC content	Number of coding sequences	NCBI Biosample ID	NCBI Accession No.
H322	4.6	44	56.7	4,255	SAMN06124518	MRXI00000000
A31	4.5	45	56.8	4,195	SAMN06124505	MRXM00000000
H1191	4.5	45	56.8	4,177	SAMN06124503	MRXQ00000000
H2399	4.5	36	56.8	4,152	SAMN06124500	MRXT00000000
H2397	4.4	143	56.8	4,136	SAMN06124502	MRXV00000000

Isolates of this ST have been identified before during surveillance studies, most notably in environmental samples from this production facility (Müller et al., 2013; Stoller et al., 2016), but were rarely reported from other studies investigating PIF and PIF environmental isolates. In order to obtain a deeper insight into the genomic organization and the possible basis of the high persistence of these strains we performed WGS and microarray analyses on these clonal as well as one more, unrelated ST83 (H2399) strain. An overview of the WGS sequence data for the four strains is given in **Table 2**. Genome sizes ranged from 4.46 to 4.55 mbp, and their % G+C was approximately 56.8. The number of coding DNA sequences ranged from 4,136 to 4,255.

Microarray analysis of strains H322, A31, H1191 and H2399 compared to 109 other *C. sakazakii* strains and one *C. malonaticus* strain is shown in **Figure 1** (with supporting information given in Supplementary Tables S1, S2). The phylogenetic tree was first developed using the over 21,000 genes captured on the microarray and then MLST data was overlaid onto the strain clades. The majority of strains grouped according to STs. The Swiss ST83 strains clustered together with ST83 strain, CsakComp15A, which was obtained from a dairy powder manufacturing facility located in the United States. Noted exceptions were ST4 *C. sakazakii* strains 18_01 and Md1g which clustered with ST8 strains and as a singleton, ST8 strain Md33g clustered as a singleton, and ST1 strain Comp62A which clustered with a ST64 cluster, respectively. These results supports the hypothesis that MA using the over 21,000 *Cronobacter* genes captured on the microarray are more resolving than the seven-allele MLST scheme. Gene differences among the strains are shown in Supplementary Table S1 and demonstrate that the number of gene differences among strains A31, H3299, H322, and H1191 was between 5 and 38 genes. Metadata for the strains used in the microarray analysis is shown in Supplementary Table S2. RMA microarray probe summarization, visualized in scatter plots, yields a much lower variance in probe sets where intensities are less than 8 (log2) (Jackson et al., 2011). By decreasing this variance a more accurate determination of gene differences between strains is achieved. A summary of representative gene differences for strains H2399 and H1191 compared to strain H322 are shown in **Figure 2**.

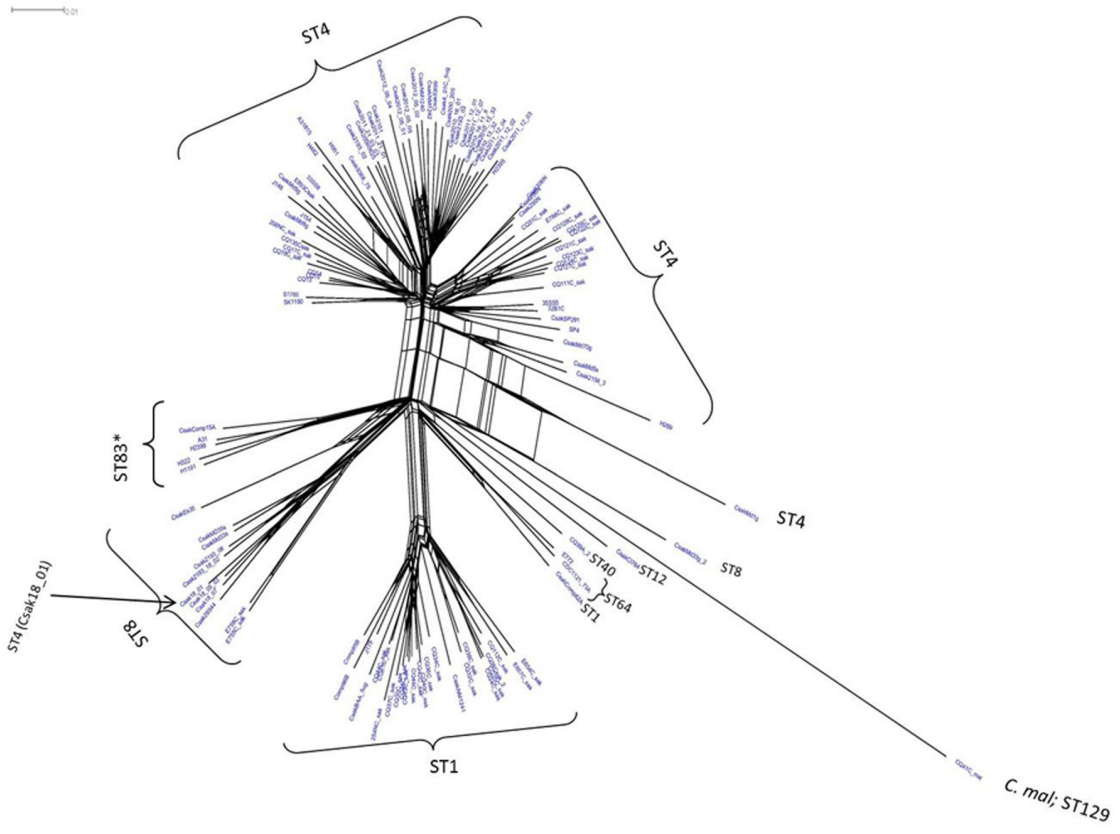


FIGURE 1 | Phylogenetic Neighbor Net (SplitsTree4) analysis of 114 *C. sakazakii* and phylogenetically related strains, which were generated from the gene-difference matrix (Supplementary Tables S1, S2) and then overlaid with sequence types (ST). The microarray experimental protocol as described by Jackson et al. (2011) and as modified by Tall et al. (2015) were used for the interrogation of the strains for the analysis. The phylogenetic tree illustrates that the *Cronobacter* microarray could clearly separate the strains according to species and respective ST. The ST83 strains (*) clustered together along with a single ST83 strain which was obtained from an United States diary powder manufacturing facility. Bar marker represents 0.01 gene differences.

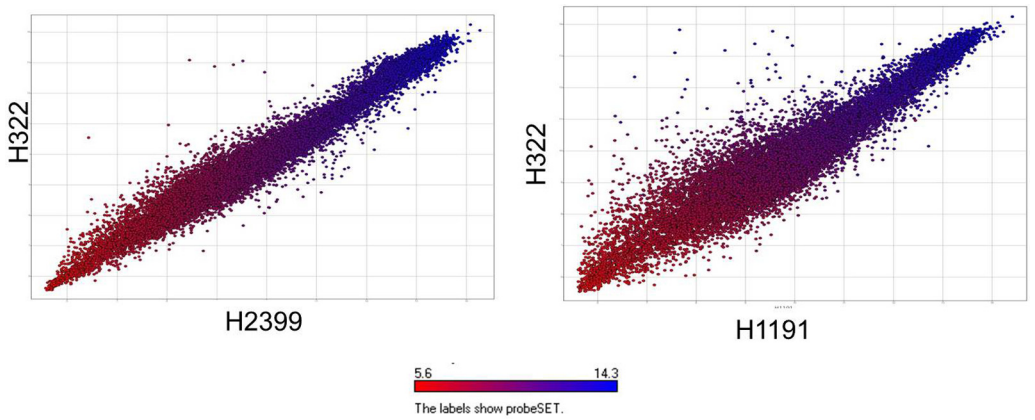


FIGURE 2 | Comparative microarray probe set summarization of two Swiss ST83 strains H2399 and H1191 compared to strain H322 using scatter plots showing RMA summarized probe set intensities from each strain's data points which are color-coded based on their intensities in H322. The *y*-axis show the RMA summarized probe set intensities from H322 (blue) and the *x*-axis shows the probe set intensities from H2399 and H1191 (red), respectively.

Homologs of 4,024 (of the 4255 genes previously identified) *C. sakazakii* BAA-894 genes conserved in ST83 and other strains used in the study were identified by local BLAST analysis as described earlier. SNPs associated with 37,711 positions across 44 genomes in 1,000 randomly chosen, conserved loci were detected using a local BLAST database and aligned to create an un-gapped dataset. A high-confidence unrooted phylogenetic tree based on the NJ algorithm in MEGA7 program is shown in **Figure 3** (supporting information is given in Supplementary Table S3) and revealed that the ST83 strains formed their own distinct cluster. Similar to the microarray-based cladistics analysis, small but distinct differences among the lineages of *C. sakazakii* strains from food, clinical and environmental samples are captured in this conserved-loci-based SNPs analysis. As expected, this high throughput multi loci SNP analysis was able to group isolates belonging to same ST into distinct clusters.

Whole genome sequencing analysis also showed that strain H322 harbors a pESA3/pCTU1-like virulence plasmid, which was previously found by whole-genome sequencing in strains *C. sakazakii* BAA-894 (NC_00978) and *C. turicensis* z3032 (NC_01328). The pESA3/pCTU1-like plasmid harbored by H322 (107,409 bp) shares 97% identity over a 75% query coverage when aligned with the pESA3 harbored by *C. sakazakii* BAA-894, and 90% identity of a 58% query coverage when aligned with *C. turicensis* z3032's pCTU1 (Franco et al., 2011). PCR analysis targeting the prevalence and distribution of plasmid targets is shown in **Table 3** which supports these genomic findings (Franco et al., 2011). Additionally, nucleotide sequences encoding plasmid partitioning proteins ParA and ParB, as well as the IncFIB plasmid conjugative transfer surface exclusion protein TraT, were found in H322 (Kucerova et al., 2010; Franco et al., 2011). Interestingly, the other four strains additionally possessed the *repA* gene, signifying that these strains also harbor a pESA3-like plasmid. Typical pESA3 plasmid genes such as *eitA*, *iucC*, *cpa* and some regions of the type six secretion system were also possessed by these strains. Additionally, strain H1191 was found to possess the *repA* gene encoded on the pESA2-like plasmid.

In addition to pESA3, the presence of a large plasmid in these ST83 genomes was also found. Detailed analysis revealed that this plasmid was significantly orthologous to the 110 kb plasmid pCS1 reported from *C. sakazakii* NCTC 8155 (Moine et al., 2016). Using BLAST analysis and manual curation, the WGS contigs representing about 80% of the pCS1 sequence (CP012254) in *C. sakazakii* strain H322, were used to create a gapped scaffold of 89 kbp (pH322_pseudomolecule). In **Figure 4**, the pH322 pseudomolecule was aligned with highly conserved pCS1-like contigs from NCIM8272 (contig 22-AWFW01000018) and Csak8399 (contig11-AWSP01000008) using an implementation of ProgressiveMauve algorithm in Geneious suite 9.1 (Darling et al., 2010; Kearse et al., 2012). The pCS1-like sequences in H322 (track 4) is shown to align with *C. sakazakii* NCTC 8155 pCS1_NCTC 8155 (track 1), *C. sakazakii* pNCIM8272 (track2) and *C. sakazakii* 8399 pCsak8399 (track 3). This analysis showed that large sequence regions within the plasmids typified in each track shared significant conservation to the reference (pCS1 plasmid CP012254). None of these sequences showed any similarity to pESA3 and/or pCTU1 virulence plasmids. In addition to the ST83 isolates from this study and the two other strains from this study as illustrated in **Figure 3**, pCS1-like sequences are present in *C. sakazakii* 558 and *C. malonaticus* 685 genomes (Supplementary Table S2) downloaded from NCBI. It is evident from this comparative analysis that the pCS1-like plasmid found in ST83 strain H322 (and others): (i) represents a new kind of high-molecular weight plasmid among *C. sakazakii* strains orthologous to the recently reported pCS1 (Moine et al., 2016); (ii) belongs to an unknown incompatibility class but contains a homolog of *parB* found in *S. enterica* serovar Typhimurium plasmid pSTM_Phi (Octavia et al., 2015); and (iii) may exist in *C. sakazakii* lineages in addition to pESA3-like plasmids.

Interestingly, the genome of H322 harbors 43 gene sequences related to efflux-pump activity, such as those from multidrug

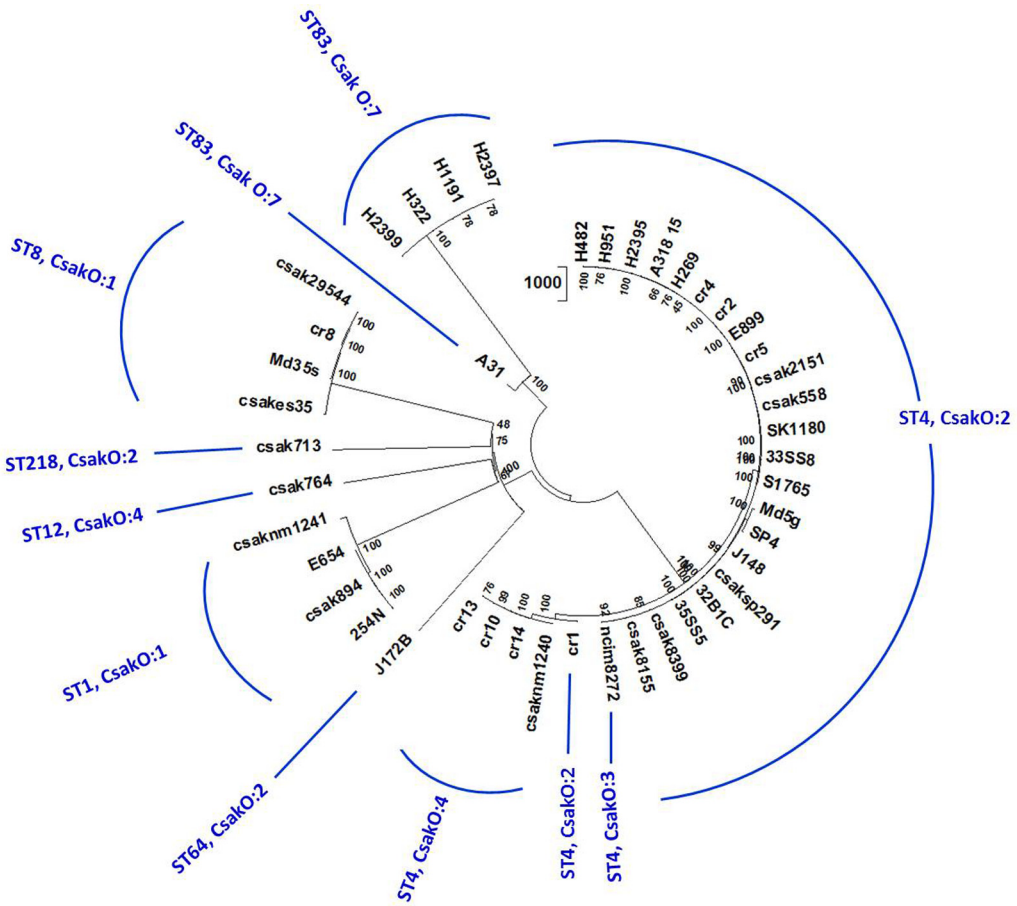


FIGURE 3 | Phylogenetic analysis of *C. sakazakii* strains of different ST: Allelic variations in conserved genes spanning the whole genome were used for this analysis. Alleles present in homologs of 1000 *C. sakazakii* strain BAA-894 chromosomal loci (reference gene set for this analysis) in 44 genomes were detected by BLAST-based workflow (Tall et al., 2015). SNPs in 37,711 positions in these genomes were concatenated and the cladogram was built using Neighbor-Joining algorithm available on MEGA7 phylogenetic suite (Kumar et al., 2016). The bootstrap consensus (values located on the nodes) in the tree are inferred from 500 replicates. 44 genomes separated into eight STs and multiple clusters reflecting microarray-based clusters. Five ST83 isolates group together with A31 showing some differences in the cluster confirming other results.

TABLE 3 | Plasmid typing results for the *C. sakazakii* serotype ST 83 strains analyzed in this study.

	PCR reaction results*														
Isolate Name	**pESA3**	**eit**	**iuc**	**cpa**	**Δcpa**	**ΔT6SS**	**T6SS IntL**	**vgrG**	**T6SSR end**	**T6SS IntR**	**Δfha**	**fha**	**dfha**	**pESA2**	**pCTU3**
A31	(+)	(+)	(+)	(+)	(+)	(–)	(+)	(–)	(–)	(+)	(+)	(–)	(–)	(–)	(–)
H2399	(+)	(+)	(+)	(+)	(+)	(–)	(+)	(–)	(–)	(+)	(+)	(–)	(–)	(–)	(–)
H2397	(+)	(+)	(+)	(+)	(+)	(–)	(+)	(–)	(–)	(+)	(+)	(–)	(–)	(–)	(–)
H1191	(+)	(+)	(+)	(+)	(+)	(–)	(+)	(–)	(–)	(+)	(+)	(–)	(–)	(+)	(–)
H322	(+)	(+)	(+)	(+)	ND	ND	(+)	(–)	(–)	(–)	(+)	(–)	ND	(–)	(–)

**Results are based on description of PCR amplification of reactions using primers and parameters described by Franco et al., (2011). For example (+) refers to an amplification of targeted gene; (–) refers to a negative amplification result, and ND refers to not determined. ND results for Δcpa and fha are due to positive PCR findings of the presence of cpa and the conserved flanking regions of fha in these strains. Negative PCR results for these targets are expected.*

resistance gene families and superfamilies such as RND, AcrR (the negatively acting repressor part of the *acrRAB* operon), MFS, PET, arsenic, DMT, and MATE. Furthermore, H322 has eleven alleles related to stress response, including: *degQ* and *degS*, and universal stress protein genes A, B, C, E, and G, as well as 25 alleles related to polysaccharide metabolism, transportation, exportation and biosynthesis. For comparison, additional genes found in each of the strains by RAST analysis are shown in Supplementary Table S4.

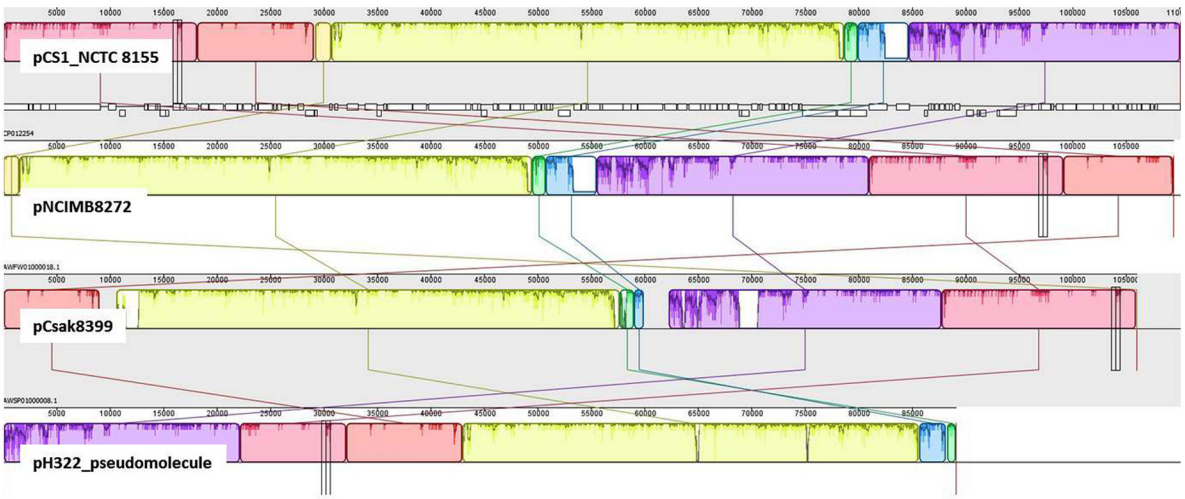


FIGURE 4 | Comparative genomic analysis to identify pCS1-like sequences: Mauve global alignment of the pCS1-like sequences in H322: (track 4) with *C. sakazakii* NCTC 8155 pCS1_NCTC 8155 (track 1), *C. sakazakii* pNCIM8272 (track2) and *C. sakazakii* 8399 pCsak8399 (track 3). H322 contigs mapping to pCS1 plasmid (CP012254) were used to create an artificial molecule to represent pH322 (track 4; pH322_pseudomolecule). This artificial scaffold was compared with pCS1 and pCS1-like plasmid contigs from strains NCIMB8272 (track2) and Csak8399 (track3). In addition to pESA3-like plasmid, isolates such as strain H322 reported in this work contain a large plasmid highly comparable to pCS1 plasmid.

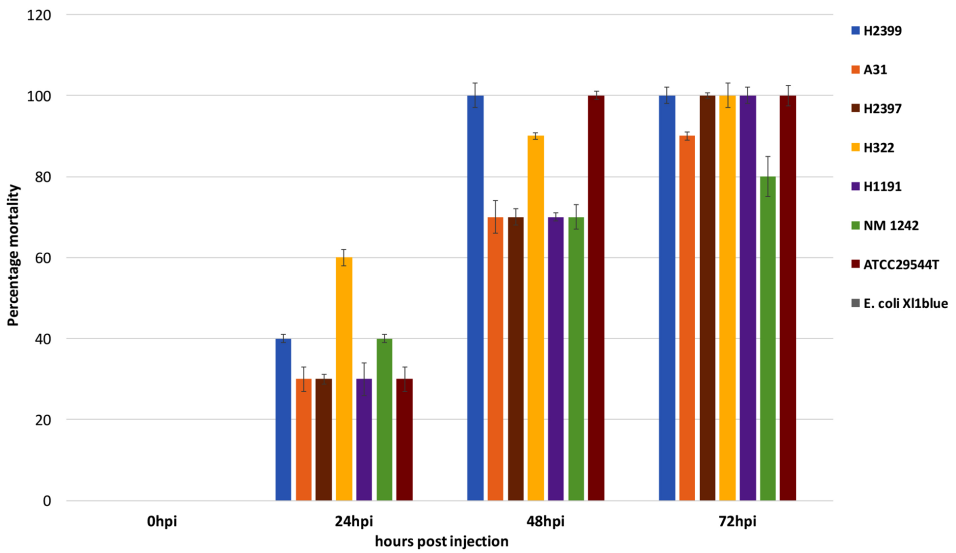


FIGURE 5 | Results of the zebrafish embryo infection experiments with five *C. sakazakii* ST83 strains, the two clinical strains ATCC29544^T and NM 1242 as well as *E. coli* Xl1 blue.

As of now, there are only two ST83 clinical isolates known which includes one from Israel (1998) and the other from China (2014) that have been submitted to the *Cronobacter* pubMLST site[3] (Jolley and Maiden, 2010). Studies on the potential virulence of *C. sakazakii* strains have been limited in the past due to the lack of suitable animal models by which strains and mutants may be analyzed in a high-throughput manner. In 2015 the zebrafish embryo model was adapted for infection studies in *Cronobacter* spp. which was intended to meet this requirement (Fehr et al., 2015; Eshwar et al., 2016). The use of this model made it possible to analyze strains of non-clinical origin for their virulence potential *in vivo*. In the current study the ST83 strains were subjected to infection studies and the results revealed high (90–100% lethality within 3 days) virulence potential for these ST83 strains (**Figure 5**). As controls, two clinical *C. sakazakii* isolates (ATCC 29544^T, NM1242) of other STs (ST8 and ST4,

[3]http://pubmlst.org/cronobacter/

respectively) as well as *E. coli* Xl1 blue were included in these experiments. While the *C. sakazakii* type strain ATCC29544^T (isolated from a child's throat) showed 100% lethality in the assay, the NM1242 (isolated from brain exudate) strain exhibited a slightly reduced lethality in zebrafish embryos. Infections with *E. coli* Xl1 blue (as well as all other controls) were asymptomatic, as expected. Although – to date – no illnesses have been linked to infections with ST83 strains, our results cannot rule out a potential risk for neonates exposed to PIF contaminated with *C. sakazakii* ST83 strains.

Infection studies with *C. sakazakii* of STs other than ST83, including *C. sakazakii* ST4 strains, which have been linked to neonatal infections and are frequently isolated from PIF and PIF environments, are currently ongoing. The availability of the H322 genome will enable its comparison with other genomes of *C. sakazakii* strains, providing more insights into genetic features encoding stress resistance, efflux pump activity, plasmid presence, and polysaccharide metabolism that are associated with this foodborne pathogen.

AUTHOR CONTRIBUTIONS

All authors (HRC, GG, AE, AS, CF-F, JG, IP, HNC, HJ, CL, FN, SF, RS, BT, and AL) contributed to the drafting of the manuscript. HRC, GG, AE, JG, IP, HNC, HJ, CL, FN, SF, RS, and BT carried out the data acquisition, analysis and interpretation of data. AE performed the zebrafish infection experiments and AE and AL interpreted the results. HRC, GG, AE, JG, IP, HNC, HJ, CL, FN, SF, and BT performed the whole genome sequencing and microarray experiments and GG interpreted the results. All authors contributed to the study concept and design; and critical revision of the manuscript for important intellectual content.

FUNDING

Funds supporting this work were obtained internally through United States FDA appropriations and the University of Zurich. Moreover, this research was also funded in part by the University of Maryland JIFSAN Program through a cooperative agreement with the FDA, #FDU001418.

ACKNOWLEDGMENTS

We would like to acknowledge the student internship programs of the International Offices of Kyungpook National University, Daegu, and Gachon University, Gyeonggi, Republic of Korea for financially supporting student interns: HJ and CL, respectively, We thank the University of Maryland, Joint Institute for Food Safety and Applied Nutrition (JIFSAN) for supporting JIFSAN interns SF and FN. We also thank the Oak Ridge Institute for Science and Education of Oak Ridge, Tennessee for sponsoring research fellow HRC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01136/full#supplementary-material

REFERENCES

Affymetrix (2014). *Expression Analysis Technical Manual, with Specific Protocols for Use with the Hybridization, Wash, and Stain Kit*. Available at: http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. doi: 10.1186/1471-2164-9-75

Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19, 185–193. doi: 10.1093/bioinformatics/19.2.185

Chase, H. R., Gopinath, G. R., Gangiredla, J., Patel, I. R., Kothary, M. H., Carter, L., et al. (2016). Genome sequences of malonate-positive *Cronobacter sakazakii* serogroup O:2, sequence type 64 strains CDC 1121-73 and GK1025, isolated from human bronchial wash and a powdered infant formula manufacturing plant. *Genome Announc.* 4:e01072-16. doi: 10.1128/genomeA.01072-16

Darling, A. E., Mau, B., and Perna, N. T. (2010). progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE* 5:e11147. doi: 10.1371/journal.pone.0011147

Eshwar, A. K., Tall, B. D., Gangiredla, J., Gopinath, G. R., Patel, I. R., Neuhauss, S. C. F., et al. (2016). Linking genomo- and pathotype: exploiting the zebrafish embryo model to investigate the divergent virulence potential among *Cronobacter* spp. *PLoS ONE* 11:e0158428. doi: 10.1371/journal.pone.0158428

Fehr, A., Eshwar, A. K., Neuhauss, S. C., Ruetten, M., Lehner, A., and Vaughan, L. (2015). Evaluation of zebrafish as a model to study the pathogenesis of the opportunistic pathogen *Cronobacter turicensis*. *Emerg. Microbes Infect.* 4:e29. doi: 10.1038/emi.2015.29

Franco, A. A., Hu, L., Grim, C. J., Gopinath, G., Sathyamoorthy, V., Jarvis, K. G., et al. (2011). Characterization of putative virulence genes on the related RepFIB plasmids harbored by *Cronobacter* spp. *Appl. Environ. Microbiol.* 77, 3255–3267. doi: 10.1128/AEM.03023-10

Grim, C. J., Gopinath, G. R., Jarvis, K. G., Sathyamoorthy, V., Trach, L. H., Chase, H. R., et al. (2015). Genome sequence of *Cronobacter sakazakii* serogroup O:4, sequence type 4 strain CDC 2009-03746, isolated from a fatal case of infantile meningitis. *Genome Announc.* 3:e00492-15. doi: 10.1128/genomeA.00492-15

Hunter, C. J., Petrosyan, M., Ford, H. R., and Prasadarao, N. V. (2008). *Enterobacter sakazakii*: an emerging pathogen in infants and neonates. *Surg. Infect.* 9, 533–539. doi: 10.1089/sur.2008.006

Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., et al. (2008). Cronobacter gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genomospecies* 1, and of three subspecies, *Cronobacter dublinensis* subsp. dublinensis subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* 58, 1442–1447. doi: 10.1099/ijs.0.65577-0

Jackson, S. A., Patel, I. R., Barnaba, T., LeClerc, J. E., and Cebula, T. A. (2011). Investigating the global diversity of *Escherichia coli* using a multi-genome DNA microarray platform with novel gene prediction strategies. *BMC Genomics* 12:349. doi: 10.1186/1471-2164-12-349

Jolley, K. A., and Maiden, M. C. J. (2010). BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. doi: 10.1186/1471-2105-11-595

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., and Forsythe, S. J. (2012a). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *Int. J. Syst. Evol. Microbiol.* 62, 1277–1283. doi: 10.1099/ijs.0.032292-0

Joseph, S., Sonbol, H., Hariri, S., Desai, P., MacClelland, M., and Forsythe, S. J. (2012b). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. *J. Clin. Microbiol.* 50, 3031–3039. doi: 10.1128/JCM.00905-12

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Kucerova, E., Clifton, S. W., Xia, X. Q., Long, F., Porwollik, S., Fulton, L., et al. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other Cronobacter species. *PLoS ONE* 5:e9556. doi: 10.1371/journal.pone.0009556

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lehner, A., Fricker-Feer, C., and Stephan, R. (2012). Identification of the recently described *Cronobacter condimenti* by an *rpoB* gene-based PCR system. *J. Med. Microbiol.* 61, 1034–1035. doi: 10.1099/jmm.0.042903-0

Lehner, A., Nitzsche, S., Breeuwer, P., Diep, B., Thelen, K., and Stephan, R. (2006). Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. *BMC Microbiol.* 6:15. doi: 10.1186/1471-2180-6-15

Moine, D., Kassam, M., Baert, L., Tang, Y., Barretto, C., Ngom Bru, C., et al. (2016). Fully closed genome sequences of five type strains of the genus Cronobacter and one *Cronobacter sakazakii* strain. *Genome Announc.* 4:e00142-16. doi: 10.1128/genomeA.00142-16

Müller, A., Stephan, R., Fricker-Feer, C., and Lehner, A. (2013). Genetic diversity of *Cronobacter sakazakii* isolates collected from a Swiss infant formula production facility. *J. Food Prot.* 76, 883–887. doi: 10.4315/0362-028X.JFP-12-521

Octavia, S., Sara, J., and Lan, R. (2015). Characterization of a large novel phage-like plasmid in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol. Lett.* 362:fnv044. doi: 10.1093/femsle/fnv044

Osaili, T., and Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. *Int. J. Food. Microbiol.* 136, 214–220. doi: 10.1016/j.ijfoodmicro.2009.08.006

Osaili, T. M., Shaker, R. R., Al-Haddaq, M. S., Al-Nabulsi, A. A., and Holley, R. A. (2009). Heat resistance of *Cronobacter* species (*Enterobacter sakazakii*) in milk and special feeding formula. *J. Appl. Microbiol.* 107, 928–935. doi: 10.1111/j.1365-2672.2009.04271.x

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Stoller, A., Stephan, R., Fricker-Feer, C., and Lehner, A. (2016). Epidemiological investigation of a powdered infant formula product batch contaminated with *Cronobacter* in a Swiss infant formula production facility. *Austin Food Sci.* 1:1028.

Stoop, B., Lehner, A., Iversen, C., Fanning, S., and Stephan, R. (2009). Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int. J. Food Microbiol.* 136, 165–168. doi: 10.1016/j.ijfoodmicro.2009.04.023

Sun, Y., Wang, M., Liu, H., Wang, J., He, X., Zeng, J., et al. (2011). Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* 77, 2209–2214. doi: 10.1128/AEM.02229-10

Tall, B. D., Chen, Y., Yan, Q. Q., Gopinath, G. R., Grim, C. J., Jarvis, K. G., et al. (2014). *Cronobacter*: an emergent pathogen using meningitis to neonates through their feeds. *Sci. Prog.* 97, 154–172. doi: 10.3184/003685014X13994743930498

Tall, B. D., Gangiredla, J., Gopinath, G. R., Yan, Q., Chase, H. R., Lee, B., et al. (2015). Development of a custom-designed, pan genomic DNA microarray to characterize strain-level diversity among *Cronobacter* spp. *Front. Pediatr.* 3:36. doi: 10.3389/fped.2015.00036

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Chase, Gopinath, Eshwar, Stoller, Fricker-Feer, Gangiredla, Patel, Cinar, Jeong, Lee, Negrete, Finkelstein, Stephan, Tall and Lehner. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 June 2017
doi: 10.3389/fmicb.2017.01138

Production of Lipopeptide Biosurfactant by a Marine *Nesterenkonia* sp. and Its Application in Food Industry

George S. Kiran[1], Sethu Priyadharsini[1], Arya Sajayan[1], Gopal B. Priyadharsini[1], Navya Poulose[1] and Joseph Selvin[2]**

[1] Department of Food Science and Technology, Pondicherry University, Puducherry, India, [2] Department of Microbiology, School of Life Sciences, Pondicherry University, Puducherry, India

Biosurfactants are smart biomolecules which have wide spread application in medicines, processed foods, cosmetics as well as in bioremediation. In food industry, biosurfactants are used as emulsion stabilizing agents, antiadhesives, and antimicrobial/antibiofilm agents. Nowadays biosurfactant demands in industries has increased tremendously and therefore new bacterial strains are being explored for large scale production of biosurfactants. In this study, an actinobacterial strain MSA31 was isolated from a marine sponge *Fasciospongia cavernosa* which showed high activity in biosurfactant screening assays such as drop collapsing, oil displacement, lipase and emulsification. Lipopeptide produced by MSA31 was found to be thermostable which was evident in differential scanning calorimetry analysis. The spectral data obtained in the Fourier transform infrared spectroscopy showed the presence of aliphatic groups combined with peptide moiety which is a characteristic feature of lipopeptides. The stability index of lipopeptide MSA31 revealed "halo-alkali and thermal tolerant biosurfactant" which can be used in the food industry. Microtiter plate assay showed 125 μg/ml of lipopeptide was effective in reducing the biofilm formation activity of pathogenic multidrug resistant *Staphylococcus aureus*. The confocal laser scanning microscopic images provided further evidences that lipopeptide MSA31 was an effective antibiofilm agent. The antioxidant activity of lipopeptide MSA31 may be due to the presence of unsaturated fatty acid present in the molecule. The brine shrimp cytotoxicity assay showed lipopeptide MSA31 was non-toxic and can be used as food additives. Incorporation of lipopeptide MSA31 in muffin showed improved organoleptic qualities compared to positive and negative control. This study provides a valuable input for this lipopeptide to be used in food industry as an effective emulsifier, with good antioxidant activity and as a protective agent against *S. aureus*.

Keywords: marine actinomycetes, lipopeptides, biosurfactant, antibiofilm, bioemulsifier

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Marcia Nitschke, University of São Paulo, Brazil
Thavasimuthu Citarasu, Centre for Marine Science and Technology, India

****Correspondence:***
George S. Kiran
seghalkiran@gmail.com;
kiran.mib@pondiuni.edu.in
Joseph Selvin
josephselvinss@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *19 March 2017*
Accepted: *06 June 2017*
Published: *28 June 2017*

Citation:
Kiran GS, Priyadharsini S, Sajayan A, Priyadharsini GB, Poulose N and Selvin J (2017) Production of Lipopeptide Biosurfactant by a Marine Nesterenkonia sp. and Its Application in Food Industry. Front. Microbiol. 8:1138. doi: 10.3389/fmicb.2017.01138

INTRODUCTION

Marine sponges are the reservoir of dense and unique marine microbial communities. The sponge tissue is a microbial niche which provides favorable conditions for the abundant growth of marine microorganisms. About 40% of sponge biomass comprises of complex microbial communities (Selvin et al., 2010). Actinomycetes are most abundant bacterial genera comprising about 20%

of microbiome in some marine sponges (Montalvo et al., 2005; Gandhimathi et al., 2008). The metabolites derived from sponge associated actinomycetes show a wide range of biological activities which include antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant, and anticancer properties (Selvin and Lipton, 2004; Bull and Stach, 2007; Pimentel-Elardo et al., 2010; Abdelmohsen et al., 2012; Blunt et al., 2013). Sponge associated actinomycetes were first reported biosurfactant producers among the group "actinomycetales" (Gandhimathi et al., 2009). Biosurfactants and bioemulsifier production has been increased in recent years as they were important green compounds which showed application in medicines, processed foods, cosmetics, and environmental cleaning (Kiran et al., 2010b).

Biosurfactants are compounds which have high surface tension reducing property along with emulsification activity whereas bioemulsifiers possess high emulsification activity with less or negligible surface tension reducing property. Biosurfactant and bioemulsifier production has increased in recent years as these biomolecules derived from actinomycetes showed tremendous application in medicine, processed foods, cosmetics and in bioremediation (Kiran et al., 2010b). Biosurfactants have multifunctional applications in food industry as emulsifiers, antiadhesives, and antimicrobial/antibiofilm agents. Biosurfactants are emulsion stabilizing agents, incorporated in food products to maintain the consistency, texture, solubilisation of fat globules, improve aroma, foaming and dispersing properties (Campos et al., 2013). Bioemulsifier is a natural food ingredient which can be used to improve the rheology of dough, increase the volume and emulsification of fat thus finds further applications in bakery and meat processing industry (Kourkoutas et al., 2004).

Considering the increased use of bioemulsifier in food industry, identification of new compounds with low or no toxicity and high emulsifying property has become essential Chemically synthesized surfactants are toxic whereas biosurfactants obtained from microbes are non-toxic and/or with least toxicity and they are highly stable at extreme temperature, pH and salinity. In food, rhamnolipid biosurfactant was used to improve the quality of the baked and confectionary products (Van Haesendonck and Vanzeveren, 2004). Incorporation of 0.10% rhamnolipid has been found to improve the texture and shelf life of muffin and croissants (Gandhi and Skebba, 2007). Bioemulsifier produced from marine *Enterobacter cloacae* has been used to enhance the viscosity of acidic food products (Shepherd et al., 1995; Iyer et al., 2006). Even though biosurfactants as bioemulsifiers have immense application in food industry, reports on their utilization in food industries are very limited.

In this study, a marine sponge associated actinomycetes *Nesterenkonia* sp. MSA31 was screened for biosurfactant production and was chemically characterized as lipopeptide derivative. The lipopeptide MSA31 showed no toxicity in brine shrimp cytotoxicity assay and acted as an antibiofilm agent against multi drug resistant *Staphylococcus aureus*. In this report, we demonstrate that incorporation of lipopeptide bioemulsifier in muffin preparation shows improved softness and its organoleptic quality.

MATERIALS AND METHODS

Isolation and Screening of Actinomycetes

The actinomycetes used in this study for biosurfactant production was isolated from a marine sponge *Fasciospongia cavernosa* collected by SCUBA diving at a depth of 10–15 m in Vizhinjam 8°22′45″N 76°59′29″E located in southwest coast of India. The sponge samples were immediately transported to the laboratory in ice box. Approximately 1 cm^3 of the sponge tissue was excised using sterile scissors and washed extensively in sterile seawater. The sponge tissue was homogenized using a tissue homogenizer. The aliquot was serially diluted in sterile seawater and plated on various media as mentioned in our previous report (Gandhimathi et al., 2009). The isolates obtained were cultured on a minimal salt media containing g/L of KH_2PO4 0.5 g, $FeSO_4$ $7H_2O$ 0.1 g, Na_2CO_3 0.2 g, L-asparagine 0.1 g, $MgSO_4$ 0.1 g, yeast extract 1 g and NaCl 1 g, pH 7. After 144 h of incubation, the culture media were centrifuged at 14,000 rpm for 20 min (Eppendorf). The filtrate obtained was screened on various screening assays including drop collapsing (Jain et al., 1991), oil displacement (Morikawa et al., 2000), lipase (Kiran et al., 2010b), haemolytic activity (Kiran et al., 2010b), surface tension measurement (dynamic tensiometer), and emulsification index (Paraszkiewicz et al., 1992). The emulsification index was determined using cell free supernatant (CFS) and sunflower oil (Goldwinner®) as substrate (1:1), the mixture was vortexed for 60 s and stability of the formed emulsion was determined after 24 h.

Identification of Biosurfactant Producer

The genomic DNA of MSA31 was isolated by CTAB/NaCl method. Universal 16S rRNA eubacterial primer (5′-GAGTTTG ATCCTGGCTCAG-3′; 5′-AGAAAGGAGGTGATCCAGCC-3′) was used for the amplification of DNA. The 16S rRNA gene sequence obtained from the producers was compared with other bacterial sequences by using NCBI BLASTn (Altschul et al., 1990, 1997) for their pair wise identities. Multiple alignments of these sequences were carried out by Clustal W 1.83 version of EBI[1] with 0.5 transition weight. Phylogenetic trees were constructed in MEGA 7 version[2] using Maximum Parsimony (MP) method.

Extraction and Purification of Biosurfactant

The isolate MSA31 was inoculated into 1 L of production media composed of minimal salt media enriched with 10 g olive oil, 10 g ammonium nitrate and 19 g NaCl and incubated at 28°C for 144 h with agitation of 180 rpm. After incubation, the CFS was obtained by centrifugation at 14,000 rpm for 20 min at 4°C (Eppendorf). The supernatant was acidified to pH 2.0 with 0.1N HCl and allowed to form precipitate by incubating overnight at 4°C. The acid precipitate was collected by centrifugation at 12,000 rpm for 30 min, 4°C.

[1]www.ebi.ac.uk/cgi-bin/clustalw/

[2]www.megasoftware.net

The precipitated biosurfactant was washed several times with sterile distilled water and the pH was adjusted to 7.0 using 0.1 N NaOH. The precipitate was resuspended in sterile distilled water and solvent optimization was performed by adding equal volume of extraction solvents such as methanol, ethyl acetate, diethyl ether and dichloromethane (v/v). The resultant aliquot was concentrated to dryness in a rotary vacuum evaporator (Yamato). The solvent extract with high emulsification activity was further purified using column chromatography on silica gel (60–120 mesh) with step wise elution with methanol and water ranging from 65 to 100% (v/v) at a flow rate of 0.5 ml/min at room temperature (27°C). The purified fraction was used for chemical characterization and identification of active molecule.

Chemical Characterization of Biosurfactant Compound

The active fraction was confirmed by the emulsification activity and the purity was checked by TLC. The TLC fractionation was performed using the solvent system for the separation of biomolecules which include proteins (*n*-butanol: acetic acid: water 45:35:20), carbohydrates (chloroform: acetic acid: water 50:30:20) and lipids (chloroform: methanol: water 60:30:10). The spots developed on the TLC plates were visualized by spraying of 50% H_2SO_4 for carbohydrates and ninhydrin for amino acids. The TLC plates were exposed in an iodine chamber to visualize the lipid fractions (Kiran et al., 2010b). To determine the functional groups the purified active column fraction was lyophilised in a lyophilizer (Yamato DC 400) and subjected to FT-IR analysis. The lyophilised active fraction was used for FTIR analysis on a Bruker IFS113v FTIR spectrometer, in the 4000–400 cm^{-1} spectral region at a resolution of 2 cm^{-1} and 50 scans. The lyophilized active fraction was investigated by using Gas chromatography (GC) (Perkin Elmer Autosystem XL GC-model Clarus-680, United States). For the structure prediction one dimensional ^1H NMR spectra were recorded by dissolving the biosurfactant at a concentration of 25 mg/ml^{-1} in deuterated DMSO and analyzed on a Bruker AVANCE III 500 MHz. C NMR was recorded on a solid state NMR (400 MHZ – JEOL-ECX-400).

Stability of Biosurfactant

The column purified fraction of biosurfactant was evaluated for its stability at different temperature, pH and salt concentrations. The stability assays were performed as per Kiran et al. (2010b). To determine the pH stability, the biosurfactant 0.4% (w/v) was dissolved in various pH ranges of buffer solutions which include 0.1 M sodium acetate buffer (pH4.0–7.0) and 0.1 M sodium phosphate buffer (pH 8.0–9.0). The aliquot was incubated at 37°C for 1 h to determine pH stability based on emulsification index. Similarly, the biosurfactant 0.4% (w/v) was dissolved in distilled water containing various concentrations of NaCl ranging between 1 and 12% and incubated at 37°C for 1 h to determine NaCl stability. Temperature stability was determined by incubating the biosurfactant at various temperatures ranging between 4 and 121°C for 1 h, and then the stability of the compound was assessed based on the emulsification index.

Thermal Gravimetric (TG) and Differential Scanning Calorimetric (DSC) Analysis

Thermal Gravimetric and Differential Scanning Calorimetric of biosurfactant were performed and determined using TA instruments, Q600 SDT and Q20 DSC (Kiran et al., 2014). Approximately 3 mg of sample was placed in an aluminum pan. The analysis was carried out over the temperature range from 0°C to 300°C at a rise in temperature of 10°C/min. The flow rate of the gas was set at 50 ml/min.

Antioxidant Activity

Antioxidant activity of the biosurfactant from MSA31 was analyzed using 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay (Turkmen et al., 2006) with necessary modifications. In this assay, 0.3 ml containing different concentrations of biosurfactant between 0.5 and 6 mg/ml were added to 3.5 ml of 99.5% ethanol containing DPPH (0.02 mM). The assay mixture was mixed well and incubated in dark at 25°C for 30 min. Butylated hydroxytoluene (BHT) was used as the positive control, ethanol was set as blank and the assays were performed in triplicates. The process of decolourization was recorded at 520 nm using Shimadzu UV-VIS spectrophotometer. The percentage of radical scavenging was calculated using the following formula.

$$AA(\%) = \left[\left(Abs._{control} - Abs._{sample}\right)/Abs._{control}\right] \times 100 \quad (1)$$

Brine Shrimp Cytotoxicity Assay

The larvae of *Artemia franciscana* were obtained by decapsulation of sterile cysts as mentioned in Kiran et al. (2016). Briefly, the cysts were aerated in sterile seawater and oxygenated continuously using aerator pumps. After 24 h incubation at room temperature (25–29°C), the freshly hatched nauplii (larvae) were collected by a micropipette. The nauplii were transferred into a 96 deep well plate with 10 nauplii per plate and different concentration of lipopeptide (25–200 μg/ml). The assay was performed in triplicates. Wells were examined under the binocular stereomicroscope (Optica) and the numbers of live and dead (non-motile) nauplii in each well were counted after 24 h.

Microtitre Plate Assay

The clinical strains of *S. aureus* were collected from Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry. The antibiogram pattern was tested as per CLSI Guidelines: Clinical and Laboratory Standards Institute (2009) to select multi drug resistant strains. *S. aureus* biofilm was allowed to form on microtitre plate wells containing lipopeptide of varying concentration (25 μg–150 μg/ml). The assay plates were prepared with 10 μl of overnight culture of *S. aureus* diluted upto growth OD of 0.005 and was inoculated into 200 μl of Luria-Bertani broth. The plates were kept under static conditions for 72 h at 37°C. After incubation the planktonic cells were removed by gentle pipetting, and the adhered cells were stained with 0.1%

crystal violet and the amount of biofilm formation was quantified at 595 nm using a microplate reader (Labnics) (Kiran et al., 2010a).

Antibiofilm Effect of Lipopeptide

The antibiofilm activity of lipopeptide was visualized using confocal laser scanning microscopy (CLSM). Effective concentration of lipopeptide 125 μg/ml was added to the Erlenmeyer flask containing LB broth (test) and the flask without lipopeptide was set as the control. The sterile glass slide was immersed into the broth using a sterile forceps. The broth was then inoculated with 1 ml of *S. aureus* overnight culture. After incubation at 37°C for 72 h, the glass slide was washed with sterile distilled water and stained with Baclight kit (Invitrogen) following manufacturer's instructions. The glass slide was then stained with equal amount of solution A and solution B (3 ml each), incubated for 15 min and observed under CLSM.

Evaluation of Lipopeptide as a Fat Replacer in Muffin Preparation

In order to evaluate the characteristics of lipopeptide on muffin preparation, the muffins were incorporated with varying concentration of lipopeptide between 0.50 and 1%. The muffin batter formulation was carried out as per Zahn et al. (2010) with necessary modifications. The ingredients used for muffin preparation include 36.57% wheat flour, 11.7% margarine, 22.30% sugar, 17.18% whole egg, 11.7% skim milk, 0.52% baking powder, and 20% water (positive control batter for muffin preparation). In the test, baking powder and egg was replaced with 0.50–1% lipopeptide. The mixture without egg was set as the negative control. The formulated dough was placed in the muffin tray and baked in a preheated oven at 180°C for 20 min until the appearance of light brown color. The prepared muffin was sealed in an air tight container for the textural analysis.

Textural Evaluation

The textural characteristics of the muffin were analyzed using a Texture analyser (TA–HDplus, Stable Micro Systems, Surrey). The analyser was pre-tested at 1.0 mm/s, test speed at 3.0 mm/s and post-speed at 10.0 mm/s, distance at 10 mm and 12.5 pps data acquisition rate, 50 kg load cell and P75 probe was used to analyze the parameters such as hardness, gumminess, chewiness, springiness, and cohesiveness.

Color Analysis of Muffin

The color of muffin was analyzed by a Hunter colorimeter (Hunter Lab Associates Inc.) using L*, a*, b* color space. The L* represents dark (0)/ light (100), while a*and b*represents red (+a) to green (+a) and yellow (+b) to blue (−b). The color was analyzed in triplicates and the mean was recorded.

RESULTS AND DISCUSSION

Isolation, Screening, and Identification of Biosurfactant Producer

A total of 33 morphologically distinct actinomycetes were isolated from the marine sponge *F. cavernosa*. All the isolates were screened for biosurfactant production. The isolate MSA31 was selected based on high emulsification activity (75%) and surface tension reducing property of 34.6 mN/m with critical micelle concentration of 18.6 μg/ml. The isolate MSA31 showed positive results in screening tests which include emulsification, drop collapsing and oil displacement. In drop collapsing test, a flat drop was observed whereas in oil displacement method, a clear diameter of 8 mm corresponding to the area of 64.24 mm^2 was observed. Emulsification activity, surface tension and the screening tests confirmed the isolate MSA31 as a biosurfactant producer. The enzymes and bioactive molecules produced by the sponge associated bacteria are economically important due to their novelty and remain active even in extremophilic conditions such as elevated salt concentration, wide range of pH, and higher temperatures. The isolate MSA31 grew optimally at increased salt concentration upto 5% and pH 6–10. Similar pH tolerance was reported for *Nesterenkonia alba* which grow optimally at pH 9–10 (Luo et al., 2009). Delgado et al. (2006) reported that *N. aethiopica*, a moderately halophilic strain can grow at salt concentration up to 3% and pH 9.0. There were a very few reports available on sponge associated actinobacteria for lipopeptide production (Gandhimathi et al., 2009; Kiran et al., 2010b). The PCR amplified KS (keto synthase) domain from the sponge associated actinobacteria envisages that the biosynthetic pathway of biosurfactants might have mediated through PKS (polyketide synthase) biosynthetic gene clusters (Selvin et al., 2016). Taxonomic affiliation based on the 16S rRNA sequence of the isolate MSA31 was retrieved from the classifier program of RDPII (Ribosomal Database Project II). The 16S rRNA sequence of the isolate MSA31 was analyzed using a megablast tool of GenBank[3]. Based on the closet matches with *Nesterenkonia* strains, the isolate MSA31 was taxonomically identified as *Nesterenkonia* sp. (**Figure 1**). The closet representative of maximum homologous (98–99%) sequences of each strains was obtained from seqmatch program of RDPII and was used for the construction of phylogenetic tree. The sequence obtained were deposited in genbank with accession number KY969127. A *Nesterenkonia* sp. isolated from Aran Bidgol lake (Iran) was used for the production of halophilic α-amylase with possible application in starch processing industries, baking, brewing, textile, and distillery industries (Shafiei et al., 2012).

Characterization of Biosurfactant

Among the various solvents used for extraction, ethyl acetate extract showed highest emulsification activity. The crude

[3]http://www.ncbi.nlm.nih.gov/

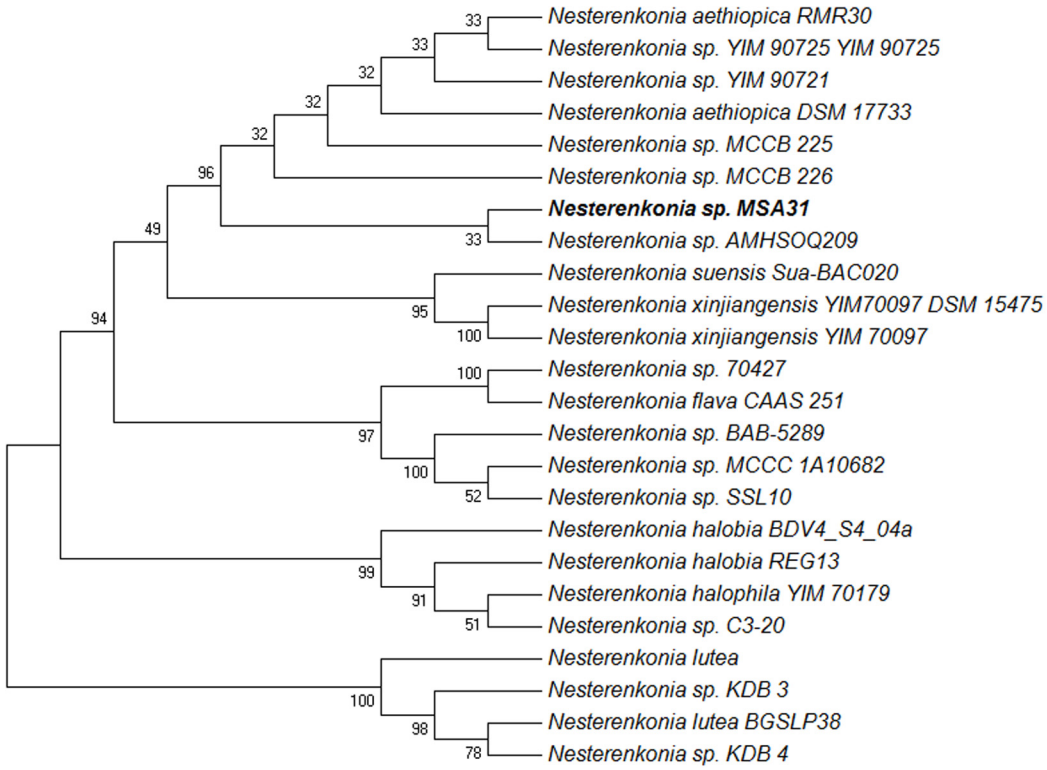


FIGURE 1 | Phylogenetic tree of MSA31 showing representatives of *Nesterenkonia* sp. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 9 most parsimonious trees (length = 89) is shown. The consistency index is (0.821429), the retention index is (0.938272), and the composite index is 0.780136 (0.770723) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

biosurfactant was purified in silica gel column chromatography with 80% methanol-water as eluting solvent. The active fractions collected from the column were checked for emulsification activity and analyzed in TLC to determine the purity of the compound. In the TLC plates, a yellow spot corresponding to the Rf value of 0.86 with chloroform: methanol: water (60:30:10) as the solvent system was identified as a biosurfactant (lipid) fraction. The FT-IR spectrum showed a strong broad peak at 3600–3200 cm^{-1} corresponding to the presence of hydrogen bonded –OH or –NH functional groups (**Figure 2**). The carboxylic groups at 1788 cm^{-1} and the sharp peak at 1630 cm^{-1} revealed the presence of amino acid zwitter ion –C = O peak or amide carbonyl peak. The absorbance at 1531 cm^{-1} was attributed to the stretching vibrations of –NH bonds. Similar absorption spectra in FT-IR was reported for lipopeptide in the literature (de Faria et al., 2011; Ghribi and Ellouze-Chaabouni, 2011; Pereira et al., 2013). Weak absorbance signals at 1444 and 1396 cm^{-1} were due to the bending vibrations of C-H bonds associated with alkyl chains. The peak appearing at 1055 cm^{-1} reveals another stretching frequency of C-O functional groups. The FT-IR spectra showed the presence of aliphatic groups combined with peptide moiety, a characteristic feature of lipopeptides. The GC-MS spectra showed the presence of aliphatic chain containing hexyl group.

^{1}H NMR Analysis

The ^{1}H NMR spectra showed broad multiplicity peaks between 8.3 and 7.5 ppm, which typify amino acid amide protons and confirm the presence of several amino acid residues in the molecule (Supplementary Figure S1). The aromatic phenyl group protons appeared as a multiplet in the range of 7.2 to 6.6 ppm and it revealed that the aromatic groups were present in the molecule. Several peaks were observed in the region of 6.6–8.3, which indicates the presence of amino acid amide protons as reported by Pereira et al. (2013). The signals appearing in the region between 5.2 and 4.2 ppm showed the presence of amino acid Hα resonance. The NMR peaks in the region of 5.2 were similar to the peaks reported earlier (Kowall et al., 1998; Liu et al., 2009). The peak at 3.2 ppm to 2.9 ppm revealed the protons attached with the acid functional group. The aliphatic $-CH_2$ protons appeared in the range of 2.4 to 1.6 ppm. The singlet peaks appeared at 1.2 and 0.9 ppm confirmed the presence of $-CH_3$, which was attached with quaternary carbon.

^{13}C NMR Spectra Analysis

In the ^{13}C NMR analysis the peak at 173 ppm revealed an $-NH_2$ group substituted quaternary carbon in the molecule, it may be due to presence of amino acid group in the molecule

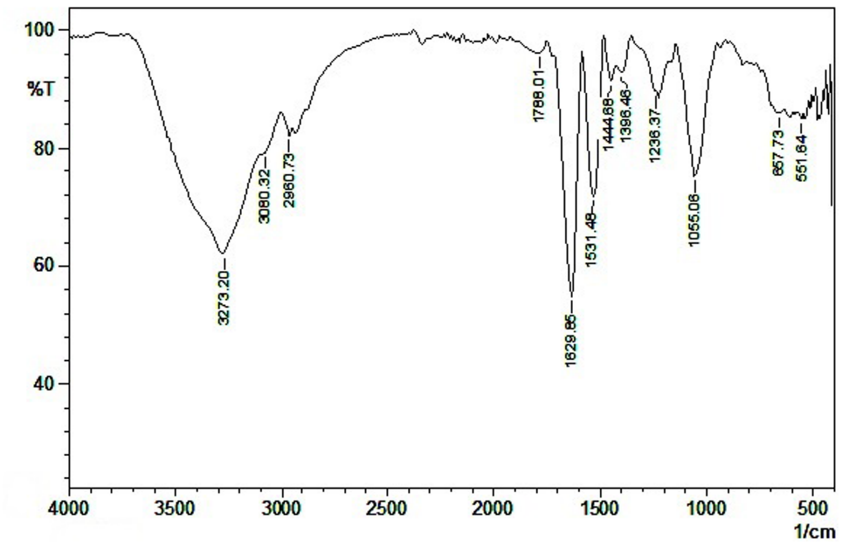


FIGURE 2 | Characterization of biosurfactant produced by MSA31 using FTIR.

(Supplementary Figure S1). The peaks at 155 ppm may be of an electronegative group attached to the aryl group, this reveals that molecule contains the electronegative groups. Peaks in the range of 136, 130, and 117 ppm showed the molecule contains aromatic groups. The peaks in the range of 73, 58, and 53 ppm revealed the presence of aliphatic groups attached to electronegative atoms, trisubstituted or tetrasubstituted carbons. The chemical shift peaks in the range of 32, 30, 25, 20, 16, 12 ppm revealed the presence of several aliphatic groups present in the molecule. The spectral data revealed the compound produced by MSA31 was a lipopeptide moiety containing aromatic aminoacid and also revealed the presence of aliphatic groups.

Stability of Biosurfactant

Stability of biosurfactant is an important criteria required for its application in food industry. The lipopeptide based biosurfactant produced by MSA31 was found to be thermo-stable in all the temperature ranges tested (**Figure 3A**). Stability was maintained even after autoclaving at 121°C and there was no change in emulsification activity even at low temperature 4°C. This property makes the lipopeptide from MSA31 suitable for ice cream and cosmetic industries. The emulsification activity (85%) of biosurfactant MSA31 was retained at 10% salt concentration and at pH of 6.0–9.0.

In TGA analysis, due to loss of water molecule a change in the mass was observed at 150°C followed by second phase of degradation at 240°C. Maximum degradation occurred at 260°C with weight loss of 50.725%. The DSC data was used to determine the phase transitions of lipopeptide from 0 to 400°C. The DSC thermogram showed exothermic peak of biosurfactant with crystallization temperature (T_c) of 80°C and the melting peaks were found at 240°C and 320°C. The thermal stability of the lipopeptide based on TGA analysis is shown in **Figure 3B**. Lipopeptide produced by MSA31 was found to be thermostable as revealed by DSC analysis. Similar thermostable lipopeptide were produced by *Nocardiopsis* sp., *Brevibacterium aureum,* and *Aneurinibacillus thermoaerophilus* MK01 (Gandhimathi et al., 2009; Kiran et al., 2010b; Sharafi et al., 2014).

Antioxidant Activity and Toxicity Evaluation

DPPH Assay

The DPPH activity of the lipopeptide from MSA31 was compared with the control BHT (**Figure 4**). A concentration dependent antioxidant activity of biosurfactant was recorded and highest activity was obtained at 6 mg/ml. Antioxidant property is an important characteristics of food products as it reduces the risk of coronary heart diseases and is also effective against degenerative diseases. The scavenging activity of lipopeptide MSA31 at 6 mg/ml was 65% which was higher than the scavenging activity recorded for *P. hubeiensis* which showed 50.3% with 10 mg/ml of mannosyl erythritol lipid (Takahashi et al., 2012). The high antioxidant activity of lipopeptide MSA31 may be due to the presence of unsaturated fatty acid present in the molecule. The acute toxicity of the lipopeptide MSA31 was evaluated using the brine shrimp cytotoxicity assay. The assay results revealed the lipopeptide was non-toxic to the brine shrimp *nauplii* in all the concentrations tested (up to 200 μg/ml). Previous reports related to the toxicity of lipopeptide were evaluated in *in vivo* toxicity assays in mice models which concluded that 2,000 mg/kg of body weight dose showed no mortality and normal behavioral indexes (Sahnoun et al., 2014). Park et al. (2006) reported that LD_{50} of surfactin in mice was above 2 g/kg. Biosurfactant produced by *Serratia marcescens* was administered to the mice orally at the dose of 5 g/kg

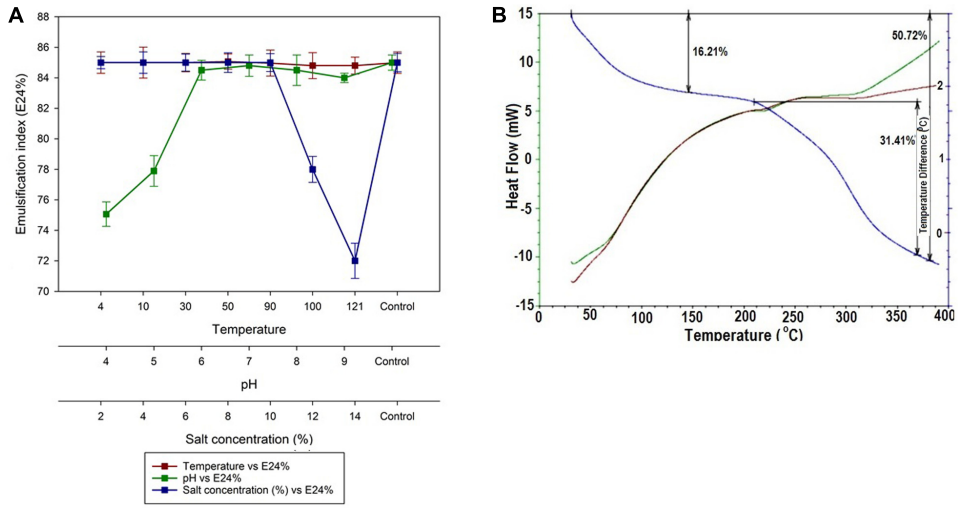


FIGURE 3 | (A) Stability of lipopeptide at increased temperature, pH and salt. The temperature stability is shown in **(B)** and the data indicates the compound was highly stable.

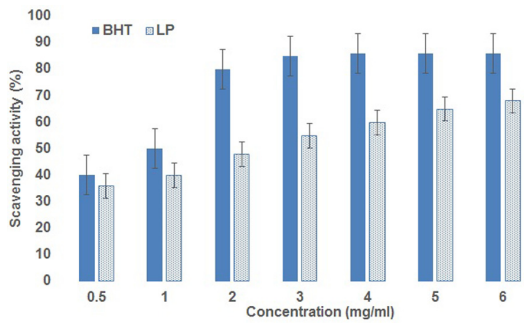


FIGURE 4 | Antioxidant activity of lipopeptide, the activity increases with the concentration of lipopeptide

and no toxic effect was noticed in the mice (Anyanwu et al., 2011). The available reports evidenced that lipopeptide and rhamnolipid biosurfactants in general were non-toxic biomolecules which can be used as food additives. According to Sahnoun et al. (2014) the lethal concentration of biosurfactant determined in the animal model was far higher than the threshold concentrations of food additives permitted by Food and Agricultural Organization and World Health Organization. In this study, brine shrimp cytotoxicity and phytotoxicity assays (Supplementary Figure S2) showed the non-toxic nature of lipopeptide biosurfactant.

Antibiofilm Effect of Lipopeptide

Microtiter plate assay showed lipopeptide MSA31 at a concentration of 125 μg/ml effectively reduced 90% of biofilm formed by *S. aureus* when compared to the control. It was observed that antibiofilm activity increases with increase in the concentration of the lipopeptide MSA31 (**Figure 5A**). The confocal microscope images evidenced that the lipopeptide was effective in controlling *S. aureus* biofilm formation (**Figures 5B,C**). The CLSM observation showed that the control biofilm was stained with green indicating live biofilm associated cells of *S. aureus* and the test biofilm was stained red indicating the dead cells of *S. aureus*. Microtiter plate assay and CLSM images showed lipopeptide at a concentration of 125 μg effectively inhibited the biofilm of MDR pathogen *S. aureus*. Reports showed that lipopeptides from *Bacillus* sp. and *Paenibacillus* sp. were known to inhibit/disperse biofilms (Price et al., 2007; Kim et al., 2009; Quinn et al., 2012).

Effect of Lipopeptide on Muffin Texture

Textural property is an important parameter in determining the quality and sensory characteristics of the muffin. The lipopeptide incorporated muffin showed reduction in hardness, chewiness and gumminess when compared to positive and negative control (**Table 1**). Among the various concentrations of lipopeptide, 0.75% was found to be effective in enhancing the softness and overall quality of the muffin. Muffin prepared using 0.75% lipopeptide had higher level of springiness, when compared to the control. According to Tess et al. (2015), a high springiness value was associated with freshness and high quality of the muffin. Thus 0.75% lipopeptide incorporated muffin was in good quality with higher springiness values. Cohesiveness is defined as the compression energy required for breaking down the food product for swallowing. The muffin prepared with 0.75% lipopeptide showed increased cohesiveness and therefore less compression energy was required to break it down. This property was an indication of soft nature of muffin. Negative control showed higher value of chewiness indicating the hard nature of muffin and 0.75% of lipopeptide incorporated muffin showed very less value of chewiness indicating the soft nature of muffin. Overall, the muffin prepared using 0.75% lipopeptide showed decreased

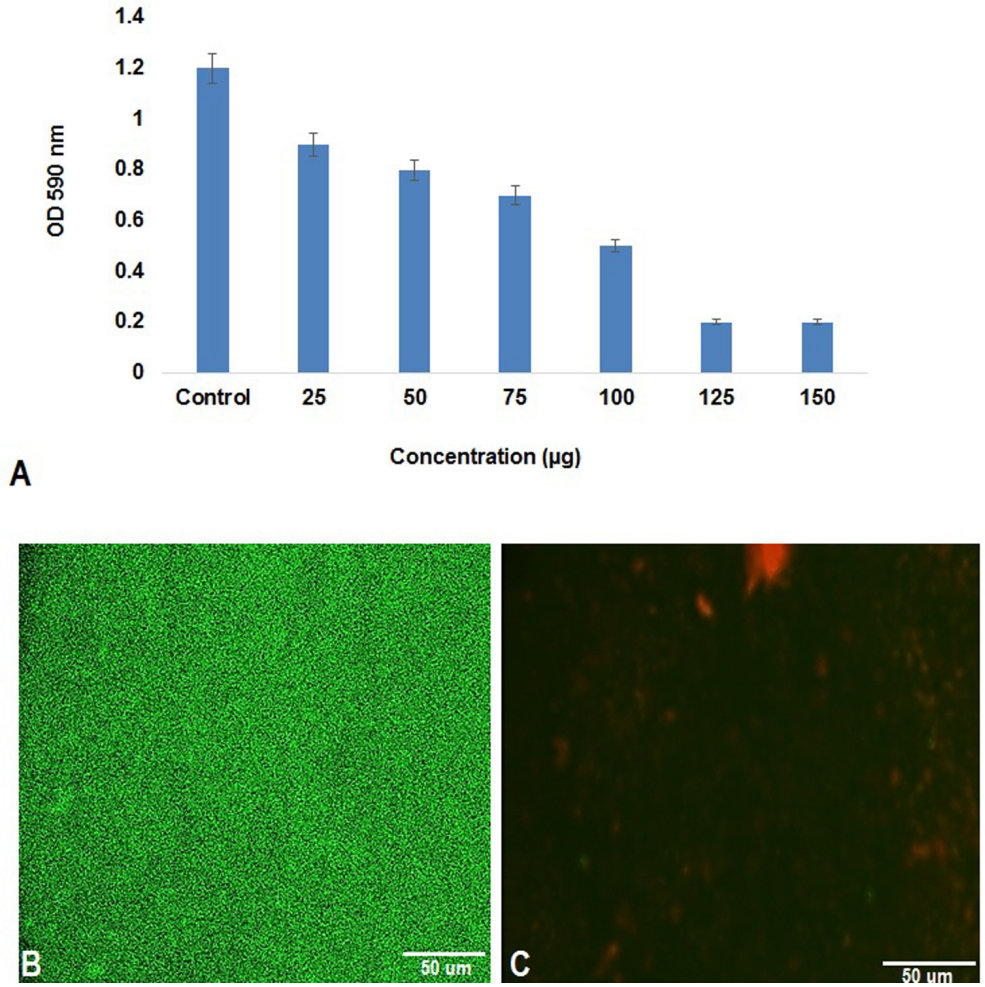


FIGURE 5 | Antibiofilm activity of lipopeptide MSA31 against MDR pathogen *S. aureus*. **(A)** Microtitre assay showing antibiofilm effect of Lipopeptide. **(B)** Is a control biofilm and **(C)** lipopeptide treated biofilm visualized by baclight staining.

hardness, chewiness and gumminess with increased springiness and cohesiveness.

Color

Color is an important factor to increase the appeal of the food products and is directly related to acceptance and taste (Chakraborty et al., 2015). The L*, a*, and b* values of prepared muffins are given in **Figure 6**. The muffin prepared by using lipopeptide showed slight increase in the b* value which indicates the yellow colouration. The b* value of lipopeptide muffin was found to be 36 when compared to the negative and positive controls of 32.37 and 34.2, respectively. The L* value was higher in the negative control (without egg and baking powder), which indicates light color of the muffin and a* value was found to be almost similar to the positive control and lipopeptide incorporated muffin. Normally in baked food products, changes in the natural color of the flour during baking is not uncommon due to Maillard reactions. The muffin prepared using lipopeptide MSA31 was light yellow in color as evident from b* value and the appeal was almost similar to the positive control. When Jambolan was incorporated into the muffin, the anthocyanin pigments in the Jambolan imparted dark color to the muffin (Singh et al., 2015).

CONCLUSION

In this study, actinobacterial strain *Nesterenkonia* sp. MSA31 was isolated from a marine sponge *F. cavernosa*. The strain MSA31 was found to be the highest biosurfactant producer among the 33 isolates screened. Based on the stability index, the lipopeptide MSA31 was characterized as "halo-alkali and thermal tolerant biosurfactant" which can be used as emulsifier and emulsion stabilizing agent in the food industry. The lipopeptide MSA31 was non-toxic, showed antibiofilm activity against a

TABLE 1 | Shows texture analysis data of the muffin, the values represented are the mean of triplicate experiments.

TPA parameters	Positive control	Negative control	0.5% LP	0.75% LP	1% LP
Hardness	1978 ± 2.0	2999 ± 3.60	1675 ± 4.0	958 ± 4.35	1345 ± 3.60
Chewiness	1128 ± 3.0	2150 ± 2.64	1115 ± 3.0	485 ± 3.60	900 ± 4.58
Gumminess	1288 ± 2.64	1925 ± 4.58	1188 ± 5.19	515 ± 3.46	855 ± 2.64
Springiness	0.99 ± 0.18	0.81 ± 0.02	0.96 ± 0.12	1.1 ± 0.17	0.98 ± 0.08
Cohesiveness	0.66 ± 0.05	0.61 ± 0.02	0.65 ± 0.05	0.72 ± 0.04	0.67 ± 0.78

The data is presented as mean ± standard deviation. The data shows that the muffin prepared using lipopeptide exhibited decreased hardness, chewiness and gumminess with increased springiness and cohesiveness values.

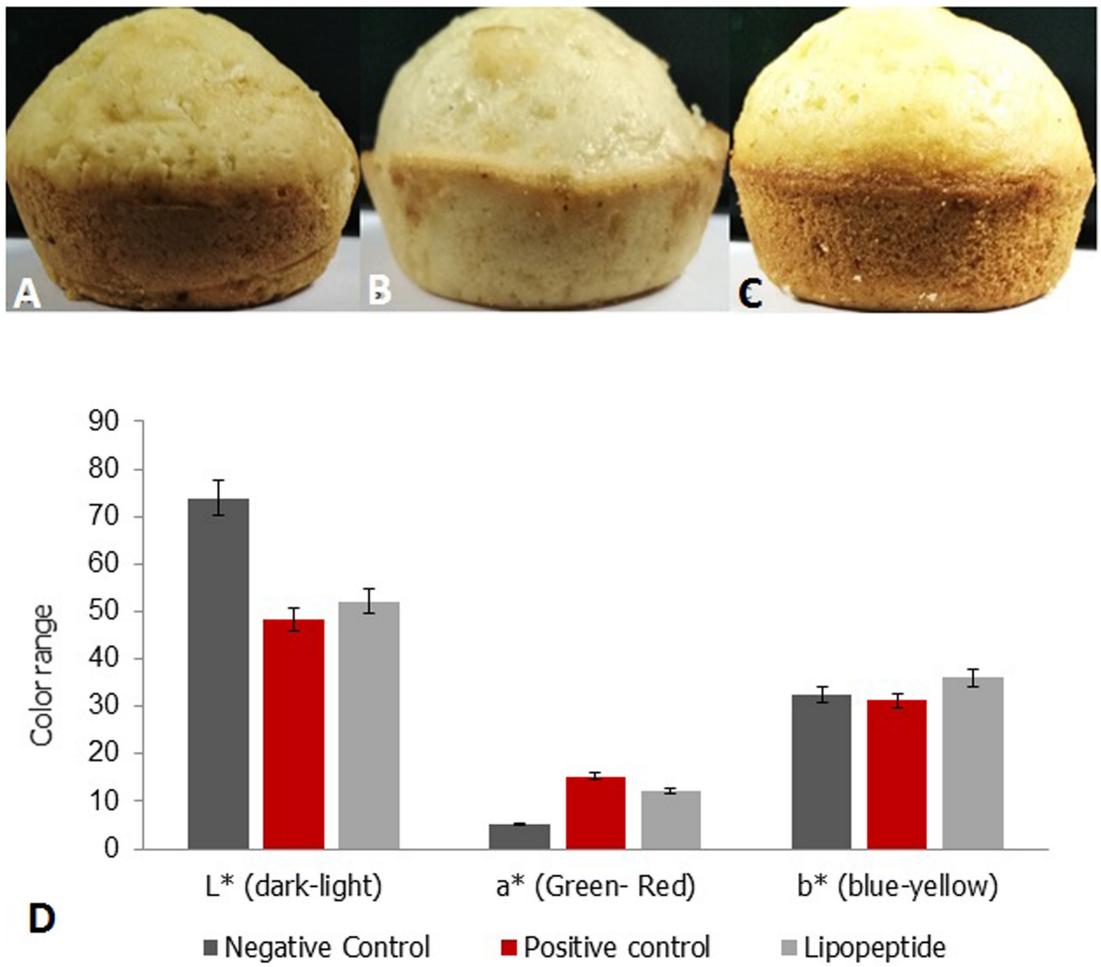


FIGURE 6 | Color analysis of muffin **(A)** Positive control muffin (egg and baking powder), **(B)** Negative control **(C)** muffin prepared using 0.75% of lipopeptide. **(D)** Color analysis of muffin L* represent the color range of lightness to darkness as per the color scale standards of the HunterLab benchtop color measurement systems (ıColorFlex EZ), a* represents green (–) to red (+), b* represents blue (–) to yellow (+). The addition of lipopeptide showed improved original color pattern of muffin to yellow color.

prominent food pathogen *S. aureus*. The emulsification property with antioxidant activity showed that the lipopeptide MSA31 can be highly beneficial to the food industry. Colors is an important factor to increase the appeal for the food products. The muffin prepared using lipopeptide showed light yellow in color, and the appeal was almost similar to the positive control. This study provided a new insight for the food industry as incorporation of lipopeptide emulsifier could improve the quality of food products with antioxidant activity as well as antibiofilm activity against *S. aureus*.

AUTHOR CONTRIBUTIONS

AS, NP, SP, and GBP performed laboratory experiments and data analysis, GSK written the manuscript and JS designed and guided the work.

ACKNOWLEDGMENTS

GSK is thankful to the Department of Science and Technology for a project grant. JS is thankful to the DBT project on the exploration of marine sponge microbiome. Finally, we thank Dr. E. Gnanamani, of Stanford University, for the interpretation of spectral data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01138/full#supplementary-material

REFERENCES

Abdelmohsen, U. R., Szesny, M., Othman, E. M., Schirmeister, T., Grond, S., Stopper, H., et al. (2012). Antioxidant and anti-protease activities of diazepinomicin from the sponge-associated *Micromonospora* strain RV115. *Mar. Drugs* 10, 2208–2221. doi: 10.3390/md10102208

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Anyanwu, C. U., Obi, S. K. C., and Okolo, B. N. (2011). Lipopeptide biosurfactant production by *Serratia marcescens* NSK-1 strain isolated from petroleum-contaminated soil. *J. Appl. Sci. Res.* 325, 79–87.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., and Prinsep, M. R. (2013). Marine natural products. *Nat. Prod. Rep.* 30, 237–323. doi: 10.1039/c2np20112g

Bull, A. T., and Stach, J. E. M. (2007). Marine actinobacteria: new opportunities for natural product search and discovery. *Trends Microbiol.* 15, 491–499. doi: 10.1016/j.tim.2007.10.004

Campos, J. M., Stamford, T. L. M., Sarubbo, L. A., Luna, J. M., Rufino, R. D., and Banat, I. M. (2013). Microbial biosurfactants as additives for food industries. *Biotechnol. Prog.* 29, 1097–1108. doi: 10.1002/btpr.1796

Chakraborty, P. S., Rao, H. N., and Mishra. (2015). Effect of combined high pressure–temperature treatments on color and nutritional quality attributes of pineapple (*Ananas comosus* L.) puree. *Innov. Food Sci. Emerg. Technol.* 28, 10–21. doi: 10.1016/j.ifset.2015.01.004

Clinical and Laboratory Standards Institute (2009). *Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20*. Wayne, PA: CLSI.

de Faria, A. F., Teodoro-Martinez, D. S., de Oliveira Barbosa, G. N., Vaz, B. G., Silva, Í. S., Garcia, J. S., et al. (2011). Production and structural characterization of surfactin (C_{14}/Leu_7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. *Process Biochem.* 46, 1951–1957. doi: 10.1016/j.procbio.2011.07.001

Delgado, O., Quillaguamán, J., Bakhtiar, S., Mattiasson, B., Gessesse, A., and Hatti-Kaul, R. (2006). *Nesterenkonia aethiopica* sp. nov., an alkaliphilic, moderate halophile isolated from an Ethiopian soda lake. *Int. J. Syst. Evol. Microbiol.* 56, 1229–1232. doi: 10.1099/ijs.0.63633-0

Gandhi, N. R., and Skebba, V. L. P. (2007). Rhamnolipid compositions and related methods of use. U.S. Patent No WO 2007095258 A2. Washington, DC: U.S. Patent and Trademark Office.

Gandhimathi, R., Arunkumar, M., Selvin, J., Thangavelu, T., Sivaramakrishnan, S., Kiran, G. S., et al. (2008). Antimicrobial potential of sponge associated marine actinomycetes. *J. Med. Mycol.* 18, 16–22. doi: 10.1016/j.mycmed.2007.11.001

Gandhimathi, R., Kiran, G. S., Hema, T. A., Selvin, J., Raviji, T. R., and Shanmughapriya, S. (2009). Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. *Bioprocess Biosyst. Eng.* 32, 825–835. doi: 10.1007/s00449-009-0309-x

Ghribi, D., and Ellouze-Chaabouni, S. (2011). Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol. Res. Int.* 2011:653654. doi: 10.4061/2011/653654

Iyer, A., Mody, K., and Jha, B. (2006). Emulsifying properties of a marine bacterial exopolysaccharide. *Enzyme Microb. Technol.* 38, 220–222. doi: 10.1016/j.enzmictec.2005.06.007

Jain, D. K., Collins-Thompson, D. L., Lee, H., and Trevors, J. T. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *J. Microbiol. Methods* 13, 271–279. doi: 10.1016/0167-7012(91)90064-W

Kim, K. M., Lee, J. Y., Kim, C. K., and Kang, J. S. (2009). Isolation and characterization of surfactin produced by *Bacillus polyfermenticus* KJS-2. *Arch. Pharm. Res.* 32, 711–715. doi: 10.1007/s12272-009-1509-2

Kiran, G. S., Lipton, A. N., Priyadharshini, S., Anitha, K., Suárez, L. E., Arasu, M. V., et al. (2014). Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine *Brevibacterium casei* MSI04 against shrimp pathogenic vibrios. *Microb. Cell Fact.* 13:114. doi: 10.1186/s12934-014-0114-3

Kiran, G. S., Priyadharshini, S., Dobson, A. D. W., Gnanamani, E., and Selvin, J. (2016). Degradation intermediates of polyhydroxy butyrate inhibits phenotypic expression of virulence factors and biofilm formation in luminescent *Vibrio* sp. PUGSK8. *npj Biofilms Microbiomes* 2:16002.

Kiran, G. S., Sabarathnam, B., and Selvin, J. (2010a). Biofilm disruption potential of a glycolipid biosurfactant from marine *Brevibacterium casei*. *FEMS Immunol. Med. Microbiol.* 59, 432–438. doi: 10.1111/j.1574-695X.2010.00698.x

Kiran, G. S., Thomas, T. A., Selvin, J., Sabarathnam, B., and Lipton, A. P. (2010b). Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. *Bioresour. Technol.* 101, 2389–2396. doi: 10.1016/j.biortech.2009.11.023

Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., and Koutinas, A. A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.* 21, 377–397. doi: 10.1016/j.fm.2003.10.005

Kowall, M., Vater, J., Kluge, B., Stein, T., Franke, P., and Ziessow, D. (1998). Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. *J. Colloid Interface Sci.* 204, 1–8. doi: 10.1006/jcis.1998.5558

Liu, X.-Y., Yang, S.-Z., and Mu, B.-Z. (2009). Production and characterization of a C 15-surfactin-O-methyl ester by a lipopeptide producing strain *Bacillus subtilis* HSO121. *Process Biochem.* 44, 1144–1151. doi: 10.1016/j.procbio.2009.06.014

Luo, H.-Y., Wang, Y.-R., Miao, L.-H., Yang, P.-L., Shi, P.-J., Fang, C.-X., et al. (2009). *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium isolated from the black liquor treatment system of a cotton pulp mill. *Int. J. Syst. Evol. Microbiol.* 59, 863–868. doi: 10.1099/ijs.0.003376-0

Montalvo, N. F., Mohamed, N. M., Enticknap, J. J., and Hill, R. T. (2005). Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek* 87, 29–36. doi: 10.1007/s10482-004-6536-x

Morikawa, M., Hirata, Y., and Imanaka, T. (2000). A study on the structure–function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta* 1488, 211–218. doi: 10.1016/S1388-1981(00)00124-4

Paraszkiewicz, K., Kanwal, A., and Dlugonski, J. (1992). Emulsifier production by steroid transforming filamentous fungus *Curvularia lunata*: growth and product characterization. *J. Biotechnol.* 92, 287–294. doi: 10.1016/S0168-1656(01)00376-5

Park, B. K., Lim, J. H., Hwang, Y. H., Kim, M. S., Song, I. B., Lee, H. G., et al. (2006). Acute oral toxicity of surfactin C in mice. *J. Toxicol. Public Health* 22, 453–458.

Pereira, J. F. B., Gudiña, E. J., Costa, R., Vitorino, R., Teixeira, J. A., Coutinho, J. A. P., et al. (2013). Optimization and characterization of biosurfactant

production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. *Fuel* 111, 259–268. doi: 10.1016/j.fuel.2013.04.040

Pimentel-Elardo, S. M., Kozytska, S., Bugni, T. S., Ireland, C. M., Moll, H., and Hentschel, U. (2010). Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. *Mar. Drugs.* 8:373. doi: 10.3390/md8020373

Price, N. P., Rooney, A. P., Swezey, J. L., Perry, E., and Cohan, F. M. (2007). Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. *FEMS Microbiol. Lett.* 271, 83–89. doi: 10.1111/j.1574-6968.2007.00702.x

Quinn, G. A., Maloy, A. P., McClean, S., Carney, B., and Slater, J. W. (2012). Lipopeptide biosurfactants from *Paenibacillus polymyxa* inhibit single and mixed species biofilms. *Biofouling* 28, 1151–1166. doi: 10.1080/08927014.2012.738292

Sahnoun, R., Mnif, I., Fetoui, H., Gdoura, R., Chaabouni, K., Makni-Ayadi, F., et al. (2014). Evaluation of *Bacillus subtilis* SPB1 lipopeptide biosurfactant toxicity towards mice. *Int. J. Pept. Res. Ther.* 20, 333–340. doi: 10.1007/s10989-014-9400-5

Selvin, J., and Lipton, A. P. (2004). *Dendrilla nigra*, a marine sponge, as potential source of antibacterial substances for managing shrimp diseases. *Aquaculture* 236, 277–283. doi: 10.1016/j.aquaculture.2004.01.021

Selvin, J., Ninawe, A. S., Kiran, G. S., and Lipton, A. P. (2010). Sponge-microbial interactions: ecological implications and bioprospecting avenues. *Crit. Rev. Microbiol.* 36, 82–90. doi: 10.3109/10408410903397340

Selvin, J., Sathiyanarayanan, G., Lipton, A. N., Al-Dhabi, N. A., Arasu, M. V., and Kiran, G. S. (2016). Ketide synthase (KS) domain prediction and analysis of iterative type II PKS gene in marine sponge-associated actinobacteria producing biosurfactants and antimicrobial agents. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00063

Shafiei, M., Ziaee, A.-A., and Amoozegar, M. A. (2012). Purification and characterization of a halophilic α-amylase with increased activity in the presence of organic solvents from the moderately halophilic *Nesterenkonia* sp. strain F. *Extremophiles* 16, 627–635. doi: 10.1007/s00792-012-0462-z

Sharafi, H., Abdoli, M., Hajfarajollah, H., Samie, N., Alidoust, L., Abbasi, H., et al. (2014). First report of a lipopeptide biosurfactant from thermophilic bacterium *Aneurinibacillus thermoaerophilus* MK01 newly isolated from municipal landfill site. *Appl. Biochem. Biotechnol.* 173, 1236–1249. doi: 10.1007/s12010-014-0928-9

Shepherd, R., Rockey, J., Shutherland, I. W., and Roller, S. (1995). Novel bioemulsifiers from microorganisms for use in foods. *J. Biotechnol.* 40, 207–217. doi: 10.1016/0168-1656(95)00053-S

Singh, J. P., Kaur, A., Shevkani, K., and Singh, N. (2015). Influence of jambolan (*Syzygium cumini*) and xanthan gum incorporation on the physicochemical, antioxidant and sensory properties of gluten-free eggless rice muffins. *Int. J. Food Sci. Technol.* 50, 1190–1197. doi: 10.1111/ijfs.12764

Takahashi, M., Morita, T., Fukuoka, T., Imura, T., and Kitamoto, D. (2012). Glycolipid biosurfactants, mannosylerythritol lipids, show antioxidant and protective effects against H_2O_2-induced oxidative stress in cultured human skin fibroblasts. *J. Oleo Sci.* 61, 457–464. doi: 10.5650/jos.61.457

Tess, M., Bhaduri, S., Ghatak, R., and Navder, K. P. (2015). Physical, textural and sensory characteristics of gluten-free muffins prepared with teff flour (eragrostis tef (zucc) trotter). *J. Acad. Nutr. Diet.* 9:A58.

Turkmen, N., Sari, F., and Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chem.* 99, 835–841. doi: 10.1016/j.foodchem.2005.08.034

Van Haesendonck, I. P. H., and Vanzeveren, E. C. A. (2004). Rhamnolipids in bakery products. U.S. Patent No W.O 2004/040984. Washington, DC: U.S. Patent and Trademark Office.

Zahn, S., Pepke, F., and Rohm, H. (2010). Effect of inulin as a fat replacer on texture and sensory properties of muffins. *Int. J. Food Sci. Technol.* 45, 2531–2537. doi: 10.1111/j.1365-2621.2010.02444.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Kiran, Priyadharsini, Sajayan, Priyadharsini, Poulose and Selvin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 July 2017
doi: 10.3389/fmicb.2017.01332

Intrinsic and Extrinsic Aspects on *Campylobacter jejuni* Biofilms

Roberta T. Melo[1,2], Eliane P. Mendonça[1,2], Guilherme P. Monteiro[1,2], Mariana C. Siqueira[1], Clara B. Pereira[1], Phelipe A. B. M. Peres[1], Heriberto Fernandez[3] and Daise A. Rossi[1,2]*

[1] Laboratory of Applied Animal Biotechnology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, [2] Laboratory of Molecular Epidemiology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, [3] Institute of Clinical Microbiology, Universidad Austral de Chile, Valdivia, Chile

Biofilm represents a way of life that allows greater survival of microorganisms in hostile habitats. *Campylobacter jejuni* is able to form biofilms *in vitro* and on surfaces at several points in the poultry production chain. Genetic determinants related to their formation are expressed differently between strains and external conditions are decisive in this respect. Our approach combines phylogenetic analysis and the presence of seven specific genes linked to biofilm formation in association with traditional microbiology techniques, using Mueller Hinton and chicken juice as substrates in order to quantify, classify, determine the composition and morphology of the biomass of simple and mixed biofilms of 30 *C. jejuni* strains. It also evaluates the inhibition of its formation by biocides commonly used in industry and also by zinc oxide nanoparticles. Genetic analysis showed high heterogeneity with the identification of 23 pulsotypes. Despite the diversity, the presence of *flaA*, *cadF*, *luxS*, *dnaJ*, *htrA*, *cbrA*, and *sodB* genes in all strains shows the high potential for biofilm formation. This ability was only expressed in chicken juice, where they presented phenotype of a strong biofilm producer, with a mean count of 7.37 log CFU/mL and an ultrastructure characteristic of mature biofilm. The composition of simple and mixed biofilms was predominantly composed by proteins. The exceptions were found in mixed biofilms with *Pseudomonas aeruginosa*, which includes a carbohydrate-rich matrix, lower ability to sessile form in chicken juice and compact architecture of the biofilm, this aspects are intrinsic to this species. Hypochlorite, chlorhexidine, and peracetic acid were more effective in controlling viable cells of *C. jejuni* in biofilm, but the existence of tolerant strains indicates exposure to sublethal concentrations and development of adaptation mechanisms. This study shows that in chicken juice *C. jejuni* presents greater potential in producing mature biofilms.

Keywords: campylobacteriosis, poultry industries, chicken juice, capacity of biofilm formation, genetic apparatus, resistance to biocides

OPEN ACCESS

Edited by:
Rosanna Tofalo, University of Teramo, Italy

Reviewed by:
Gerardo Manfreda, Università di Bologna, Italy
Jordi Rovira, University of Burgos, Spain

****Correspondence:***
Roberta T. Melo
roberta-melo@hotmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *25 March 2017*
Accepted: *30 June 2017*
Published: *18 July 2017*

Citation:
Melo RT, Mendonça EP, Monteiro GP, Siqueira MC, Pereira CB, Peres PABM, Fernandez H and Rossi DA (2017) Intrinsic and Extrinsic Aspects on Campylobacter jejuni Biofilms. Front. Microbiol. 8:1332. doi: 10.3389/fmicb.2017.01332

INTRODUCTION

Campylobacter jejuni is one of the pathogens most commonly involved in food-borne gastroenteritis worldwide. It infects about one million people in the United States each year and in Europe this rate reaches more than 200,000 (Scallan et al., 2011; European Food Safety Authority, 2015). In addition, an estimated number of 1/1,000 clinical cases may result in more severe neurological conditions, including Guillain-Barré Syndrome (Nachamkin et al., 1998).

The main reservoir of this microorganism is the intestinal tract of birds and other endothermic animals, and is often isolated in chicken meat. Generally, consumption of this undercooked meat is the cause of human host infection (Guyard-Nicodeme et al., 2013). The risk is consistent with the high levels of contamination found in studies conducted in Europe, USA and United Kingdom, which shows contamination higher than 70% in chicken carcass flocks (Batz et al., 2012; Lawes et al., 2012; European Food Safety Authority, 2015).

Due to the large number of reported cases of campylobacteriosis, it has become necessary to use epidemiological typing, method that allows the characterization and discrimination of bacterial strains. The data obtained in these investigations can be used by public health surveillance in identifying the causes of food outbreaks (Nakari, 2011). Among these methods, PFGE, pulsed-field gel electrophoresis, is considered the gold standard in bacterial epidemiological analyzes, since it allows a high discriminatory power compared to other techniques (Goering, 2010).

The paradox between the rigorous growth conditions of *C. jejuni* in the laboratory and the ubiquity as an effective and constant pathogen in chicken samples represents one of the most notable characteristics of *C. jejuni* (Mihaljevic et al., 2007). One of the strategies that *C. jejuni* can use to overcome its fragility in the face of environmental hostility is the ability to form biofilms. These structures represent a mode of growth and survival, in which the bacterial transits from free living to sessile form, attached to a biotic or abiotic surface surrounded by a viscous matrix that protects from stressful environmental conditions (Kostakioti et al., 2013). These communities increase the survival of this microorganism under unfavorable conditions, such as the presence of antibiotics and chemical agents (Trachoo and Frank, 2002; Joshua et al., 2006; Ica et al., 2012; Drozd et al., 2014).

A serious problem in the chicken processing industries is the insufficient removal of organic material composed of a complex mixture of carbohydrates, proteins, lipids, and sugars (Chmielewski and Frank, 2007) of the surfaces, which provides an ideal medium for microorganisms to multiply and survive. This environment assists in bacterial fixation to surfaces by altering the physicochemical properties of the surface and by the greater availability of nutrients (Dat et al., 2010; Hwang et al., 2012). Trying to simulate the nutritional conditions on the abiotic surfaces during processing, a model system with "chicken juice" (Brown et al., 2014) is used, based on the supplementation of culture medium with defrosted filter-sterilized poultry exudates (Birk et al., 2006).

The extracellular matrix is an essential component of bacterial biofilms, and normally, corresponds for more than 90% of the dry mass of a biofilm (Flemming and Wingender, 2010). In addition, it allows the cells to remain hydrated and metabolically active, imprisoning nutrients and liquids near the bacterial cells. It also reduces the access of large molecules, such as antimicrobials (Billings et al., 2013), allowing bacterial persistence, beyond being structurally important, once it maintains the biofilm shape and ensures its cohesion (Sutherland, 2001). Knowing the composition and architecture of the extracellular matrix of biofilms is important, as it helps in the use of tools that improve efficiency and disinfection strategies.

The molecular mechanisms that regulate biofilm formation of *C. jejuni* are still poorly understood. Some of the genes involved in the process include the ones responsible for cell motility (*flaA*) (Reuter et al., 2010), cell adhesion (*cadF*), quorum-sensing (*luxS*) (Plummer, 2012) and stress response (*dnaJ*, *cbrA*, *htrA*, and *sodB*) (Oh and Jeon, 2014).

The biofilm formation is flagella-mediated at the first moment of the adhesion, together with the proteins involved in cell adhesion, although its functionality is not crucial (Svensson et al., 2014). Detection of quorum-sensing markers indicates ability of binding between cells, development and detachment of biofilm (Plummer, 2012). Already the markers involved in the stress response play a decisive role, contributing to a superexpression of the capacity of formation of sessile cells (Oh and Jeon, 2014).

The aim of this study was to carry out a phylogenetic analysis on *C. jejuni* strains isolated from chicken carcasses destined for national market and also to exportation, followed by a qualitative and quantitative study on the formation of biofilms, including molecular aspects involving the presence of specific genes, the architecture and composition of these structures and also the interaction of these strains in mixed biofilms under conditions with and without supplementation with chicken juice. Finally, the objective was to evaluate the performance of different chemical agents in the removal of *C. jejuni* bacterial biomass to establish control strategies at industry.

MATERIALS AND METHODS

Strains and Growth Conditions

The study was conducted with 30 *C. jejuni* strains from the analysis of 280 cooled chicken carcasses ready for commercialization from September to November of 2015, from a Brazilian poultry exporting industry, with a complete production cycle.

Isolation was previously performed according to International Standards Organization (2006) and identification of the species was done by multiplex PCR according to Harmon et al. (1997). After confirmation, the strains were stored at −80°C in UHT skimmed milk.

To perform subcultures for reactivation, the strains were seeded for 48 h in Bolton broth (Oxoid) supplemented with 5% of defibrinated ram blood (Laborclin) at 37°C in microaerophilic conditions (Probac), followed by plaque peal in CCDA Agar (*Campylobacter* Blood-Free Selective Agar Base) (Oxoid) incubated under the same conditions (International Standards Organization, 2006).

In the adhesion and biofilm assays, the strains were cultured for 48 h under microaerophylia at 37°C in 20 mL of Mueller Hinton broth (MH) (Difco), using as inoculum the culture present in the plates of CCDA Agar. In parallel, these assays were performed using 20 mL of Mueller Hinton broth supplemented with 5% of chicken juice (Birk et al., 2004)—equivalent to the 100% concentration according to Brown et al. (2015)—to simulate the conditions of industry. After growing in both conditions, the suspensions were adjusted to an $OD_{600} = 0.22$ to

0.28, corresponding to a count of 10^4 CFU/mL. The cells were centrifuged (5,000 rpm, 10 min, 4°C) and washed twice (0.9% NaCl) before the beginning of cultures for adhesion and biofilm assays.

The scanning electron microscopy (SEM) analysis was performed for simple (only *C. jejuni*) and mixed biofilms (*C. jejuni* paired with *Salmonella* sp., *Escherichia coli*, *Pseudomonas aeruginosa* or *Staphylococcus aureus*). The inoculum preparation, growth conditions and incubation were the same as previously described for *Campylobacter*: microaerophilic atmosphere at 37°C for 48 h. Both, in the SEM and in the biofilm matrix composition assay, only three *C. jejuni* strains, phylogenetically distinct by PFGE and with different classifications of BFI (Biofilm Formation Index) were used.

In phylogenetic analysis by PFGE, the bacteria were cultured at 42°C overnight in a brain and heart infusion agar (BHI agar) with 5% of defibrinated sheep blood under microaerophilic conditions. The present culture on the agar was resuspended in saline (0.85% NaCl) until reaching $OD_{610} = 0.570–0.820$ for carrying out the enzymatic digestion process.

Controls used in the study were *C. jejuni* strains (ATCC 33291, NCTC 11351, and IAL 2383) and *C. coli* (ATCC 43478). For mixed cultures, *Escherichia coli* strain (ATCC 25922), *P. aeruginosa* (PAO 1), *Salmonella* Enteritidis (ATCC 13076), and *Staphylococcus aureus* (ATCC 25923) were used.

Adhesion Test

The adhesion test was performed according to Sulaeman et al. (2009) with modifications. Briefly, 200 μl of the bacterial suspension containing 10^4 cells prepared in MH broth and MH with 5% of chicken juice was added in 96-well plates. After incubation for 4 h at 37°C under microaerophylia, the adherent bacteria were washed twice with 0.9% NaCl solution and collected in wells filled with the same solution by scraping during 90 s. The obtained cell suspension was serially diluted and seeded in CCDA agar for enumeration in CFU. All strains were evaluated in triplicate and in three independent replicates.

Qualitative Biofilm Formation Test

Biofilms were formed as described by Kudirkiene et al. (2012), with modifications. Briefly, 200 μl of the bacterial suspension containing 10^4 cells prepared in MH broth and MH with 5% of chicken juice was added in 96-well plates. For biomass formation, the plates were incubated for 48 h at 37°C in microaerophilic conditions.

After incubation, the media were removed, the wells were washed twice with 0.9% NaCl solution and dried for 30 min at 55°C. Total biomass was measured by fixation with 0.1% Crystal Violet (LaborClin) for 5 min, followed by elution with alcohol-acetone solution, containing 80% of ethanol and 20% of acetone (Synth®). The eluted dye was removed from each well and placed in a new 96-well microtiter plate for reading at OD_{595} (BA–bacteria adhered). The assays were done with eight replicates for each strain in three replicates. For the determination of the Biofilm Formation Index, the following formula was used:

$$BFI = \frac{BA - PC}{BS}$$

Where BFI represents the final result regarding the Biofilm Formation Index, BA the optical density obtained in the mixture of bacteria adhered, PC the absorbance value in the control wells without microorganisms, BS the optical density (OD_{600}) of the suspended cultures in MH and MH with 5% of chicken juice (Naves et al., 2008). The final classification followed **Table 1**.

Quantitative Biofilm Formation Test

The number of sessile cells was determined by counting in CFU. After biofilm formation as described in the previous item, the wells were washed twice with a 0.9% NaCl solution, and the biomass was removed by scraping the wells for 90 s. The obtained cell suspension was serially diluted and plated on CCDA agar plates to obtain the number of CFU. All the assays were performed in triplicate, on three independent occasions.

Identification of Specific Genes

The genomic DNA was extracted by the Wizard Genomic DNA Purification Kit (Promega), following the protocol provided by the manufacturer. Purified DNA (10 ng) was used as template for all PCR reactions. The PCR conditions and primers used in this study are described in **Table 2**.

The studied genes were *flaA* (motility), *cadF* (intracellular colonization), *luxS* (quorum-sensing mechanism), *dnaJ* (thermotolerance), *htrA* (aids in growth under stress), *cbrA* (resistance to osmotic shock), and *sodB* (tolerance to oxidative stress).

PCR reactions were performed using the GoTaq® Green Master Mix kit (Promega) according to the manufacturer's instructions. The amplified products were subjected to 1.5% agarose gel electrophoresis using the TBE 0.5x runner buffer (Invitrogen) and as a molecular weight standard of 100 pb marker (Invitrogen).

Biofilm Inhibition Test

To examine the interaction between *C. jejuni* biofilms with biocides components and nanoparticles, the protocol described by Lu et al. (2012) was used. The chemical compounds tested were: Chlorhexidine 1% solution, Sodium Hypochlorite 1%, Peracetic Acid 0.8% and Zinc Oxide (ZnO) nanoparticles 6 mmol/L.

Ten colonies grown in CCDA plates were diluted in 10 mL of 0.9% NaCl solution, adjusted according Mc Farland scale 0.5. From this solution, a 100 μL aliquot (corresponding to 10^7 cells), was inoculated onto a sterile cellulose membrane with 0.45 μm of porosity and 47 mm of diameter on a *Brucella* agar plate (Difco) enriched with 5% of defibrinated sheep blood. The plates were incubated at 37°C in microaerophilia and in every 24 h the membrane was transferred to a new plate, during 3 days.

Subsequently, the membrane was placed in a flask containing 20 mL of MH broth with respective concentrations of

TABLE 1 | Classification of biofilm formation index.

Strong	Medium	Weak	Nonexistent
≥1.10	0.70–1.10	0.35–0.69	<0.35

TABLE 2 | PCR conditions, nucleotide sequences and *amplicon* sizes for the specific *Campylobacter jejuni primers* used in this study.

Genes	Primers	Sequence 5′ → 3′	Size (pb)	DNA (ng)	Primer (pmol)	PCR Conditions	References
flaA	flaA-F	ATGGGATTTCGTATTAACAC	1728	50	10	94°C – 10 min; 30 cicles: 94°C – 1 min, 47°C – 1 min, 72°C – 1 min; 72°C – 10 min	Hanel et al., 2004
	flaA-R	CTGTAGTAATCTTAAAACATTTTG					
cadF	cadFI-F2B	TTGAAGGTAATTTAGATATG	400	40	40	94°C – 10 min; 30 cicles: 94°C – 1 min, 47°C – 1 min, 72°C – 1 min; 72°C – 10 min	Zheng et al., 2006
	cadFI-R1B	CTAATACCTAAAGTTGAAAC					
luxS	luxS-1	AGGCAAAGCTCCTGGTAAGGCCAA	1080	50	10	94°C – 3 min; 30 cicles: 94°C – 30 s, 55°C – 1 min, 72°C – 1 min; 72°C – 10 min	Elvers and Park, 2002
	luxS-2	GGATCCGTATAGGTAAGTTCATTTTTGCTCC					
dnaJ	dnaJ F	AAGGCTTTGGCTCATC	720	20	20	95°C – 2 min; 30 cicles: 94°C – 1 min, 46°C – 1 min, 72°C – 1 min; 72°C – 5 min	Datta et al., 2003
	dnaJ R	CTTTTTGTTCATCGTT					
htrA	htrA F	TAATACGACTCACTATAGGGTAAGTTTAGCA AGTGCTTTATTTGC	1393	10	10	95°C – 1 min; 35 cicles: 95°C – 30 s, 50°C – 1 min, 72°C – 1 min; 72°C – 5 min	Datta et al., 2003
	htrA R	AAAACCATTGCGATATACCCAAACT					
cbrA	cbrA F	TAATACGACTCACTATAGGGTCAACTCTATC CTTGCCATTATCTT	1165	10	10	95°C – 1 min; 35 cicles: 95°C – 30 s, 50°C – 1 min, 72°C – 1 min; 72°C – 5 min	Biswas et al., 2011
	cbrB R	GTAGATATTGCTTTTGGTTTTGCTG					
sodB	sodB F	ATGATACCAATGCTTTTGGTGATTT	638	20	20	95°C – 2 min; 30 cicles: 94°C – 1 min, 46°C – 1 min, 72°C – 1 min; 72°C – 5 min	Biswas et al., 2011
	sodB R	TAATACGACTCACTATAGGGCATTTGCATAA AAGCTAACTGATCC					

the chemical compounds. The flasks were incubated in microaerophylia at 37°C for 24 h. Subsequently, the membrane was washed three times with phosphate buffer (PBS), followed by treatment in 25 mL of 0.1% trypsin for 15 min at room temperature. Thereafter, the resulting solution of the incubation underwent serial dilutions for further counting.

Biofilm Stability Test

The biofilm stability assay was performed according to the protocol described by Chaignon et al. (2007), with some modifications. Biofilms were formed into 96-well plates as described above. After 48 h of growth, the culture medium was removed, the wells were washed twice with sterile 0.9% NaCl solution and then filled with 200 µl of a proteinase K solution (Invitrogen, USA) on concentration of 1 mg/ml in 20 mM Tris (pH 7.5) and 100 mM NaCl or 200 µl of a 10 mM sodium metaperiodate solution (Sigma-Aldrich, USA) prepared in 50 mM acetate buffer (pH 4.5). The plates were incubated for 2 h at 37°C. After treatment, the biofilms were washed with 200 µL of sterile 0.9% NaCl and stained with 1% crystal violet. The absorbance was evaluated on a plate reader at 595 nm with an alcohol-ketone solution containing 80% of ethanol and 20% of acetone (Synth®), as white. The experiment was performed in biofilms of three *C. jejuni* strains formed with chicken juice. All assays were performed in eight wells, on three independent occasions.

Scanning Electron Microscopy

The preparation of the material for analysis in SEM was done according to Brown et al. (2014) with modifications. Simple and mixed biofilms in the MH and chicken juice media were formed in glass beads with a diameter of 5 mm, respecting the growth conditions described for *Campylobacter*: microaerophilic atmosphere at 37°C for 48 h. After biomass formation, the samples were fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M buffer PBS (pH 7.4) overnight at 4°C. The fixative was removed, and the samples washed three times with PBS buffer. The beads were post-fixed with 1% osmium tetroxide for 2 h and washed three times with PBS buffer. The beads were dehydrated in a series of ethanol solutions (30, 40, 50, 60, 70, 80, and 90% and then three times at 100%) for 20 min for each step.

The samples were dried in CPD (Critical Drying Point) (CPD 030, Baltec, Germany) using liquid carbon dioxide as the transition fluid. The samples were coated with a 20 nm thick layer of gold (SCD 050, Baltec, Germany) and visualized on MEV VP Zeiss Supra 55 SEM FEG operating at 5 kV.

PFGE

The isolates were typed by PFGE according to the protocol described in the Center Disease and Control (2013). Digestion of the intact genomic DNA was done with 30 U of the enzyme *Sma* I (Invitrogen) for 2 h at 25°C. The DNA fragments were separated on 1% agarose gel (SeaKem Gold) in 0.5X TBE buffer in the CHEF DRIII (Bio-Rad) apparatus, for a period of 18 h, with the following parameters, 200 v, 120° angle, Gradient of 6 v/cm and buffer temperature of 14°C.

The gels were stained with ethidium bromide and photographed under UV light. The analysis for dendrogram formation was performed using GelCompare II software. The comparison of the band patterns was performed by the UPGMA analysis method, using the Dice similarity coefficient.

Statistical Analysis

The obtained results were analyzed using GraphPad Prism, version 6.0. Qualitative and quantitative biofilm formation tests

were evaluated using simple variance analysis (ANOVA). For the biofilm inhibition test, ANOVA was used to compare the results of the control with the resistant strains in the test groups, and to analyze the counts between the control strains and tests separately. For the simple comparisons of two variables, in the biofilm stability test, Student's *t*-test was used. All tests were performed at a confidence level of 95%.

RESULTS

Ability to Adhere to the Abiotic Surface

The adhesion assays were conducted in Mueller Hinton and Muller Hinton supplemented with chicken juice media with an initial bacterial concentration of approximately 10^3 CFU/well. The indices found showed that the ability to adhere to the polystyrene varied according to the strain.

The results showed that all tested strains had adhesion capacity when inoculated in Muller Hinton (MH) and MH + 5% of chicken juice, but there was a reduction in counts ($p < 0.05$) compared to the initial inoculum when the strains were held at Mueller Hinton.

In Mueller Hinton, 46.7% (14/30) of the strains showed medium adhesion pattern, with a count above the average in all the tests, and 53.3% (16/30) of the strains showed weak adhesion by presenting lower values than those obtained in the general mean of the initial inoculum ($p < 0.05$), as shown in **Figure 1**.

The values were significantly higher ($p < 0.05$) when the strains were inoculated in the chicken juice, with all 30 (100%) strains classified as strongly adherent, because they had higher counts than the initial inoculum.

The ability of these strains to adhere strongly in conditions similar to those present in the avian industry (chicken juice) helps to explain their survival and persistence in the slaughterhouse. The 4-h incubation period was sufficient for the initial establishment of the biofilm structure, and thus could act as a constant source of contamination in the industry.

The nutritive particles available in chicken juice can form a thin layer above the surface of the polystyrene wells and on glass surfaces that facilitate this bacterial adhesion (Li, 2016).

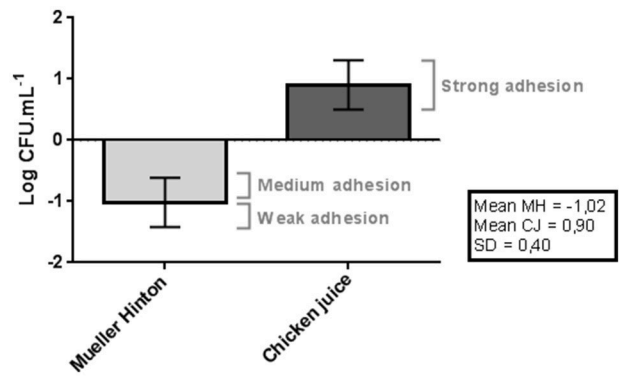


FIGURE 1 | Difference between adhesion and the mean of initial inoculum obtained in the counts (log of CFU/mL) of *C. jejuni* in the Mueller Hinton and Chicken juice. Error bars indicate the standard deviation for the means of the counts obtained for each strain at three repetitions. SD indicates the standard deviation used in the classification of the strains: Poor adherence (counts below the mean in MH up to 1 SD), Average adherence (higher than average counts in MH up to 1 SD), Strong adherence (higher than average counts in MH Up to more than 1 SD).

Classification and Quantification of *C. jejuni* Biofilms

All of *C. jejuni* strains (100%) were capable of forming strong biofilms when supplemented with chicken juice by the crystal violet test (**Table 3**). The same did not occur when in the presence of Mueller Hinton, where none of the strains presented a strong producer profile under this condition. The inclusion of chicken juice promoted a significant increase in bacterial biomass, increasing in average 1.70 the Biofilm Formation Index (BFI) when compared to the value found in non-supplemented samples.

In the condition not supplemented with chicken juice, 10/30 (33.3%) strains did not form biofilms, 13/30 (43.3%)

TABLE 3 | Classification of strains, according to the BFI (Biofilm Formation Index), under the different enrichment conditions.

Identification	Mueller hinton (MH)		MH + 5% of Chicken juice	
	BFI	Classification	BFI	Classification
F 03	0.476	Weak	1.926	Strong
F 45	0.261	Nonexistent	2.199	Strong
F 48	0.119	Nonexistent	2.243	Strong
F 51	0.280	Nonexistent	2.105	Strong
F 67	0.721	Medium	2.2665	Strong
F 68	0.388	Weak	2.558	Strong
F 80	0.326	Nonexistent	2.114	Strong
F 84	0.625	Weak	2.226	Strong
F 85	0.417	Weak	2.086	Strong
F 87	0.310	Nonexistent	1.937	Strong
F 88	0.482	Weak	1.988	Strong
F 100	0.304	Nonexistent	1.632	Strong
F 127	0.897	Medium	2.338	Strong
F 138	0.576	Weak	2.362	Strong
F 140	0.847	Medium	2.190	Strong
F 157	0.841	Medium	2.302	Strong
F 163	0.393	Weak	2.377	Strong
F 164	0.560	Weak	2.343	Strong
F 172	0.513	Weak	2.344	Strong
F 175	0.491	Weak	2.165	Strong
F 206	0.320	Nonexistent	2.178	Strong
F 211	0.327	Nonexistent	2.437	Strong
F 236	0.677	Weak	1.864	Strong
F 240	0.917	Medium	2.658	Strong
F 246	0.492	Weak	2.141	Strong
F 247	0.970	Medium	2.339	Strong
F 248	0.266	Nonexistent	2.143	Strong
F 253	0.325	Nonexistent	2.429	Strong
F 255	0.644	Weak	2.423	Strong
F 256	0.964	Medium	2.761	Strong

were classified as weak producer and 7/30 (23.3%) as medium producer, according to **Table 1**.

These data shows that surfaces in contact with organic matter inside the industry may harbor these biofilms, since there is a constant presence of chicken juice during the processing of chicken carcasses. If hygiene measures are not frequent and sufficiently conducted, the exudate present in the chicken carcass guarantees conditions for *C. jejuni* maintenance.

The data obtained in the biofilm counts proved the differences obtained in the tests with crystal violet. Starting from a constant initial inoculum ($p > 0.05$) in all assays, it was observed that in both, the adhesion and biofilm formation, had a significant increase in the counts and in bacterial multiplication in the presence of chicken juice when compared to the counts in Mueller Hinton (**Table 4**).

The results obtained in the adhesion assays showed that in relation to the initial inoculum there was a significant reduction in the counts when the strains were inoculated in MH and a significant increase in the chicken juice ($p < 0.05$). This fact reveals the difficulty of maintaining and developing the initial structure of the biofilm in Mueller Hinton.

The values obtained in the adhesion (4 h) in chicken juice were similar to those found after 48 h (biofilm) in MH, indicating another evidence of the superiority of chicken juice in the establishment of the sessile form of *C. jejuni*. In addition, the high counts detected in chicken juice after biofilm formation, suggests that in this condition the biofilm may be well established.

Genetic Repertoire Related to Sessile Form of *C. jejuni*

Analysis of the seven genes potentially required for the formation of strong biofilms in *C. jejuni* showed that all strains (100%) have the potential to form strong biofilms, since all the genes evaluated were identified in the 30 strains.

These findings are consistent with the results obtained in the counts and the colorimetric test (crystal violet) of the biofilms of *C. jejuni* in the presence of chicken juice. This supplement probably allowed expression of genes associated with the transition to sessile form, including the studied genes.

Chemical Agents Reduce *C. jejuni* Biofilm

The use of chemical agents had a high potential for elimination of viable cells from *C. jejuni* biofilms. For all the used products, was a significant reduction in the bacterial counts in relation to the untreated biofilm. In 17/30 (56.7%) of biofilms, total elimination of the microorganism was observed for all chemical agents tested.

Figure 2 shows the counts obtained in the untreated biofilms, which obtained a mean value of 6.41 Log CFU/mL ($p > 0.05$), and the resistant biofilms treated with the different products that showed growth after 24 h in contact.

The inclusion of sodium hypochlorite 1% allowed the survival of 6/30 (20.0%) of the strains, with a mean count of 1.30 log CFU/mL. This value did not differ between strains ($p > 0.05$), and demonstrated a reduction of about 5.11 log cycles relative to untreated biofilm.

There was also no difference in counts after the use of chlorhexidine 1% ($p > 0.05$) for 4/30 (13.3%) strains tolerant to this agent. The mean count of 1.33 log CFU/mL after treatment showed a mean decrease of 5.08 log cycles compared to the control.

For peracetic acid and ZnO nanoparticles (NPs), the reduction in the number of CFUs varied significantly ($p < 0.05$) among the tolerant strains, indicating that persistence in the presence of these agents may be a characteristic strain-dependent.

Seven of the 30 strains (23.3%) in the sessile form survived in the presence of peracetic acid with counts varying from 1.34 to 2.16 log of CFU/mL. Therefore, the reduction was from 4.25 to 5.07 log cycles compared to the control.

In the presence of ZnO NPs, it was observed that 13/30 (43.3%) strains were tolerant and presented the highest log of CFU/mL, alternating from 2.09 to 4.07. The decrease was from 2.34 to 4.32 log cycles in relation to the control.

In general, chlorhexidine 1%, sodium hypochlorite 1% and peracetic acid 0.8% presented equivalent efficiency in the control of *C. jejuni* biofilm ($p > 0.05$), due to the high number of biofilms removed and by the low counts obtained for the resistant strains. ZnO NPs presented the lowest treatment efficacy ($p < 0.05$).

Table 5 shows the resistance profiles of the agents obtained for the 13 strains that showed growth after the biofilm inhibition test.

TABLE 4 | Counts obtained in the assays for adhesion analysis and biofilm formation in the 30 strains of *C. jejuni*.

Mediums	Mean counts ± standard deviation (Log CFU/mL)		
	Initial inoculum	Adhesion	Biofilm
Mueller Hinton	4.15 ± 0.29 Aa	3.13 ± 0.35 aB	5.30 ± 0.38 aC
Chicken juice	4.10 ± 0.32 Aa	5,00 ± 0.49 bB	7.37 ± 0.23 bC

Different lowercase letters in the columns and different capital letters on the lines indicate significant difference.

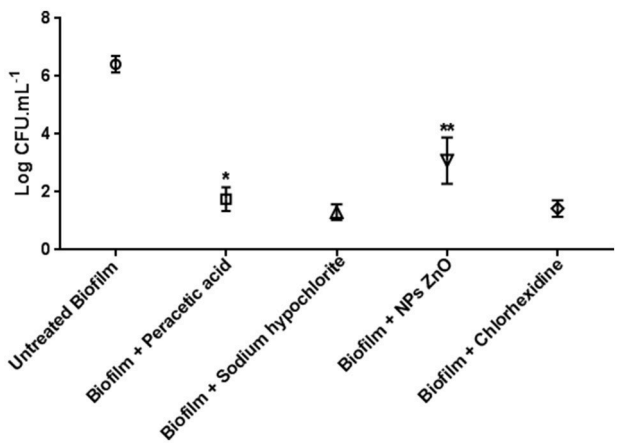


FIGURE 2 | Biofilm counts of *C. jejuni* (log CFU/mL) in the control test (30 strains) and maintained for 24 h in peracetic acid solution 0.8% (7 strains), sodium hypochlorite 1%, ZnO NP 6 mM (13 strains), and chlorhexidine 1% (4 strains). *$p < 0.01$; **$p < 0.001$ using one way Anova for the counts in the samples of the same treatment.

The presence of biofilms resistant to disinfectant agents shows that there are probably intrinsic or extrinsic adaptive mechanisms that allow their survival. According to **Table 5**, two strains showed resistance profile VI (F206 and F246), that is, they are tolerant to all agents and may characterize a problem in the industry due to the difficulty of eliminating them and the risk of dissemination of this characteristic to other strains.

TABLE 5 | Resistance profiles to chemical agents tested on 13 biofilms of *C. jejuni*.

Chemical agents				Profile	Number of strains
ZnO NPs				I	4 (30.8%)
ZnO NPs	Chlorhexidine			II	1 (7.7%)
Peracetic acid	ZnO NPs			III	2 (15.4%)
Hypochlorite	ZnO NPs	Chlorhexidine		IV	1 (7.7%)
Peracetic acid	Hypochlorite	ZnO NPs		V	3 (23.1%)
Peracetic acid	Hypochlorite	ZnO NPs	Chlorhexidine	VI	2 (15.4%)

Structure and Composition of *C. jejuni* and Mixed Biofilms

At the SEM assay we observed changes in biomass formed for the three types of biofilm patterns identified in the MH: F80 (unable to produce biofilm), F255 (weak producer) and F256 (medium producer). In addition, difference in yield was noted when the substrate for its growth was supplemented with chicken juice (**Figure 3**).

Figure 3A shows the presence of isolated bacteria, indicating the inability to form biofilm in this condition.

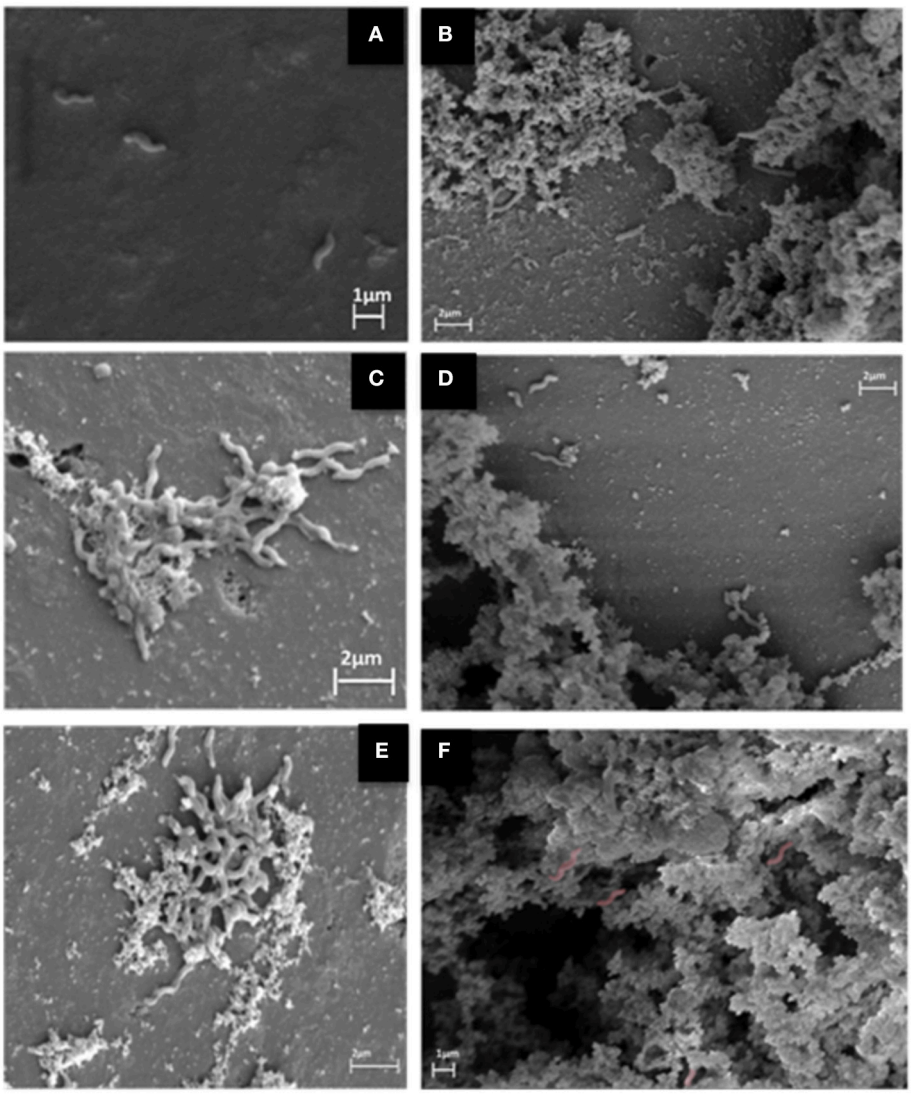


FIGURE 3 | SEM images for three *C. jejuni* strains with different BFI (biofilm formation index) in MH (**A**, nonexistent; **C**, weak; and **E**, medium), and strong producers in chicken juice **(B,D,F)** (Computerized staining in *C. jejuni* in **F**).

In 4c and 4e the initial formation of biofilm is observed, with primary production of extracellular matrix. Already in 3b, 3d, and 3f (in chicken juice) there is formation of the mature biofilm, with a tridimensional structure of the evident matrix.

The composition assay performed with proteinase K and sodium metaperiodate promoted protein degradation and carbohydrate oxidation (**Figure 4**).

In both MH and chicken juice biofilms, the proteinase treatment almost completely removed the biomass formed by the three strains tested. However, the carbohydrate oxidant showed little or no effect (F255 in MH) on the biofilm produced by these strains.

For the mixed biofilm assays of *C. jejuni* with *Escherichia coli*, *P. aeruginosa*, *Salmonella* Enteritidis and *Staphylococcus aureus* the SEM demonstrated competitive disadvantage of the three strains of *C. jejuni* tested. The predominance of the other species was clear as shown in **Figure 5**, indicating the selection pressure exerted by the other species on *C. jejuni*.

In **Figure 6** was possible to verify the alteration in the biomass formed by the crystal violet method compared to the control group composed of simple biofilm of only *C. jejuni*.

In practically all the tests, there was a higher production of biomass in relation to the control. The exception is in the mixed biofilms with *P. aeruginosa* that exhibited a different behavior from that found for the other species. In the presence of MH, the biofilm production was exacerbated ($p < 0.001$), but in chicken juice the biomass was significantly lower, showing that some factor present in chicken juice could inhibit the transition to sessile form in this specie.

This fact was also observed in SEM, by the formation of a denser biomass in MH when compared to chicken juice (**Figures 5E,F**).

The assays concerning to the composition of the matrix for mixed cultures are probably more related to the other species and not, in fact, to *C. jejuni*. For all mixed biofilms the composition was predominantly proteic, except for mixed biofilms with *P. aeruginosa* whose presence of carbohydrates was more evident (data not shown).

Genetic Diversity of *C. jejuni*

Twenty three pulsotypes (A-V) were identified by PFGE, being 17 of them characterized as distinct profiles (**Figure 7**).

Five profiles (A, I, K, M, and U) were classified as clusters with homology higher than 80%, composed of strains with the same genotype. The M-type pulse was designated as a clone because it showed 100% of similarity.

The K, M, and U pulsotypes, grouped isolated strains on the same date, indicating possible cross-contamination among the samples. However, the A and I pulses presented isolated strains at different dates suggesting the persistence of this genotype in the industry, probably due to the biofilm formation.

DISCUSSION

Biofilms of *C. jejuni*

During the last decade, *C. jejuni* has been regularly presented as the leading cause of bacterial foodborne infections in Europe and the USA. Given the importance to public health of this zoonosis, it is relevant to understand the survival mechanisms adopted by this pathogen.

One of the mysteries of the genus *Campylobacter* is that it is a pathogenic microorganism that survives successfully in the host and industrial environment under stressful conditions, and paradoxically is a mandatory microaerophilic that survives poorly under controlled laboratory conditions. In addition, in comparison to other agents causing foodborne disease, such as *E. coli* and *Salmonella* spp., *C. jejuni* needs a low infective dose (500–800 CFU) to cause disease in the host (Black et al., 1988). Although this may contribute to infection, it is still unclear what allows the bacteria to survive during transmission under adverse conditions.

Survival in a biofilm would be an explanation to protect bacteria from various environmental stresses, antimicrobial agents and/or disinfectants and the immune response of the host.

In this study we found that these structures represent a reservoir of cells and that the level of biofilm formation by *C. jejuni* is clearly increased under conditions similar to those found in the industry with the presence of chicken juice.

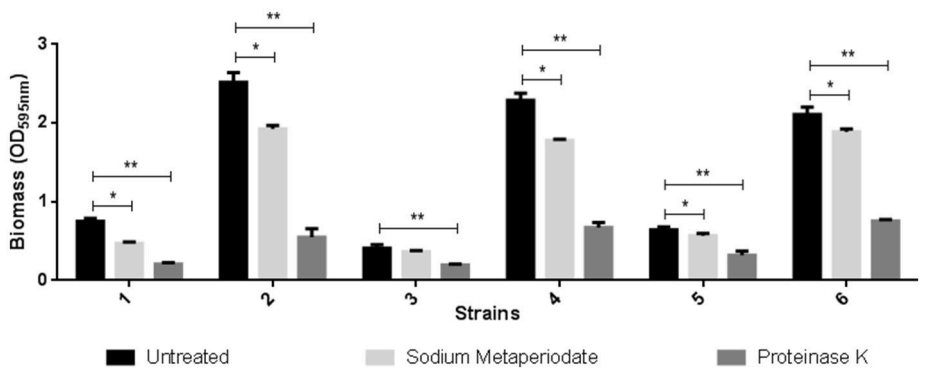


FIGURE 4 | Effect of treatment with sodium metaperiodate and proteinase K on the biofilm of three *C. jejuni* strains. Results represent means with standard deviation (error bars) of three independent experiments. 1 (F 80 in Mueller Hinton), 2 (F 80 in chicken juice), 3 (F 255 in Mueller Hinton), 4 (F 255 in chicken juice), 5 (F 256 in Mueller Hinton), and 6 (F 256 in Chicken juice). *$p < 0.05$; **$p < 0.001$ using one way Anova.

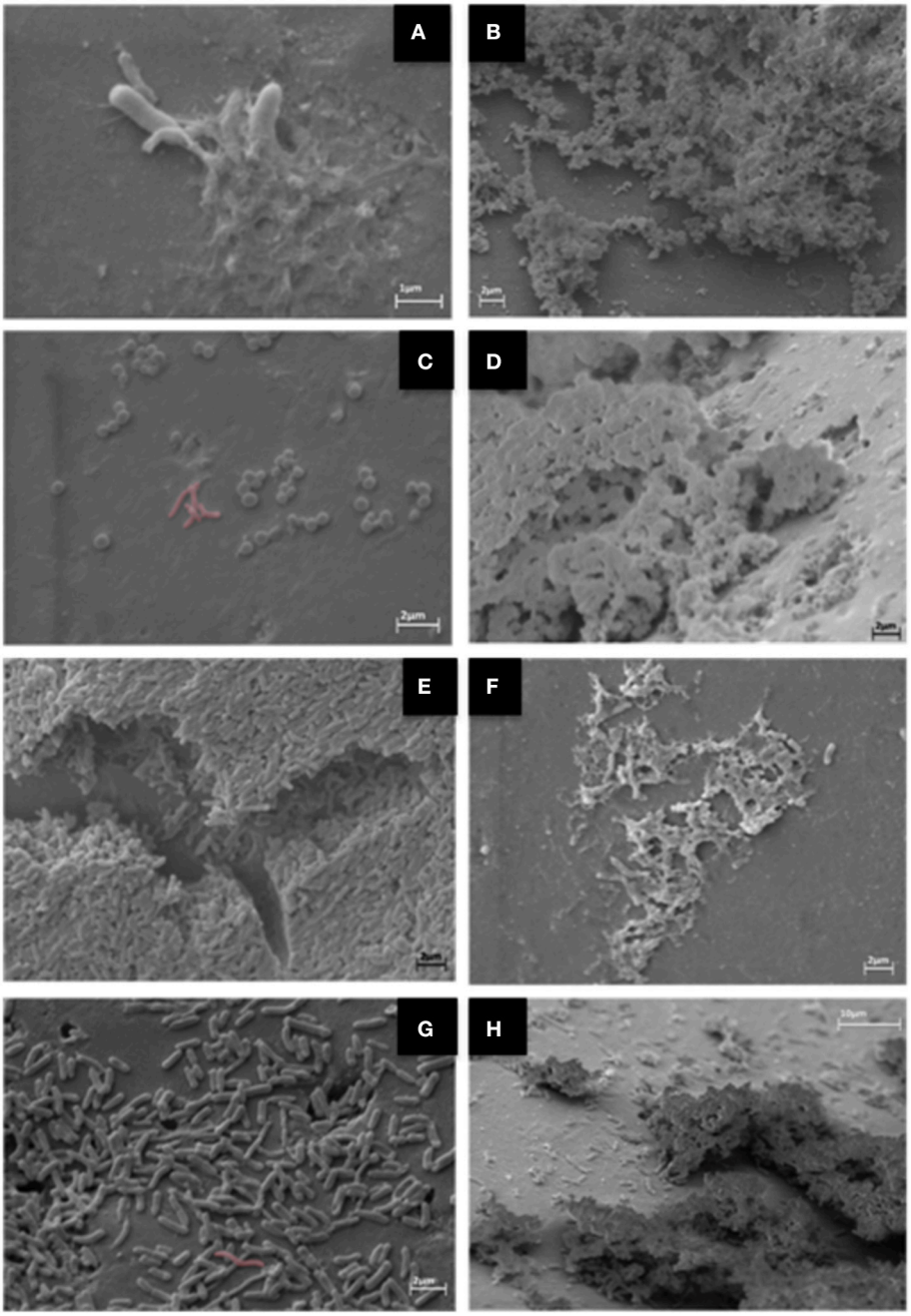


FIGURE 5 | SEM images for mixed biofilms. *C. jejuni* with *Salmonella* Enteritidis in MH **(A)** and in Chicken Juice (CJ) **(B)**, with *Staplylococcus aureus* in MH **(C)**, and in CJ **(D)**, with *Pseudomonas aeruginosa* in MH **(E)** and in CJ **(F)** and with *Escherichia coli* in MH **(G)** and in CJ **(H)**. Computerized staining in *C. jejuni* in **(C)** and **(G)**.

The detection of viable cells in significant quantities in biofilms formed in chicken juice corroborates the idea that survival and persistence in the production chain may represent the main problem of contamination in final product. Despite the use of microaerophylia for this study, it is known that the mature biofilm can provide an adequate environment for microaerophilic growth allowing the ideal conditions for maintenance and dissemination of this pathogen (Reuter et al., 2010).

The biofilm formation involves the succession of several steps, starting with initial adhesion. For this reason, *C. jejuni*'s ability to adhere to a inert surface was investigated, in order to subsequently assess their ability to initiate and develop the biofilm. The adhesion capacity was variable and lower in the 30 strains tested in MH. The delayed adhesion profile may indicate less ability to acquire the sessile form, but may also be related to the need for a prolonged period of contact with the surface to lead to a stronger future adhesion (Turonova et al., 2015). In contrast,

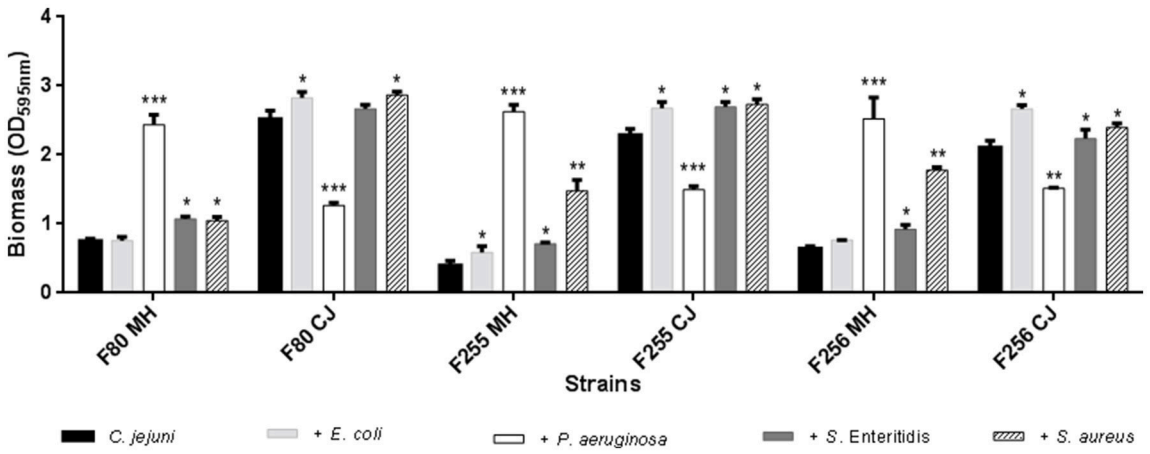


FIGURE 6 | Changes in biomass of mixed biofilms of *C. jejuni* with *E. coli*, with *P. aeruginosa*, with *S. Enteritidis* and with *S. aureus*, separately. Results represent means with standard deviation (error bars) of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ using Student's *T*-test for comparisons with the control (*C. jejuni*).

in chicken juice the counts showed high adhesion capacity for all strains. The medium supplemented with chicken juice allowed a better condition for adhesion to the inert surface (Li, 2016).

The results obtained in both colorimetric and quantitative tests revealed the superiority of chicken juice in relation to MH.

Chicken carcass exudates contain a complex mixture of carbohydrates, proteins, lipids, and sugars (Chmielewski and Frank, 2007), providing an ideal medium for the proliferation and survival of bacteria. The accumulation of these organic materials allows the formation of micro-layers on the surfaces that aid in bacterial fixation, together with greater availability of nutrients (Hwang et al., 2012).

Thus, in the industrial environment, the presence of meat-based exudates may exacerbate the problem of contamination by *C. jejuni*. Our results add and are consistent with the findings of Brown et al. (2015) who also detected the efficiency of chicken juice at different concentrations in the biofilm production for five *Campylobacter* strains.

Genetic Apparatus of *C. jejuni*

Once the phenotypic characterization was performed concerning the sessile kind of living, analysis of the specific genes revealed that all strains possess the genes required to develop a biofilm.

Thus, gene identification in the strains of *C. jejuni* did not explain the differences in the classification of the biofilms formed in MH. In contrast, the identification of all the genes surveyed in all strains is consistent with the strong producer character obtained in chicken juice. Therefore, chicken juice is likely to provide all the necessary conditions for expression of the genetic potential recorded by the presence of *flaA*, *cadF*, *luxS*, *dnaJ*, *htrA*, *cbrA*, and *sodB* genes and this same ability is not detected in MH.

The genes linked to quorum-sensing, adhesion, adverse conditions and motility were all previously described as important for the acquisition of the sessile form (Kalmokoff et al., 2006; Svensson et al., 2009; Howlett et al., 2012; Sulaeman et al., 2012; Avila-Ramirez et al., 2013; van Alphen et al., 2014).

There are reports that flagellar expression is required for the formation of biofilms by *C. jejuni* (Lehtola et al., 2006; Reeser et al., 2007), including *flaA* and *flaB* genes (Reuter et al., 2010). However, the absence of these characteristics does not completely prevent the acquisition of the sessile form. The advantage in the expression of this characteristic is due to the initial fixation, biofilm structuring, orientation to a pre-existing biofilm in addition to the correlation with other non-flagellar extracellular proteins that contribute indirectly to the sessile lifestyle (Howard et al., 2009; Kim et al., 2015).

Numerous genes in *Campylobacter* were previously described as mediators of adhesion *in vitro*. Among them, the *cadF* gene that encodes the binding proteins CadF fibronectin (Konkel et al., 2010).

Several enzymes and proteins are already described by the involvement in bacterial protection against oxidative stress, whose action is related to peroxide or superoxide detoxification. Among them, the enzyme superoxide dismutase (SodB) appears as a major regulator in *C. jejuni* (Flint et al., 2014; Kim et al., 2015).

Some quorum-sensing systems have already been detected in *Campylobacter*. The production of AI-1 (acyl-homoserine autoinducer) represents one of these mechanisms, which accumulates in the extracellular environment and diffuses freely in the bacterial cytoplasm, which at high levels binds to a cellular transcription enhancer (*luxS*) that encodes the luciferase, a metabolic key enzyme in the SAM recycling pathway (S-adenosylmethionine). This metabolite is essential in the performance of important biosynthetic reactions, such as the methylation of bacterial DNA, the synthesis of polyamines and bacterial vitamins. The most important performance of the *luxS* gene is associated with the synthesis of a new AI called autoinducer-2 (AI-2). Increased bacterial population growth also promotes elevation of AI-2 concentrations in the environment. The *luxS* gene acts in the formation of several molecular compounds, which together are called AI-2 variants. These

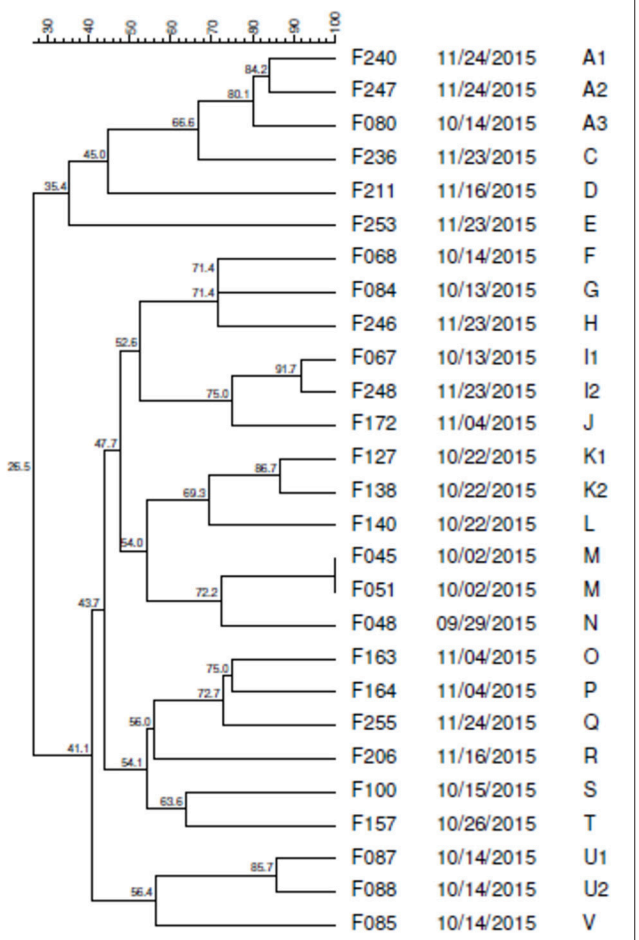


FIGURE 7 | Dendrogram generated by computerized analysis (Gel Compare II) of DNA profiles of *C. jejuni* strains, based on pulsed field electrophoresis (PFGE). The analysis was performed by the Dice/UPGMA method (tolerance parameter of 0.5%, optimization of 0.5%, homology ≥ 80%).

molecules have potential for recognition and inclusion of mixed populations and of the same species in biofilms (Xavier and Bassler, 2005).

Much of *C. jejuni* has functional LuxS enzymes and is capable of producing AI-2. However, the presence of nutrients is necessary for the production of AI-2, and these are found in foods, such as milk and chicken juice, even when the microorganisms are kept under adverse conditions, such as in oxidative stress and in low temperatures (Ligowska et al., 2011; Parveen and Cornell, 2011; Tazumi et al., 2011; Plummer, 2012).

Strategies for the Elimination of Viable Cells of Sessile *C. jejuni*

In the poultry industry investigated, the chemical agents: peracetic acid 0.8%, sodium hypochlorite 1% and chlorhexidine 1%; are used by the quality control team. On the other hand, ZnO NPs, represent a potential sanitizing agent for experimental use, with no usual application in hygiene in the food producing industries.

The results showed that the three agents used in the industry routine were more effective in elimination, although 9/30 (30.0%) of the strains were identified to be tolerant to at least one of them. In contrast, ZnO NPs showed less efficacy with 13/30 (43.3%) resistant strains and with counts higher than the other agents.

The presence of tolerant strains to different sanitizers suggests that the use of these agents in the routine of the industrial environment in an inadequate way can result in the sublethal exposure to these biocides, representing a real risk for the adaptation of these bacteria, besides positively influencing the production of biofilms (Keeratipibul and Techaruwichit, 2012; Techaruvichit et al., 2016).

As for ZnO NPs it is possible that tolerant bacteria have already acquired characteristics that confer this resistance, such as the presence of efflux pumps, ZnO resistance genes and the ability to maintain intact the integrity of membrane. This characteristic has already been identified in *Escherichia coli* and *Enterococcus faecium* (Mileyeva-Biebesheimer, 2011).

Although the use of chemical compounds provides benefits in disinfection, they have the limitation of not destroying the residual structures of the biofilm matrix that may facilitate their resurgence or maintenance (Ohsumi et al., 2015). Thus, special efforts are required for the complete removal of highly adherent biofilms adapted to *C. jejuni* biocides (Techaruvichit et al., 2016).

Probably, the effectiveness in the control is possible by the association of hygiene plans with different agents, respecting the periods between cleaning, besides strategies, like the periodical rotation of biocides.

Architecture and Constitution of *C. jejuni* and Mixed Biofilms

For the three *C. jejuni* strains under sessile form in the glass beads, with MH substrate plus chicken juice, it was observed in SEM that the structure of the biofilm was quite similar, with a more expanded and stable architecture, besides the presence of irregular coverage along the surface of the sphere, consistent with the presence of several macrocolonies. Differently, in MH, this pattern varied according to the strain, so that the most developed structure observed was the presence of microcolonies that indicate the immature stage of the biofilm.

A study by Bronnec et al. (2016) compared the ultrastructure of two strains of *C. jejuni* in biofilm under microaerophilic and aerobiose conditions. The authors concluded that the differences revealed the formation of mature and immature biofilm, being a strain-dependent characteristic.

The variations in the architecture of the formed biofilms can have relation not only with the nutrient available to the bacterium, but also because it is a strain-dependent character. Turonova et al. (2015) showed that *C. jejuni* NCTC 11168 produces biofilm with multilayer type structure, while *C. jejuni* 81–176 was able to form finger-like biofilm with an open ultrastructure.

The capacity to form biofilm with open ultrastructure composed of wells and channels was identified in the three strains of *C. jejuni* tested in the presence of chicken juice. This type

of heterogeneous structure gives the characteristic of a mature biofilm, which allows the formation of interconnected fluxes that aid in the access to nutrients for the cellular aggregates and in the drainage of the metabolic residues (Donlan and Costerton, 2002).

The composition assays allowed to identify that all strains reduced biomass with treatment with sodium metaperiodate and proteinase K, the last one being more significant. Thus, the treatment of biomass with products of proteolytic action can be considered an effective mechanism for partial degradation, allowing a better penetration of antimicrobial agents into the matrix. Although the use of proteinase K is expensive in the poultry industry, the effectiveness of the tests opens the prospects for the chemical industry to the development of other similar proteolytics and of lower cost, since they will probably not require the necessary purity to be used in molecular techniques.

Considering the proteic nature of biofilms, it is possible that the association of potent proteolytics in association with sanitizers is an adequate strategy in the prevention of *C. jejuni* biofilms.

The centesimal composition of MH and chicken juice was compared and it was found that the analysis of 100 mL of chicken juice has 2.79% of protein and 0.06% of carbohydrates. MH contains 1.85% protein and 0.2% of carbohydrate. Even with only 5% of chicken juice in the trials, the presence of a higher protein build-up added to the existence of blood and other unassessed components may have provided *C. jejuni* not only with the microaerophilic condition required for this microorganism, as well as a greater presence of iron, important conditions for its metabolism and consequent survival and multiplication, which may have had a positive influence on biofilm formation.

For the mixed biofilms it was observed that there was an increase in the formed biomass. This increase was significant depending on the microorganism to which the interaction occurred and the medium used. In addition, there was variability in the composition of the formed biofilm.

The competitive disadvantage of *C. jejuni* visualized in the SEM indicates that probably the identified variations in biomass and in the constitution may be more related to the characteristics of the other species than to the interaction itself.

SEM images demonstrated that the configuration of mixed biofilms presented the same pattern found in the monospecific biofilm of *C. jejuni*, in both MH and chicken juice. The exception was restrict to the interaction with *P. aeruginosa* that presented in addition a more compact and flat conformation with the presence of well delimited pores, and it was also identified a higher biomass in MH in comparison with chicken juice, that presented a significant difference ($p < 0.001$) in the colorimetric assay.

The predominance of the other species in detriment of *C. jejuni*, in mixed biofilms, may be related to the biofilm formation time, since *C. jejuni* is a fastidious and demanding specie. In addition, the prevalence of other species in mixed biofilms has also been described previously and may indicate the existence of selection pressure exerted under *C. jejuni* in the first days.

According to Culotti and Packman (2015) only after 3 days of formation of the mixed biofilm of *C. jejuni* and *P. aeruginosa* was it possible to observe the presence of dispersed and discrete colonies of *C. jejuni*, which were present only on the surface of the biofilm formed by *P. aeruginosa*. In addition, the authors also detected that there was a predominance of *P. aeruginosa* biofilm morphology that remained unchanged in the *C. jejuni* presence and exhibited the same typical characteristics of the simple *P. aeruginosa* biofilm.

Several authors have already stated that both, co-inoculation and the inclusion of *C. jejuni* in pre-established biofilms facilitates subsequent growth of the sessile form of this agent (Zhang et al., 2013; Culotti and Packman, 2014, 2015).

Aswathanarayan and Vittal (2013) have suggested that different bacterial species secrete enzymes that modify the composition of extracellular polymeric substance (EPS) of biofilms in response to external stresses, resulting in changes in the biofilm architecture in a specific environment. In this way, the inclusion of different species in two substrates (MH and chicken juice) promoted these modifications.

The exception found in mixed biofilms with *P. aeruginosa* in chicken juice may represent a specific characteristic of this specie. Many animal macromolecules have been reported with the ability to form an adherent film, but not always capable of improving biofilm formation. For example, bovine serum albumin reduces formation of biofilms in *S. aureus* (Xu et al., 2008) and *Burkholderia cepacia* (Hwang et al., 2012). On the other hand, it is important for adhesion in *Cronobacter* (Healy et al., 2010). These differences also correlate with changes in the ability to express absorption proteins, leading to a variability in time to biofilm formation (Brown et al., 2015). In addition, the composition of the *P. aeruginosa* biofilm matrix is predominantly of polysaccharides, mainly alginate (Mann and Wozniak, 2012), which confers a differentiated structure, which can be detected in SEM and may represent another explanation for difficulty in adherence in the presence of chicken juice.

Genotyping

The high heterogeneity found in *C. jejuni* strains is due to the fact that most of them are naturally competent to take the DNA present in the environment and promote recombination in their genome, that is, they execute the transformation mechanism effectively, due to production of extracellular DNAse (Clark et al., 2014).

The presence of strains with high percentage of phylogenetic similarity in different flocks and in the same one, was also reported by other authors who stated that slaughter conditions may be the main responsible for the presence of strains with a high degree of homology in samples from the same flock, such as the equipment used in animal processing and cross-contamination (Petersen and Wedderkopp, 2001; Workman et al., 2008).

Our approach has shown that the ability of *C. jejuni* in developing a structured biofilm is highly variable depending on the strain when maintained in MH. However, when there is supplementation with chicken juice, all strains present a strong biofilm producer pattern. The chicken juice allows a greater fixation of *C. jejuni* as it assigns a surface more conditioned to bacterial adhesion.

Genome analysis revealed the high potential of strains in the acquisition of sessile lifestyle, phenotypically proven in chicken juice. Its variable behavior in MH and chicken juice, apparently results from modifications in the expression of genes involved in stress response, adhesion and biofilm formation.

The existence of tolerant strains to the tested biocides and most used in the poultry industry suggests the existence of exposure to sublethal concentrations, representing a real risk for the development of adaptation mechanisms.

The ultrastructure of simple and mixed biofilms showed the early maturity range when in chicken juice compared to MH. However, in biofilms with *P. aeruginosa* this pattern is inverted, probably due to the particular characteristics of this species.

Identification of the predominantly protein composition of *C. jejuni* biomass and also in mixed biofilms may aid in the future development of agents of action with proteolytic approach as a prevention and strategy of control. However, it is noteworthy that in mixed culture with *P. aeruginosa* there is predominance of a polysaccharide matrix.

Phylogenetic diversity was evidenced by the presence of 23 pulsotypes, which confirms the intrinsic characteristic of *C. jejuni* to easily recombine its genome by gene transformation.

AUTHOR CONTRIBUTIONS

RM: Elaborated the project, put into practice the techniques that were not yet standardized by the team and conducted the analysis of the results, application of statistics in all tests and discussion of the study. EM: Responsible for molecular analyzes, including elaboration of the PFGE dendrogram and in the preparation of samples for analysis in SEM. GM: Manipulation of *Campylobacter* strains for biofilm formation and inhibition tests, including control of the initial inoculum. MS: Performed the replicates of the tests involving the quantification and classification of *C. jejuni* biofilms. CP: Performed the replicates of the biofilm inhibition tests. PP: Molecular analyzes to evaluate the presence of specific genes. HF: Helped in the discussions of the work and in the inclusion of new ideas. DR: Guidance in the writing of the results and discussion and correction of the final paper.

FUNDING

We thank FAPEMIG for the aid to purchase consumables and CNPq for the provision of funds for the granting of a scholarship and for the purchase of equipment and consumables essentials in the execution of the study.

ACKNOWLEDGMENTS

To CNPq and FAPEMIG for the provision of financial resources for the execution of the study and to the exporting company responsible for granting the samples.

REFERENCES

Aswathanarayan, J. B., and Vittal, R. R. (2013). "Microbial biofilms and their control by various antimicrobial strategies," in *Microbial Pathogens and Strategies for Combating them: Science, Technology and Education*, ed A. Méndez-Vilas (Badajoz: Formatex), 124–133.

Avila-Ramirez, C., Tinajero-Trejo, M., Davidge, K. S., Monk, C. E., Kelly, D. J., and Poole, R. K. (2013). Do globins in microaerophilic *Campylobacter jejuni* confer nitrosative stress tolerance under oxygen limitation? *Antioxid. Redox Signal.* 18, 424–431. doi: 10.1089/ars.2012.4750

Batz, M. B., Hoffmann, S., and Morris, J. G. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *J. Food Prot.* 75, 1278–1291. doi: 10.4315/0362-028X.JFP-11-418

Billings, N., Millan, M. R., Caldara, M., Rusconi, R., Tarasova, Y., Stocker, R., et al. (2013). The extracelular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* 9:e1003526. doi: 10.1371/journal.ppat.1003526

Birk, T., Ingmer, H., Andersen, M. T., Jorgensen, K., and Brondsted, L. (2004). Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. *Lett. Appl. Microbiol.* 38, 66–71. doi: 10.1046/j.1472-765X.2003.01446.x

Birk, T., Rosenquist, H., Brondsted, L., Ingmer, H., Bysted, A., Christensen, B. B., et al. (2006). A comparative study of two food model systems to test the survival of *Campylobacter jejuni* at −18 degrees C. *J. Food Prot.* 69, 2635–2639. doi: 10.4315/0362-028X-69.11.2635

Biswas, B., Hannon, S. J., Townsend, H. G. G., Andrew, P., and Allan, B. J. (2011). Genes coding for virulence determinants of *Campylobacter jejuni* in human clinical and cattle isolates from Alberta, Canada, and their potential role in colonization of poultry. *Int. Microbiol.* 14, 25–32. doi: 10.2436/20.1501.01.132

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., and Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157, 472–479. doi: 10.1093/infdis/157.3.472

Bronnec, V., Turonova, H., Bouju, A., Cruveiller, S., Rodrigues, R., Demnerova, K., et al. (2016). Adhesion, biofilm formation, and genomic features of *Campylobacter jejuni* Bf, an atypical strain able to grow under aerobic conditions. *Front. Microbiol.* 7:1002. doi: 10.3389/fmicb.2016.01002

Brown, H. L., Reuter, M., Hanman, K., Betts, R. P., and van Vliet, A. H. M. (2015). Prevention of biofilm formation and removal of existing biofilms by extracellular DNAses of *Campylobacter jejuni*. *PLoS ONE* 10:e0121680. doi: 10.1371/journal.pone.0121680

Brown, H. L., Reuter, M., Salt, L. J., Cross, K. L., Betts, R. P., van Vliet, A. H. M., et al. (2014). Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 80, 7053–7060. doi: 10.1128/AEM.02614-14

Center Disease and Control (2013). *Standardized Laboratory Protocol for Molecular Subtyping of Campylobacter jejuni by Pulsed Field Gel Electrophoresis (PFGE)*. Atlanta, GA: PulseNet USA. The National Molecular Subtyping Network for Foodborne Disease Surveillance.

Chaignon, P., Sadovskaya, I., Ragunah, C. H., Ramasubbu, N., Kaplan, J. B., Jabbouri, S., et al. (2007). Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl. Microbiol. Biotechnol.* 1, 125–132. doi: 10.1007/s00253-006-0790-y

Chmielewski, R. A. N., and Frank, J. F. (2007). "Inactivation of Listeria monocytogenes biofilms using chemical sanitizers and heat," in *Biofilms in the Food Environment*, ed H. P. Blaschek, H. H. Wang, and M. E. Agle (London: Blackwell Publishing, Ltd.), 73–104.

Clark, C. G., Chong, P. M., McCorrister, S. J., Mabon, P., Walker, M., and Westmacott, G. R. (2014). DNA sequence heterogeneity of *Campylobacter jejuni* CJIE4 prophages and expression of prophage genes. *PLoS ONE* 9:e95349. doi: 10.1371/journal.pone.0095349

Culotti, A., and Packman, A. I. (2014). *Pseudomonas aeruginosa* promotes *Escherichia coli* biofilm formation in nutrient-limited medium. *PLoS ONE* 9:e107186. doi: 10.1371/journal.pone.0107186

Culotti, A., and Packman, A. I. (2015). *Pseudomonas aeruginosa* facilitates *Campylobacter jejuni* growth in biofilms under oxic flow conditions. *FEMS Microbiol. Ecol.* 91:fiv136. doi: 10.1093/femsec/fiv136

Dat, N. M., Hamanaka, D., Tanaka, F., and Uchino, T. (2010). Surface conditioning of stainless steel coupons with skim milk solutions at different pH values and its effect on bacterial adherence. *Food Control.* 21, 1769–1773. doi: 10.1016/j.foodcont.2010.06.012

Datta, S., Niwa, H., and Itoh, K. (2003). Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. *J. Med. Microbiol.* 52, 345–348. doi: 10.1099/jmm.0.05056-0

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193. doi: 10.1128/CMR.15.2.167-193.2002

Drozd, M., Chandrashekhar, K., and Rajashekara, G. (2014). Polyphosphate-mediated modulation of *Campylobacter jejuni* biofilm growth and stability. *Virulence* 5, 680–690. doi: 10.4161/viru.34348

European Food Safety Authority (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J.* 13:162. doi: 10.2903/j.efsa.2015.3991

Elvers, K. T., and Park, S. F. (2002). Quorum sensing in *Campylobacter jejuni*: detection of a luxS encoded signaling molecule. *Microbiology* 148, 1475–1481. doi: 10.1099/00221287-148-5-1475

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Flint, A., Sun, Y. Q., Butcher, J., Stahl, M., Huang, H., and Stintzi, A. (2014). Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. *Infect. Immun.* 82, 2266–2275. doi: 10.1128/IAI.01528-13

Goering, R. V. (2010). Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infect. Genet. Evol.* 10, 866–875. doi: 10.1016/j.meegid.2010.07.023

Guyard-Nicodeme, M., Tresse, O., Houard, E., Jugiau, F., Courtillon, C., Manaa, K. E., et al. (2013). Characterization of *Campylobacter* spp. transferred from naturally contaminated chicken legs to cooked chicken slices via a cutting board. *Int. J. Food Microbiol.* 164, 7–14. doi: 10.1016/j.ijfoodmicro.2013.03.009

Hanel, I., Muller, J., Muller, W., and Schulze, E. (2004). Correlation between invasion of Caco-2 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni*. *Vet. Microbiol.* 101, 75–82. doi: 10.1016/j.vetmic.2004.04.004

Harmon, K. M., Ramson, G. M., and Wesley, I. V. (1997). Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. *Mol. Cell. Probes.* 11, 195–200. doi: 10.1006/mcpr.1997.0104

Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., et al. (2010). *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. *Foodborne Pathog. Dis.* 7, 339–350. doi: 10.1089/fpd.2009.0379

Howard, S. L., Jagannathan, A., Soo, E. C., Hui, J. P., Aubry, A. J., Ahmed, I., et al. (2009). A *Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis and colonization of chickens. *Infect. Immun.* 77, 2544–2556. doi: 10.1128/IAI.01425-08

Howlett, R. M., Hughes, B. M., Hitchcock, A., and Kelly, D. J. (2012). Hydrogenase activity in the foodborne pathogen *Campylobacter jejuni* depends upon a novel ABC-type nickel transporter (NikZYXWV) and is SlyD-independent. *Microbiology* 158, 1645–1655. doi: 10.1099/mic.0.054130-0

Hwang, G., Kang, S., El-Din, M. G., and Liu, Y. (2012). Impact of conditioning films on the initial adhesion of *Burkholderia cepacia*. *Colloids Surf. B Biointerfaces* 91, 181–188. doi: 10.1016/j.colsurfb.2011.10.059

Ica, T., Caner, V., Istanbullu, O., Nguyen, H. D., Ahmed, B., Call, D. R., et al. (2012). Characterization of mono- and mixed-culture *Campylobacter jejuni* biofilms. *Appl. Environ. Microbiol.* 78, 1033–1038. doi: 10.1128/AEM.07364-11

International Standards Organization (2006). *10272-1: Microbiology of Food and Animal Feeding Stuffs–horizontal Method for Detection and Enumeration of Campylobacter spp. Part 1: Detection Method*. Geneva: ISO.

Joshua, G. W., Guthrie-Irons, C., Karlyshev, A. V., and Wren, B. W. (2006). Biofilm formation in *Campylobacter jejuni*. *Microbiology* 152, 387–396. doi: 10.1099/mic.0.28358-0

Kalmokoff, M., Lanthier, P., Tremblay, T. L., Foss, M., Lau, P. C., Sanders, G., et al. (2006). Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188, 4312–4320. doi: 10.1128/JB.01975-05

Keeratipibul, S., and Techaruwichit, P. (2012). Tracking sources of Listeria contamination in a cooked chicken meat factory by PCRRAPD-based DNA fingerprinting. *F. Control.* 27, 64–72. doi: 10.1016/j.foodcont.2012.02.026

Kim, J., Park, C., and Kim, Y. (2015). Role of flgA for flagellar biosynthesis and biofilm formation of *Campylobacter jejuni* NCTC11168. *J. Microbiol. Biotechnol.* 25, 1871–1879. doi: 10.4014/jmb.1504.04080

Konkel, M. E., Larson, C. L., and Flanagan, R. C. (2010). *Campylobacter jejuni* FlpA binds fibronectin and is required for maximal host cell adherence. *J. Bacteriol.* 192, 68–76. doi: 10.1128/JB.00969-09

Kostakioti, M., Hadjifrangiskou, M., and Hultgren, S. J. (2013). Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harbor Perspect. Med.* 3:a010306. doi: 10.1101/cshperspect.a010306

Kudirkiene, E., Cohn, M. T., Stabler, R. A., Strong, P. C. R., Serniene, L., Wren, B. W., et al. (2012). Phenotypic and genotypic characterizations of *Campylobacter jejuni* isolated from the broiler meat production process. *Curr. Microbiol.* 65, 398–406. doi: 10.1007/s00284-012-0170-z

Lawes, J. R., Vidal, A., Clifton-Hadley, F. A., Sayers, R., Rodgers, J., Snow, L., et al. (2012). Investigation of prevalence and risk factors for *Campylobacter* in broiler flocks at slaughter: results from a UK survey. *Epidemiol. Infect.* 140, 1725–1737. doi: 10.1017/S0950268812000982

Lehtola, M. J., Pitkanen, T., Miebach, L., and Miettinen, I. T. (2006). Survival of *Campylobacter jejuni* in potable water biofilms: a comparative study with different detection methods. *Water Sci. Technol.* 54, 57–61. doi: 10.2166/wst.2006.448

Li, J. (2016). *The Effects of Meat Juice on Biofilm Formation of Campylobacter and Salmonella*. Master's thesis. University of British Columbia.

Ligowska, M., Cohn, M. T., Stabler, R. A., Wren, B. W., and Andbrondsred, L. (2011). Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int. J. Food Microbiol.* 5, 111–115. doi: 10.1016/j.ijfoodmicro.2010.08.027

Lu, X., Samuelson, D. R., Rasco, B. A., and Konkel, M. E. (2012). Elucidation of the antimicrobial effect of diallyl sulfide on *Campylobacter jejuni* biofilms. *J. Antimicrob. Chemother.* 67, 1915–1926. doi: 10.1093/jac/dks138

Mann, E. E., and Wozniak, D. J. (2012). Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiol. Rev.* 36, 893–916. doi: 10.1111/j.1574-6976.2011.00322.x

Mihaljevic, R. R., Sikic, M., Klancnik, A., Brumini, G., Mozina, S. S., Abram, M., et al. (2007). Environmental stress factors affecting survival and virulence of *Campylobacter jejuni*. *Microb. Pathog.* 43, 120–125. doi: 10.1016/j.micpath.2007.03.004

Mileyeva-Biebesheimer, O. (2011). *An Investigation into Metal Oxide Nanoparticle Toxicity to Bacteria in Environmental Systems using Fluorescence Based Assays*. master's thesis. Toledo, OH: University of Toledo Digital Repository.

Nachamkin, I., Allos, B. M., and Ho, T. (1998). *Campylobacter* species and Guillain-Barré syndrome. *Clin. Microbiol. Rev.* 11, 555–567.

Nakari, U. M. (2011). *Identification and Epidemiological Typing of Campylobacter Strains Isolated from Patients in Finland*. Helsinki: National Institute for Health and Welfare (THL). 126. ISBN 978-952-245-465-2 (printed); ISBN 978-952-245-466-9 (pdf).

Naves, P., Del Prado, G., Huelves, L., Gracia, M., Ruiz, V., and Blanco, J. (2008). Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. *J. Appl. Microbiol.* 105, 585–590. doi: 10.1111/j.1365-2672.2008.03791.x

Oh, E., and Jeon, B. (2014). Role of alkyl hydroperoxide reductase (AhpC) in the biofilm formation of *Campylobacter jejuni*. *PLoS ONE* 9:e87312. doi: 10.1371/journal.pone.0087312

Ohsumi, T., Takenaka, S., Wakamatsu, R., Sakaue, Y., Narisawa, N., Senpuku, H., et al. (2015). Residual structure of *Streptococcus mutans* biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. *PLoS ONE* 10:e0116647. doi: 10.1371/journal.pone.0116647

Parveen, N., and Cornell, K. A. (2011). Methylthio adenosine/S-adenosylhomocysteine nucleosidase, acritical enzyme for bacterial metabolism. *Mol. Microbiol.* 79, 7–20. doi: 10.1111/j.1365-2958.2010.07455.x

Petersen, L., and Wedderkopp, A. (2001). Evidence that certain clones of *Campylobacter jejuni* persist during successive broiler flock rotations. *Appl. Environ. Microbiol.* 67, 2739–2745. doi: 10.1128/AEM.67.6.2739-2745.2001

Plummer, P. J. (2012). LuxS and quorum-sensing in *Campylobacter*. *Front. Cell. Infect. Microbiol.* 2:22. doi: 10.3389/fcimb.2012.00022

Reeser, R. J., Medler, R. T., Billington, S. J., Jost, B. H., and Joens, L. A. (2007). Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73, 1908–1913. doi: 10.1128/AEM.00740-06

Reuter, M., Mallett, A., Pearson, B. M., and van Vliet, A. H. M. (2010). Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76, 2122–2128. doi: 10.1128/AEM.01878-09

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.P11101

Sulaeman, S., Hernould, M., Schaumann, A., Coquet, L., Bolla, J. M., Dé, E., et al. (2012). Enhanced adhesion of *Campylobacter jejuni* to abiotic surfaces is mediated by membrane proteins in oxygen-enriched conditions. *PLoS ONE* 7:e46402. doi: 10.1371/journal.pone.0046402

Sulaeman, S., Le Bihan, G., Rossero, A., Federighi, M., De, E., and Tresse, O. (2009). Comparison between the biofilm initiation of *Campylobacter jejuni* and *Campylobacter coli* strains to an inert surface using BioFilm Ring Test. *J. Appl. Microbiol.* 108, 1303–1312. doi: 10.1111/j.1365-2672.2009.04534.x

Sutherland, I. W. (2001). The biofilm matrix–an immobilized but dynamic microbial environment. *Trends Microbiol.* 9, 222–227. doi: 10.1016/S0966-842X(01)02012-1

Svensson, S. L., Davis, L. M., MacKichan, J. K., Allan, B. J., Pajaniappan, M., Thompson, S. A., et al. (2009). The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. *Mol. Microbiol.* 71, 253–272. doi: 10.1111/j.1365-2958.2008.06534.x

Svensson, S. L., Pryjma, M., and Gaynor, E. C. (2014). Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*. *PLoS ONE* 9:e106063. doi: 10.1371/journal.pone.0106063

Tazumi, A., Negoro, M., Tomiyama, Y., Misawa, N., Itoh, K., Moore, J. E., et al. (2011). Uneven distribution of the *luxS* gene with in the genus *Campylobacter*. *Brit. J. Biomed. Sci.* 68, 19–22. doi: 10.1080/09674845.2011.11732836

Techaruvichit, P., Takahashi, H., Kuda, T., Miya, S., Keeratipibul, S., and Kimura, B. (2016). Adaptation of *Campylobacter jejuni* to biocides used in the food industry affects biofilm structure, adhesion strength, and cross-resistance to clinical antimicrobial compounds, *Biofouling* 32, 827–839. doi: 10.1080/08927014.2016.1198476

Trachoo, N., and Frank, J. F. (2002). Effectiveness of chemical sanitizers against *Campylobacter jejuni*-containing biofilms. *J. Food Prot.* 65, 1117–1121. doi: 10.4315/0362-028X-65.7.1117

Turonova, H., Briandet, R., Rodrigues, R., Hernould, M., Hayek, N., and Stintzi, A. (2015). Biofilm spatial organization by the emerging pathogen *Campylobacter jejuni*: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6:709. doi: 10.3389/fmicb.2015.00709

van Alphen, L. B., Wenzel, C. Q., Richards, M. R., Fodor, C., Ashmus, R. A., Stahl, M., et al. (2014). Biological roles of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*. *PLoS ONE* 9:e87051. doi: 10.1371/journal.pone.0087051

Workman, S. N., Mathison, G. E., and Lavoie, M. C. (2008). An investigation of sources of *Campylobacter* in a poultry production and packing operation in Barbados. *Int. J. Food Microbiol.* 121, 106–111. doi: 10.1016/j.ijfoodmicro.2007.10.014

Xavier, K. B., and Bassler, B. L. (2005). Interference with AI-2-mediated bacterial cell-cell communication. *Nature.* 437, 750–753. doi: 10.1038/nature03960

Xu, C.-P., Boks, N. P., de Vries, J., Kaper, H. J., Norde, W., Busscher, H. J., et al. (2008). *Staphylococcus aureus*-fibronectin interactions with and without fibronectin-binding proteins and their role in adhesion and desorption. *Appl. Environ. Microbiol.* 74, 7522–7528. doi: 10.1128/AEM.00948-08

Zhang, W., Sileika, T., and Packman, A. (2013). Effects of fluid flow conditions on interactions between species in biofilms. *FEMS Microbiol. Ecol.* 84, 344–354. doi: 10.1111/1574-6941.12066

Zheng, J., Meng, J. H., Zhao, S. H., Singh, R., and Song, W. X. (2006). Adherence to and invasion of human intestinal epithelial cells by *Campylobacter jejuni* and *Campylobacter coli* isolates from retail meat products. *J. Food Prot.* 69, 768–774. doi: 10.4315/0362-028X-69.4.768

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Melo, Mendonça, Monteiro, Siqueira, Pereira, Peres, Fernandez and Rossi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 July 2017
doi: 10.3389/fmicb.2017.01311

Modeling the Combined Effect of Pressure and Mild Heat on the Inactivation Kinetics of *Escherichia coli*, *Listeria innocua*, and *Staphylococcus aureus* in Black Tiger Shrimp (*Penaeus monodon*)

Barjinder P. Kaur[1]* *and P. Srinivasa Rao*[2]

[1] Department of Food Engineering, National Institute of Food Technology Entrepreneurship and Management, Sonepat, India, [2] Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur, Kharagpur, India

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Zhao Chen, Clemson University, United States
Alexandra Lianou, Agricultural University of Athens, Greece
Dario De Medici, Istituto Superiore di Sanità, Italy

***Correspondence:**
Barjinder P. Kaur
barjinderpkaur@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 December 2016*
Accepted: *28 June 2017*
Published: *24 July 2017*

Citation:
Kaur BP and Rao PS (2017) Modeling the Combined Effect of Pressure and Mild Heat on the Inactivation Kinetics of Escherichia coli, Listeria innocua, and Staphylococcus aureus in Black Tiger Shrimp (Penaeus monodon). Front. Microbiol. 8:1311. doi: 10.3389/fmicb.2017.01311

The high-pressure inactivation of *Escherichia coli*, *Listeria innocua*, and *Staphylococcus aureus* was studied in black tiger shrimp (*Penaeus monodon*). The processing parameters examined included pressure (300 to 600 MPa) and temperature (30 to 50°C). In addition, the pressure-hold period (0 to 15 min) was investigated, thus allowing both single-pulse pressure effects (i.e., zero holding time) and pressure-hold effects to be explored. *E. coli* was found to be the most sensitive strain to single-pulse pressure, followed by *L. innocua* and lastly *S. aureus*. Higher pressures and temperatures resulted in higher destruction rates, and the value of the shape parameter (β') accounted for the downward concavity ($\beta' > 1$) of the survival curves. A simplified Weibull model described the non-linearity of the survival curves for the changes in the pressure-hold period well, and it was comparable to the original Weibull model. The regression coefficients (R^2), root mean square error (RMSE), accuracy factor (A_f), bias factor (B_f), and residual plots suggested that using linear models to represent the data was not as appropriate as using non-linear models. However, linear models produced good fits for some pressure–temperature combinations. Analogous to their use in thermal death kinetics, activation volume (V_a) and activation energy (E_a) can be used to describe the pressure and temperature dependencies of the scale parameter (δ, min), respectively. The V_a and E_a values showed that high pressure and temperaturefavored the inactivation process, and *S. aureus* was the most baro-resistant pathogen.

Keywords: black tiger shrimp, high-pressure processing, inactivation, pathogens, weibull, log-linear

INTRODUCTION

High-pressure processing (HPP) offers an attractive alternative to heat pasteurization as a means to produce preservative-free, microbiologically safe, and stable foods (Pavuluri and Kaur, 2014). Over the last decade, significant progress has been made in the high-pressure pasteurization of foods, and several commercial products treated with HPP are now available on the market in several countries (Balasubramaniam et al., 2008). The high level of interest in this novel technology is due

to its ability to destroy or inactivate foodborne microorganisms and enzymes in food with minimal change in the organoleptic properties and nutritional quality of the food. Pressures ranging from 300 to 600 MPa can inactivate most pathogenic and spoilage vegetative cells, yeasts, and molds (Smelt, 1998). Moreover, it is economically beneficial to use lower pressure in combination with mild heat (30–50°C) (Buzrul et al., 2008).

For effective use of high-pressure technology for food preservation, it is necessary to study the interactions between the processing parameters (pressure, time, and temperature) and to determine the optimum conditions for obtaining desirable levels of microbial destruction while maintaining a high degree of nutritional quality and good flavor and texture (Kaur and Rao, 2016).

Several studies have demonstrated the efficacy of HPP for inactivating a wide spectrum of Gram-negative and Gram-positive bacteria in suspensions, as well as in solid food items. HPP inactivates microorganisms by acting against multiple targets, including intracellular and membrane-bound enzymes (Wouters et al., 1998). However, the rate and pattern of HPP-induced microorganism inactivation is quite variable and influenced by the processing conditions, medium composition, and microorganism type/strain. Therefore, there is a need for accurate prediction of the inactivation behavior of foodborne microorganisms, as well as accurate characterization of their resistance to HPP. Kinetic modeling is helpful for determining the most efficient processing parameters and for the prediction of the HPP effects on microbial inactivation and product shelf-life. It is also an effective way of carrying out a risk assessment and simulation of the HPP process (Smelt et al., 2002).

Although simple first-order-type inactivation curves do sometimes occur for pressure-treated cells (Basak et al., 2002; Riahi et al., 2003; Dogan and Erkmen, 2004; Ramaswamy et al., 2008), significant deviations from linearity (such as, sigmoidal curves, curves with shoulders, and tailing) have been reported by multiple researchers (Garriga et al., 2002; Chen and Hoover, 2003; Buzrul and Alpas, 2004; Rajan et al., 2006; Ahn et al., 2007; Rendueles et al., 2011). A number of models have been proposed to describe these non-linear survival curves, such as, the Baranyi, Weibull, modified Gompertz, log-logistic, and quasi-chemical models. Among these models, the most simple and flexible is the Weibull model, and it is reported to fit the experimental data better than the other models (Panagou et al., 2007; Serment-Moreno et al., 2014).

Black tiger shrimp (*Penaeus monodon*) is a commercially important shrimp species, persistently in demand in the global market due to its distinct flavor, texture, and high nutritional value (Pushparajan and Soundarapandian, 2010). In past decades, there have been many issues associated with shrimp exported from India due to high bacteria counts and the presence of pathogenic bacteria such as, *E. coli* and *Listeria monocytogenes. L. monocytogenes* is particularly problematic for the food industry as it is widespread in the environment and can grow in a broad range of temperatures (Jofré et al., 2009). Crustaceans have also been linked with food poisoning attributed to *S. aureus* because of the ubiquity of this organism in the processing environment combined with the degree of handling required for processing these food types (Nicolaides, 2009).

Thus, the present study was undertaken with the aim of investigating the inactivation kinetics of *E. coli* O157:H7 ATCC 43895 (a Gram-negative bacterium) and two Gram-positive bacteria, namely *L. innocua* ATCC 33090 (a nonpathogenic indicator microorganism for *L. monocytogenes*) and *S. aureus* ATCC 29213, in black tiger shrimp at various pressure–temperature (*P*–*T*) combinations. These three foodborne pathogens have been reported to be resistant to pressure treatment (Guan et al., 2006; Buzrul et al., 2008; Ruiz-Espinosa et al., 2013).

Abbreviations: HPP, High-pressure processing; E_a, Activation energy; MPa, Megapascal; N_0, Initial microbial counts in the control samples; N_{PE}, Number of cells that survived after single-pulse pressurization; N_t, Number of cells that survived after pressure treatment for time t (min); SPPE, Single-pulse pressure effect; V_a, Activation volume.

MATERIALS AND METHODS

Preparation of Bacterial Cultures

Pure cultures *of E. coli* O157:H7 ATCC 43895, *L. innocua* ATCC 33090, and *S. aureus* ATCC 29213 were procured in freeze-dried form from Merck Ltd India. The bacteria were activated by inoculating tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (product number: M011; Himedia Laboratories, India) with each of the pure cultures, and they were then incubated at 37°C for 24 h. Stock cultures were prepared by mixing the activated cultures with sterilized 80% glycerol (v/v) in a 1:1 ratio in Eppendorf tubes, which were stored at −40°C in a deep freezer until further analysis. The purity of the cultures was determined by Gram staining and microscopic observation. Each inoculum was prepared by inoculating 100 mL of sterile TSBYE with 1 mL of the thawed stock culture, and it was then incubated at 37°C for 24–30 h to obtain a count of 10^9 colony-forming units (CFU) mL^{-1}.

Preparation and Inoculation of Samples

Freshly harvested black tiger shrimp weighing 25–35 g were obtained from Shankarpur coast, West Bengal, India, and transported to the laboratory within 4 h under chilled conditions (with a sample-to-ice ratio of 1:1). The shrimp were washed with chilled water, deheaded, and shelled. Before inoculation, the samples were tested for the presence of *E. coli*, *Listeria*, and *S. aureus* according to the method proposed by the APHA (2001), which is described later in Section Enumeration.

After ensuring the absence of these organisms, test samples were inoculated with the activated bacteria, according to the method described by Anang et al. (2007). Whole shrimp samples were dipped into each of the prepared inocula (10^9 CFU mL^{-1}) individually at room temperature (27°C) for 15 min. The ratio of sample to culture suspension was 1:2 (w/v), which allowed complete immersion of the samples. The initial bacterial cell counts in the shrimp samples after dipping were approximately 10^7 CFU g^{-1}. Thereafter, the samples were left to dry in a sterile cabinet for 1 h. The inoculated samples were packed in ethylene vinyl alcohol films (thickness: 110 ± 1.0 μm), with three

shrimps per pouch. The samples were double packed to ensure no direct contact with the pressure-transmitting medium during the HPP.

HPP

The high-pressure treatment was performed in a lab-scale HPP unit (model: S-IL-100-250-09-W; Stansted Fluid Power Systems, UK), with aqueous monopropylene glycol 30% (v/v) as the pressure-transmitting medium. The samples were processed at four pressures, 300, 400, 500, and 600 MPa, and four temperatures, 30, 40, 50, and 60°C. For each *P*–*T* combination, the following six pressure-hold periods were investigated: 0, 3, 6, 9, 12, and 15 min. The single-pulse pressurization (0-min holding time) involved pressurization of the sample to the desired level followed by immediate depressurization. The rate of pressurization was fixed at 300 MPa min^{-1} and the depressurization was achieved in < 10 s. After *P*–*T* treatment, the pouches were kept at 4°C for 24 h to allow the pressurized cells to recover from the pressure stress effect before analysis. For each *P*–*T* combination, three pouches (with three shrimp per pouch) were processed. The experiments were performed independently twice and, for each processing condition, three samples were analyzed.

Enumeration

Microbiological analyses were performed according to the method described by the APHA (2001). A 10-g sample was aseptically cut from each sample lot and macerated with 90 mL peptone water (0.1%) in a sterile glass mortar. The homogenate was serially diluted using 0.1% sterile peptone water and plated onto appropriate culture media. For ease of handling, the direct pour plate method was adopted. *E. coli* was enumerated on Violet Red Bile Agar and *L. innocua* on Listeria-Selective Agar Base (PALCAM). The plates were incubated at 37°C for 24 h for *E. coli* and 48 h for *L. innocua*. To examine the surviving *S. aureus* populations, samples were inoculated on Baird Parker Agar and the plates incubated at 37°C for 48 h. Black colonies with clear zones, which were presumed to be *S. aureus*, were counted.

Kinetics Analysis

The pressure-induced destruction of the microorganisms was analyzed in terms of the single-pulse pressure effect (SPPE) and the pressure-hold effect. First, the SPPE was determined by calculating the logarithmic difference between the initial microbial counts in the control samples (N_0) and the number of cells that survived after single-pulse pressurization (N_{PE}) as follows:

$$SPPE = Log_{10}(N_0) - Log_{10}(N_{PE}) \quad (1)$$

Second, a modified Weibull model (Equation 2; (Chakraborty et al., 2015)) was fitted to the data on survivor fractions corresponding to the different pressure-hold periods using Origin Pro version 8.0 (OriginLab Corporation, Northampton, MA, USA).

$$Log_{10}\frac{N_t}{N_{PE}} = -\left(\frac{t}{\delta'}\right)^{\beta'} \quad (2)$$

where N_t is the number of microorganisms that survived after pressure treatment for time t (min), N_{PE} is the number of cells that survived after single-pulse pressurization, and δ' and β' represent the scale parameter (min) and shape parameter (dimensionless), respectively. $\beta' < 1$ denotes upward concavity, $\beta' > 1$ represents downward concavity, and $\beta' = 1$ corresponds to linear (first-order) kinetics. The concavity can be used to interpret the inactivation resistance of the bacterial population: (a) homogenous ($\beta' = 1$), (b) increasing resistance ($\beta' < 1$), or (c) decreasing resistance as a result of accumulated damage in the population ($\beta' > 1$) (Serment-Moreno et al., 2014).

The best-fit values of δ' and β' were determined for each species at each of the *P*–*T* combinations. It has been reported that the shape parameter (β') represents the microorganism behavior index (Fernandez et al., 2002). Therefore, within the ranges of the processing parameters evaluated in this study, for each species, β was fixed at a uniform value using the method described by Chakraborty et al. (2015). In brief, for a pre-assumed value of β',

TABLE 1 | Estimated PE values (Log PE) for *E. coli*, *L. innocua* and *S. aureus* in black tiger shrimp.

Bacterial species	Temperature(°C)	Pressure (MPa)			
		300	400	500	600
E. coli	30	0.90 ± 0.04^{aA}	1.15 ± 0.03^{aA}	1.56 ± 0.10^{aA}	1.75 ± 0.09^{aA}
	40	2.74 ± 0.09^{aB}	3.17 ± 0.11^{abB}	3.99 ± 0.08^{bB}	4.29 ± 0.17^{bB}
L. innocua	30	0.88 ± 0.04^{aA}	1.09 ± 0.06^{aA}	1.46 ± 0.14^{aA}	1.58 ± 0.08^{aA}
	40	2.65 ± 0.15^{aB}	2.99 ± 0.14^{aB}	4.01 ± 0.23^{bB}	4.39 ± 0.11^{bB}
S. aureus	30	0.75 ± 0.05^{aA}	0.90 ± 0.07^{aA}	1.23 ± 0.05^{aA}	1.29 ± 0.08^{aA}
	40	2.09 ± 0.14^{aB}	2.69 ± 0.2^{abB}	3.73 ± 0.09^{bB}	4.01 ± 0.17^{bB}
	50	4.39 ± 0.11^{aC}	4.81 ± 0.21^{abC}	5.19 ± 0.25^{bC}	5.61 ± 0.20^{bC}

All values are means ± standard deviations of data from two independent experiments. Different superscripts (a, b) in the same row indicate significant difference (P < 0.05). Different superscripts (A, B, C) in the same column indicate significant difference (P < 0.05).

the δ' values were calculated using Equation (3) for the five non-zero pressure-hold periods (with three replications for each *P*–*T* combination).

$$\delta' = \left[\frac{t}{\left\{ -Log_{10}\left(\frac{N_t}{N_{PE}}\right)^{\frac{1}{\beta'}} \right\}} \right] \quad (3)$$

Next, the logarithm of the fractional count value ($-\text{Log}_{10}\text{N}_t/\text{N}_{PE}$) was recalculated using Equation (2) at the average value of δ' (δ'_{avg}, min) for the relevant *P*–*T* combination. For each species, the value of β' was varied from 0 to 2 and the value with the minimum cumulative sum of the square of errors (SSE; according to Eq. 4) was designated as the uniform β for the entire *P*–*T* domain tested.

$$SE = \sum \left[\left(Log_{10}\frac{N_t}{N_{PE}} \right)_{\delta'_{avg}} - \left(Log_{10}\frac{N_t}{N_{PE}} \right)_{experimental} \right]^2 \quad (4)$$

After fixing the β value at a single value for the entire *P*–*T* domain, the δ values were recalculated for all the conditions tested. Analogous to thermal death kinetics, using δ to represent the decimal reduction time (*D*-value, min), the rate of destruction (k, min^{-1}) was calculated according to Equation (5).

$$k = \left(\frac{2.303}{\delta^{\beta}} \right)^{\frac{1}{\beta}} \quad (5)$$

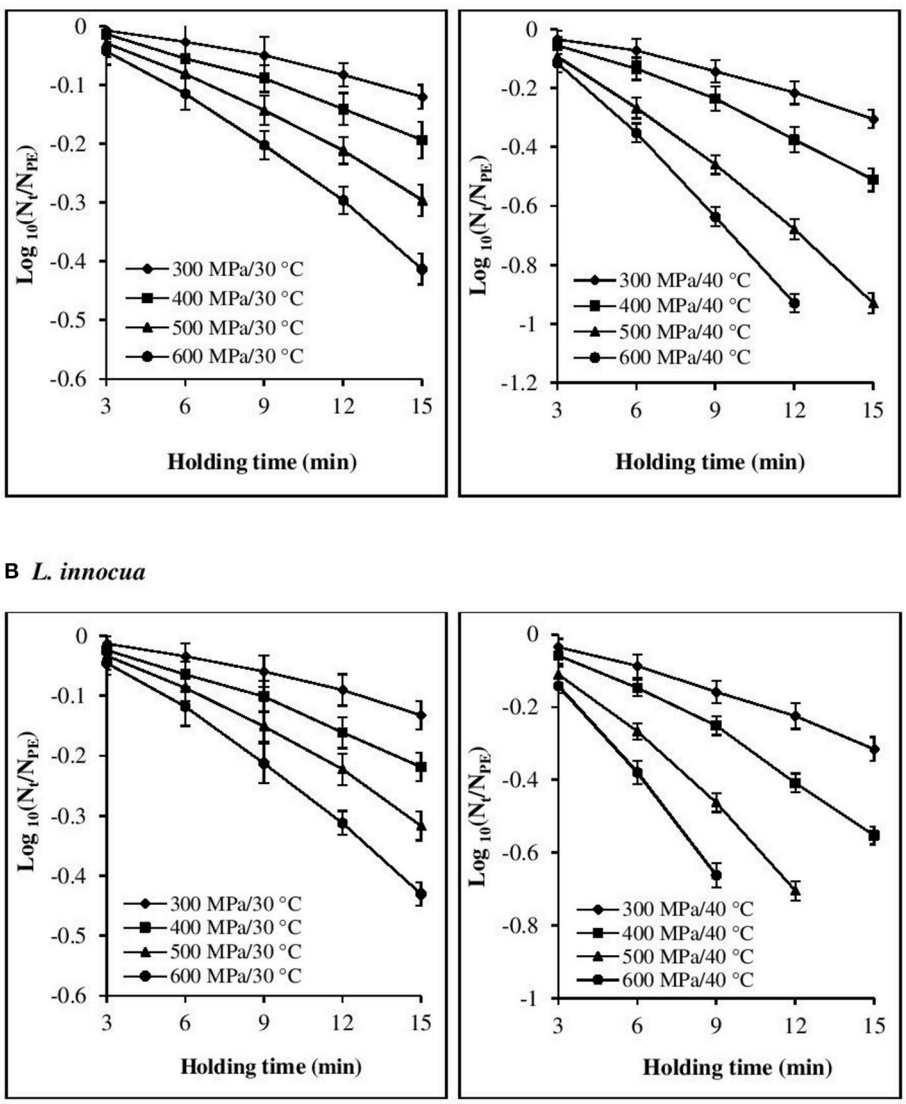


FIGURE 1 | Effect of different pressure-temperature treatments on the inactivation of **(A)** *E. coli* and **(B)** *L. innocua* in black tiger shrimp.

The pressure sensitivity of k at a fixed temperature was quantified using the activation volume (V_a, cm^3mol^{-1}), which was calculated using the Eyring equation (Equation 6).

$$\text{Ln } k = \text{Ln } k_{ref,P} + \frac{V_a}{RT}\left[P_{ref} - P\right] \quad (6)$$

where $k_{ref,P}$ is the rate constant (min^{-1}) at the reference pressure, P_{ref} (450 MPa, the midpoint of the pressure axis).

The temperature sensitivity of k at a fixed pressure was quantified using the activation energy (E_a, kJ mol^{-1}). This was calculated using the Arrhenius equation (Equation 7) in which the logarithmic function of k is directly proportional to the reciprocal of the absolute temperature (T, K).

$$\text{Ln } k = \text{Ln } k_{ref,T} + \frac{E_a}{R}\left[\frac{1}{T_{ref}} - \frac{1}{T}\right] \quad (7)$$

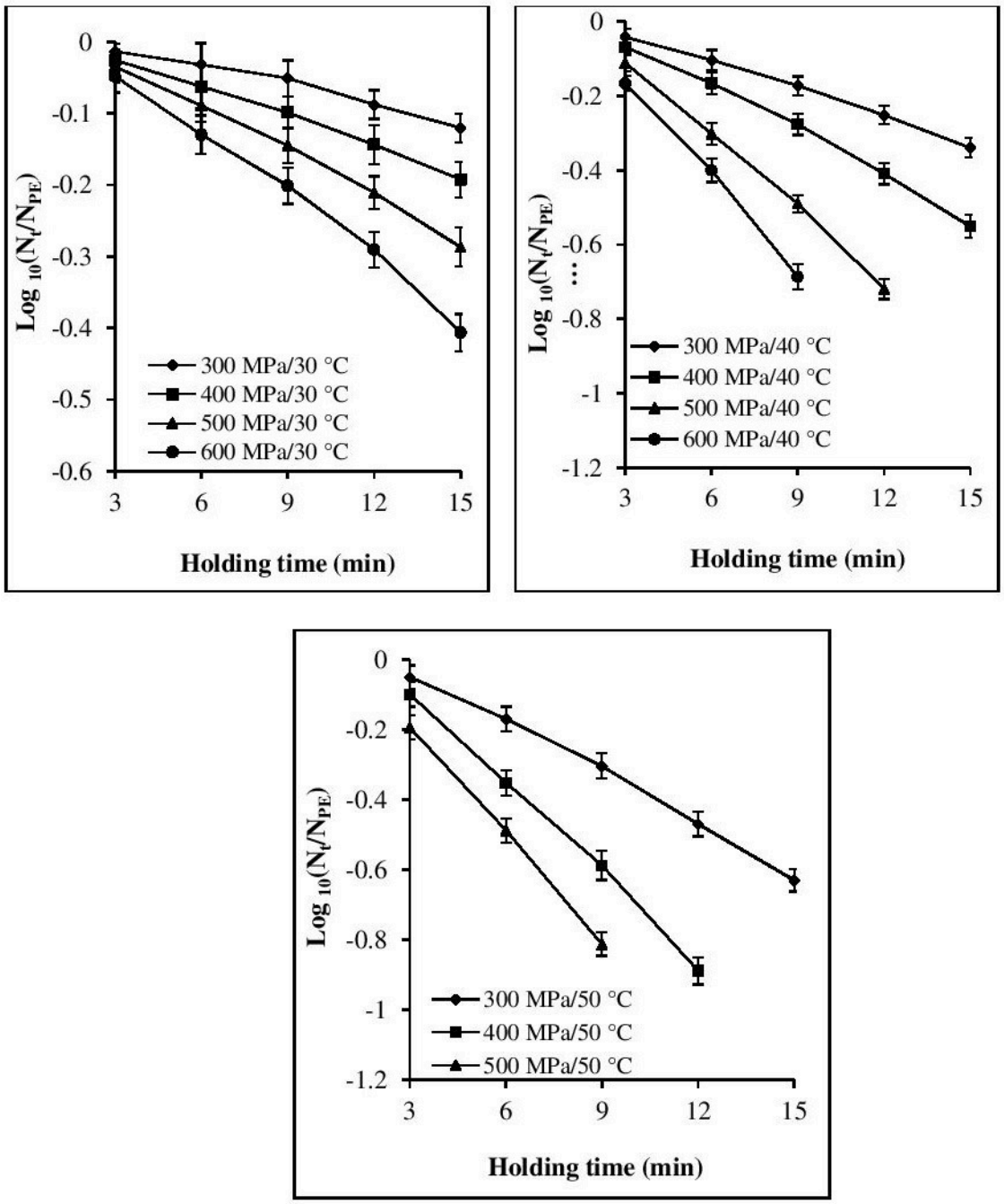


FIGURE 2 | Effect of different pressure-temperature treatments on the inactivation of *S. aureus* in black tiger shrimp.

where $k_{ref,T}$ is the rate constant (min^{-1}) at the reference temperature, T_{ref} (318 K, the midpoint of the temperature axis).

The regression analyses for calculating the rate constants, V_a, E_a, SSE, and regression coefficients (R^2) were performed in Microsoft Excel 2007 (Microsoft Corp., USA). Non-linear curve fitting for k as a function of V_a or E_a was performed using Origin Pro version 8.0.

Statistical Analysis

An analysis of variance (ANOVA) was carried out using SPSS for Windows version 17 (SPSS Inc., Chicago, IL, USA). The difference between the pairs of means was evaluated using Tukey's test, with a P–value < 0.05 being considered statistically significant.

RESULTS AND DISCUSSION

Effect of Single-Pulse Pressurization

A significant SPPE ($P < 0.05$) was observed, both with increases in pressure and temperature, indicating the efficacy of single-pulse pressurization for lowering microbial counts (**Table 1**). For processing parameters of 300–600 MPa/30–60°C, the SPPE values ranged from 0.90 to 4.29 Log_{10} cycles, 0.88 to 4.39 Log_{10} cycles, and 0.75 to 5.61 Log_{10} cycles for *E. coli*, *L. innocua*, and *S. aureus*, respectively. The effect of temperature was observed to be statistically more significant ($P < 0.05$) than that of pressure. Among the three microorganisms studied, *E. coli* exhibited the greatest sensitivity to single-pulse pressure, followed by *L. innocua* and lastly *S. aureus*. Ramaswamy et al. (2008) reported similar findings, with greater inactivation by pulse pressurization of *E. coli* O157:H7 compared to *L. monocytogenes* Scott A.

The reductions in the bacterial counts induced by single-pulse pressurization were due to the formation of cavitation voids in the bacterial cells as a result of rapid pressurization and depressurization, which led to physical disruption and death of the bacteria (Hiremath and Ramaswamy, 2011). The amount of disruption to the cell wall is dependent on the processing conditions (Ramaswamy et al., 2003). The depressurization magnitude has been reported to have a greater effect on the cell wall than the pressurization magnitude (Hayakawa et al., 1994). The increase in pressure come up time (i.e., the time required to reach the desired pressure) caused the microorganisms to be exposed to pressurization stress for an extra period of time, which increased the extent of inactivation. In addition, the greater reduction in bacterial counts at higher temperature was due to thermal lethality, which thus aided the damage caused by the pressure treatment (Chakraborty et al., 2014).

Isobaric–Isothermal Inactivation Kinetics of Pathogens

The inactivation rate increased with increases in each of the three processing parameters, viz. pressure, temperature, and time (**Figures 1**, **2**). The inactivation rates of the three pathogens during isobaric–isothermal cycles decreased in the following order: *L. innocua* > *E. coli* > *S. aureus*. Several researchers have also reported that *S. aureus* was the most baro-resistant pathogen in their studies (Yuste et al., 2004; Jofré et al., 2009; Cebrián et al., 2016).

At 30°C, the reduction in the count of all three pathogens was <5 Log CFU g^{-1}. The maximum inactivation, 5 Log CFU g^{-1} was obtained for *L. innocua* at 600 MPa/15 min. *S. aureus*

TABLE 2 | Shape factor (β_l, β', and β) obtained after model fitting at different pressure-temperature combinations for different microorganisms in black tiger shrimp.

Bacterial species	Pressure (MPa)	The log-linear model		The Weibull models							
		$\beta_l = 1$		30°C		40°C		50°C		Overall β at SSE_{min}	
		β_l	Adj R^2 at 30, 40, 50°C	β'	Adj R^2	β'	Adj R^2	β'	Adj R^2	β	Adj R^2 at 30, 40, 50°C
E. coli	300	1[a]	0.90, 0.91	1.45 ± 0.05[b]	0.99	1.49 ± 0.07[b]	0.97	ND	ND	1.48[b]	0.99, 0.98
	400	1[a]	0.89, 0.86	1.44 ± 0.07[b]	0.98	1.46 ± 0.02[b]	0.94	ND	ND	1.48[b]	0.97, 0.97
	500	1[a]	0.78, 0.80	1.51 ± 0.11[b]	0.99	1.42 ± 0.02[ab]	0.95	ND	ND	1.48[b]	0.96, 0.97
	600	1[a]	0.76, 0.81	1.52 ± 0.02[b]	0.99	1.43 ± 0.05[a]	0.97	ND	ND	1.48[b]	0.98, 0.97
L. innocua	300	1[a]	0.87, 0.87	1.41 ± 0.09[b]	0.99	1.39 ± 0.05[b]	0.98	ND	ND	1.40[b]	0.96, 0.98
	400	1[a]	0.88, 0.86	1.39 ± 0.05[b]	0.98	1.41 ± 0.05[b]	0.97	ND	ND	1.40[b]	0.98, 0.98
	500	1[a]	0.85, 0.83	1.43 ± 0.04[b]	0.98	1.39 ± 0.11[b]	0.99	ND	ND	1.40[b]	0.98, 0.99
	600	1[a]	0.85, 0.85	1.40 ± 0.04[b]	0.97	1.39 ± 0.06[b]	0.97	ND	ND	1.40[b]	0.97, 0.98
S. aureus	300	1[a]	0.85, 0.89, 0.86	1.33 ± 0.08[b]	0.99	1.31 ± 0.07[b]	0.96	1.34 ± 0.07[b]	0.98	1.30[b]	0.97, 0.98, 0.97
	400	1[a]	0.82, 0.80, 0.82	1.29 ± 0.10[b]	0.98	1.32 ± 0.03[b]	0.98	1.28 ± 0.07[b]	0.97	1.30[b]	0.98, 0.99, 0.99
	500	1[a]	0.87, 0.81, 0.81	1.32 ± 0.02[b]	0.99	1.30 ± 0.06[b]	0.97	1.30 ± 0.07[b]	0.98	1.30[b]	0.98, 0.98, 0.99
	600	1[a]	0.86, 0.78, 0.81	1.35 ± 0.03[b]	0.99	1.31 ± 0.05[b]	0.98	ND	ND	1.30[b]	0.98, 0.98, 0.99

All values are means ± standard deviations of data from two independent experiments. Different superscripts (a, b) in the same row indicate significant difference ($P < 0.05$). ND, not determined.

exhibited the highest resistance to pressure, as a minimum processing intensity of 500 MPa/9 min/50°C was required for its complete destruction. However, in the case of *L. innocua*, no detectable levels were observed after treatment at 500 MPa/15 min/40°C and 600 MPa/12–15 min/40°C. In contrast, *E. coli* was completely destroyed at 600 MPa/15 min/40°C. At 50°C, neither organism was detected for any of the pressure-holding time conditions. Enhancement of pathogen viability loss with increases in pressure and temperature has been reported for multiple products, including broth (Alpas et al., 2000), milk (Gao and Jiang, 2005), juices (Van Opstal et al., 2003; Bayindirli et al., 2006), and ham (Tassou et al., 2008). The inactivation rates achieved in these studies varied due to the differences in treatment conditions, bacterial strain, test substrate, and enumeration medium.

Model Fitting for the Isobaric Period

Visual inspection of the isothermal–isobaric inactivation kinetic curves of the studied pathogens suggested that the curves were non-linear ($\beta \neq 1$) in most cases (**Figures 1**, **2**). Therefore, fitting a straight line to the data points was deemed inappropriate as it would result in considerable errors. Non-linearity in the survival curve might arise from the adaptability of bacterial subpopulations, with variable resistance to the applied stress, which also depends on the surrounding conditions (Bevilacqua et al., 2015).

At all the *P*–*T* combinations, the logarithmic survival curves showed the same trend, with downward concavity ($\beta' > 1$). The downward concavity suggested that the microbial cells became less resistant with increased pressure-hold periods, which might be due to the separation of the bacterial cells into clumps (Chakraborty et al., 2015). Based on the minimum SSE values obtained, the β values were set at 1.48, 1.4, and 1.3 for *E. coli*, *L. innocua*, and *S. aureus*, respectively, for the entire *P*–*T* domain used in the study. The β values were significantly different ($p < 0.05$) from β_l; however, there was no significant difference in comparison to β' (**Table 2**). Using fixed β values resulted in simpler models, and the reduction in the number of parameters is expected to have resulted in more reliable predictions.

TABLE 3 | Comparison of goodness-of-fit of linear and Weibull models for the survival curves of *E. coli*, *L. innocua* and *S. aureus* in black tiger shrimp.

Bacterial species	Temperature (°C)	Pressure (MPa)	The log-linear model			The fitted Weibull model			The simplified Weibull model		
			RMSE	A_f	B_f	RMSE	A_f	B_f	RMSE	A_f	B_f
E. coli	30	300	0.211	1.259	1.159	0.004	1.023	1.017	0.001	1.007	1.003
		400	0.062			0.007			0.002		
		500	0.081			0.009			0.002		
		600	0.141			0.008			0.005		
	40	300	0.231	1.233	1.168	0.010	1.021	0.998	0.008	1.013	1.005
		400	0.050			0.003			0.004		
		500	0.192			0.010			0.007		
		600	0.133			0.010			0.005		
L. innocua	30	300	0.092	1.259	1.209	0.002	1.011	0.979	0.002	1.008	0.999
		400	0.024			0.004			0.002		
		500	0.015			0.004			0.005		
		600	0.022			0.002			0.003		
	40	300	0.026	1.232	1.193	0.005	1.007	0.987	0.004	1.002	0.997
		400	0.145			0.030			0.017		
		500	0.056			0.007			0.008		
		600	0.043			0.003			0.003		
S. aureus	30	300	0.016	1.190	1.065	0.003	1.008	0.986	0.007	1.009	1.002
		400	0.073			0.004			0.006		
		500	0.122			0.001			0.001		
		600	0.091			0.002			0.004		
	40	300	0.073	1.174	1.146	0.001	1.002	0.980	0.002	0.998	0.991
		400	0.044			0.003			0.004		
		500	0.056			0.007			0.005		
		600	0.053			0.003			0.002		
	50	300	0.022	1.182	1.157	0.005	1.008	0.985	0.007	1.003	0.999
		400	0.097			0.030			0.011		
		500	0.110			0.007			0.008		

The comparison of the model parameters obtained for the log-linear and both Weibull models at different temperatures is presented in **Tables 2, 3**. The simplified Weibull model produced fits comparable to the original Weibull model. The goodness of fit of the models was compared by computing the adjusted R^2 and RMSE, along with the accuracy factor (A_f) and bias factor (B_f) (Ross, 1996), which are shown in Equations (8) and (9), respectively, in which N represents the number of k-values estimated.

$$A_f = 10^{\frac{\sum\left|Log\left(\frac{k_{predicted}}{k_{observed}}\right)\right|}{N}} \quad (8)$$

$$B_f = 10^{\frac{\sum Log\left(\frac{k_{predicted}}{k_{observed}}\right)}{N}} \quad (9)$$

Generally, higher R^2 values, smaller RMSE values, and A_f and B_f values that are closer to 1 indicate a better model fit. When β' was fixed at 1 for the entire P–T domain, the adjusted R^2 for all the microbial groups varied between 0.76 and 0.91. In contrast, the fitted Weibull curves each had an adjusted $R^2 \geq 0.94$ and very small RMSE values (the maximum, 0.03, was obtained for both *L. innocua* and *S. aureus*). However, the simplified Weibull models had adjusted R^2 and RMSE values ranging from 0.97 to 0.99 and 0.001 to 0.017, respectively.

The adequacy of the model fitting was investigated using residual plots (**Figure 3**) and calculating the correlation between the predicted and experimental values for the linear and non-linear models at the fixed β values (**Figure 4**). Residual plots indicate whether a model is fully appropriate for the data being analyzed. The residual plots strongly suggested that the linear regression function was not appropriate, as there was curvature

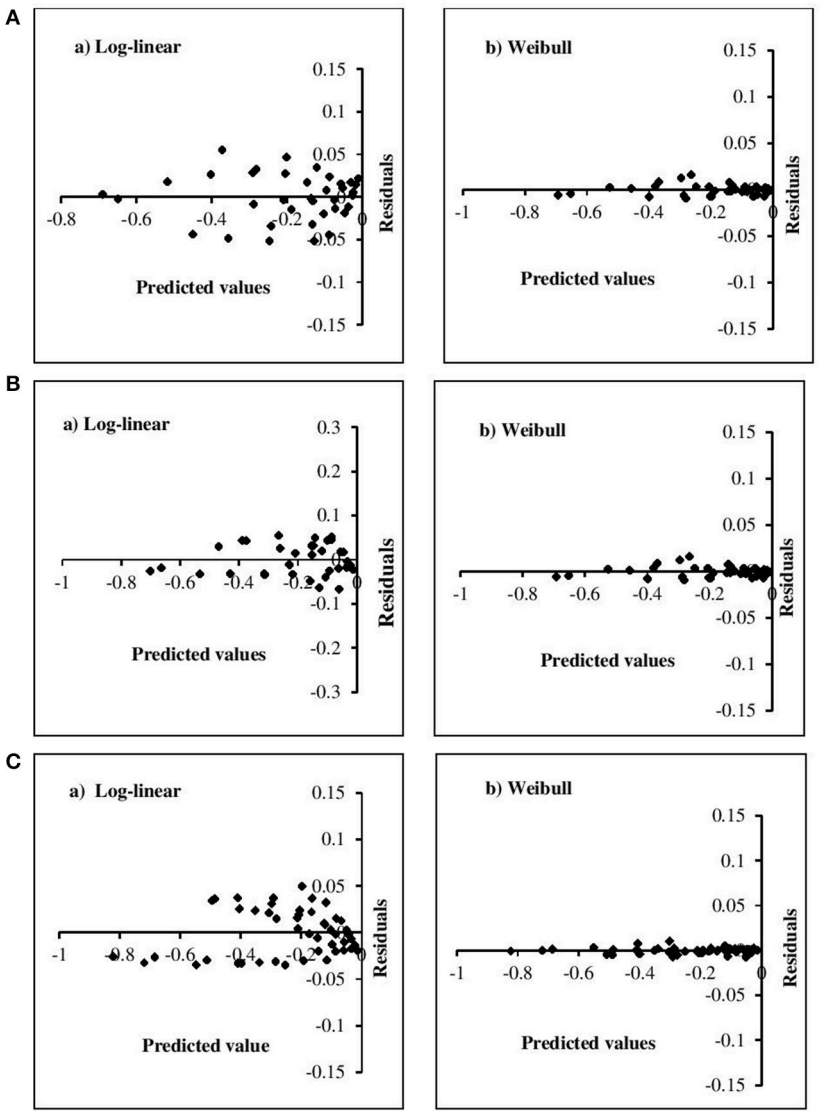


FIGURE 3 | Residual plots for the inactivation of **(A)** *E. coli*, **(B)** *L. innocua*, **(C)** *S. aureus* for log-linear and simplified Weibull model.

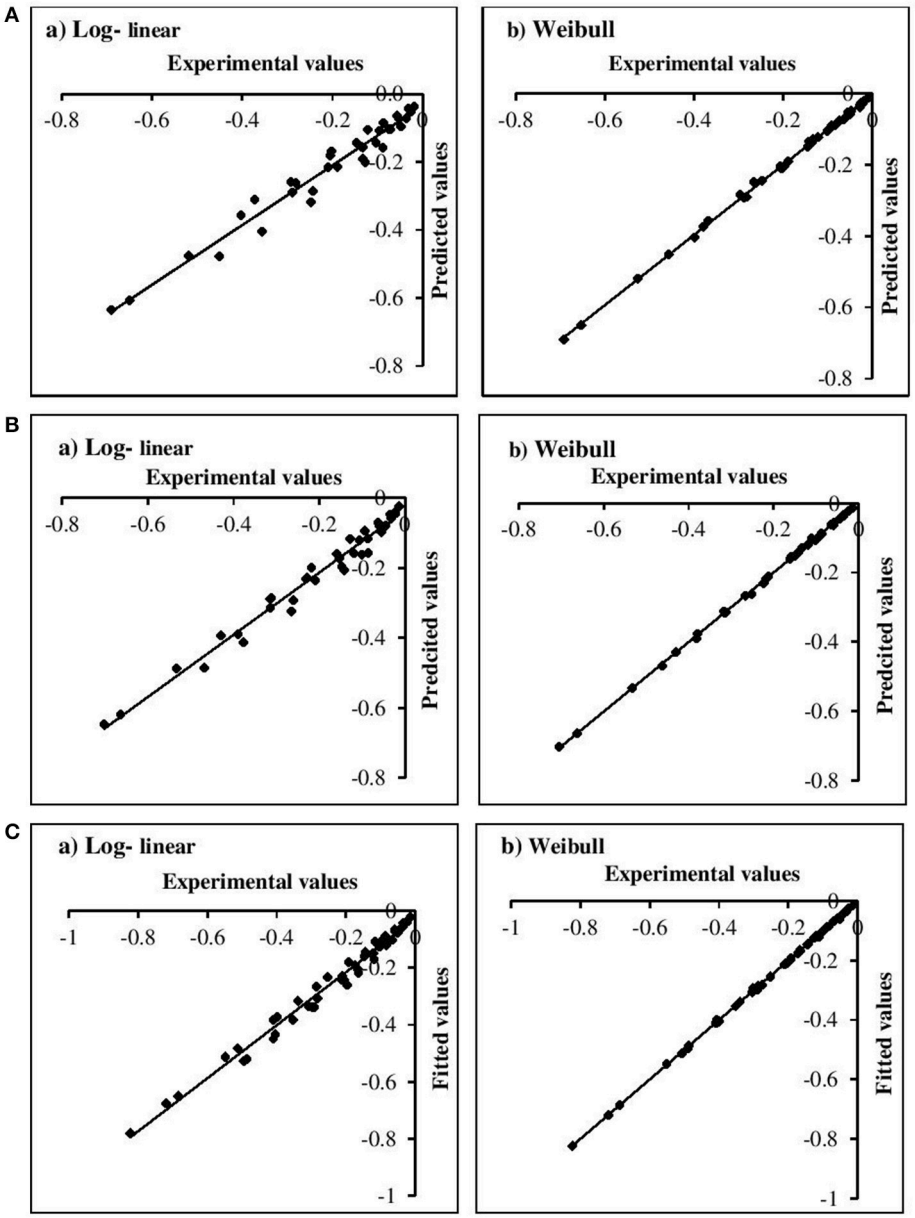


FIGURE 4 | Correlation between experimental and predicted values for **(A)** *E. coli*, **(B)** *L. innocua*, **(C)** *S. aureus* for log-linear and simplified Weibull model.

in the data and the residuals departed from 0 in a systematic fashion. However, for the non-linear models, the residuals were distributed randomly, falling within a horizontal band centered around 0. Moreover, the correlation between the predicted and experimental values also indicated a close relationship between these values for the non-linear models, which proved to be more appropriate than the log-linear models. In addition, the Weibull model has previously been successfully used to describe microbial kinetics for various HPP products (Van Opstal et al., 2003; Pilavtepe-Çelik et al., 2009; Serment-Moreno et al., 2016), with the inactivation curves demonstrating upward or downward concavity.

Effect of HPP on the Scale Parameter (δ) and Rate Constant (k)

The deviation of the refitted scale parameters (δ values) from the corresponding δ' values was also investigated (**Table 4**). At the fixed β values, the δ values for all three pathogens were almost comparable to the δ' values. However, at $\beta = 1$, the difference between the δ_l and δ values was significant ($P < 0.05$). A non-linear relationship between the δ values and the applied pressure and temperature was observed. Therefore, using first-order kinetics would lead to an underestimation or overestimation of the decimal reduction time.

TABLE 4 | Scale factors (δ', δ, and δ_l) obtained after model fitting at different pressure-temperature combinations for different pathogens in black tiger shrimp.

Bacterial species	Pressure (MPa)	δ values at different temperatures (min)								
		30°C			40°C			50°C		
		δ'	δ	δ_l	δ'	δ	δ_l	δ'	δ	δ_l
E. coli	300	57.46 ± 3.24^{aA}	55.15 ± 2.60^{aA}	141.94 ± 9.74^{bA}	32.08 ± 0.94^{cA}	35.29 ± 1.64^{cA}	57.58 ± 3.24^{aA}	ND	ND	ND
	400	45.92 ± 2.07^{aB}	46.29 ± 1.14^{aB}	83.38 ± 6.31^{bB}	23.28 ± 1.31^{cB}	23.58 ± 0.88^{cB}	31.57 ± 1.33^{dB}	ND	ND	ND
	500	34.19 ± 2.24^{aC}	35.25 ± 2.21^{aC}	58.33 ± 4.77^{bC}	15.35 ± 0.90^{cC}	15.54 ± 1.00^{cC}	18.90 ± 1.54^{dC}	ND	ND	ND
	600	27.50 ± 1.10^{aD}	28.12 ± 1.19^{aD}	42.10 ± 2.78^{bD}	12.10 ± 0.84^{cD}	12.35 ± 0.91^{cD}	14.85 ± 1.25^{cD}	ND	ND	ND
L. innocua	300	53.85 ± 3.21^{aA}	52.15 ± 4.40^{aA}	128.41 ± 7.14^{bA}	34.97 ± 1.22^{cA}	34.31 ± 1.89^{cA}	52.07 ± 3.04^{aA}	ND	ND	ND
	400	44.75 ± 3.07^{aB}	43.48 ± 3.22^{aB}	75.15 ± 3.24^{bB}	23.18 ± 1.14^{cB}	23.25 ± 2.04^{cB}	30.81 ± 2.71^{dB}	ND	ND	ND
	500	33.79 ± 2.00^{aC}	34.47 ± 2.34^{aC}	52.64 ± 2.48^{bC}	15.50 ± 1.07^{cC}	15.11 ± 1.11^{cC}	18.55 ± 0.94^{cC}	ND	ND	ND
	600	27.46 ± 1.13^{aD}	27.43 ± 1.16^{aD}	38.20 ± 1.04^{bD}	12.10 ± 0.83^{cD}	12.09 ± 1.00^{cD}	14.54 ± 0.76^{cD}	ND	ND	ND
S. aureus	300	61.46 ± 4.59^{aA}	63.72 ± 4.66^{aA}	139.27 ± 8.14^{bA}	36.72 ± 2.22^{cA}	37.31 ± 1.24^{cA}	47.50 ± 2.11^{dA}	21.45 ± 3.04^{eA}	20.08 ± 2.24^{eA}	29.05 ± 3.22^{fA}
	400	54.06 ± 0.04^{aB}	53.26 ± 2.26^{aB}	83.41 ± 5.02^{bB}	27.60 ± 1.06^{cB}	29.83 ± 1.55^{cB}	36.34 ± 2.94^{dB}	14.81 ± 2.54^{eB}	14.48 ± 1.32^{eB}	17.33 ± 1.10^{eB}
	500	38.76 ± 0.04^{aC}	39.43 ± 2.43^{aC}	56.47 ± 4.66^{bC}	17.47 ± 1.64^{cC}	17.86 ± 1.79^{cC}	19.76 ± 1.13^{cC}	7.82 ± 1.84^{dCA}	7.30 ± 0.89^{dC}	10.39 ± 1.07^{eC}
	600	29.48 ± 0.04^{aD}	30.44 ± 3.06^{aD}	40.37 ± 3.13^{bD}	14.01 ± 1.44^{cD}	15.54 ± 1.94^{cD}	16.88 ± 2.84^{cD}	ND	ND	ND

All values are means ± standard deviations of data from two independent experiments. Different superscripts (a, b, c, d, e, f) in the same row indicate significant difference ($P < 0.05$). Different superscripts (A, B, C, D) in the same column indicate significant difference ($P < 0.05$).

TABLE 5 | Inactivation rate constants, activation volume, and activation energy values for *E. coli*, *L. innocua* and *S. aureus* using Eq. (5–7).

Bacterial species	Pressure (MPa)	*k*-value ($\times 10^{-2}$ min^{-1})			E_a (kJ mol^{-1})
		30°C	40°C	50°C	
E. coli	300	3.19 ± 0.11^{aA}	4.98 ± 0.27^{bA}	ND	44.63 ± 2.11^{A}
	400	3.80 ± 0.47^{aAB}	7.46 ± 0.69^{bAB}	ND	47.87 ± 3.24^{A}
	500	4.99 ± 0.31^{aBC}	11.31 ± 1.37^{bBC}	ND	61.75 ± 4.46^{B}
	600	6.31 ± 0.66^{aC}	14.23 ± 1.89^{bC}	ND	64.71 ± 5.04^{B}
$-V_a$ (cm^3 mol^{-1})		5.65 ± 0.04^{a}	8.11 ± 0.04^{b}	ND	
L. innocua	300	3.48 ± 0.68^{aAB}	5.29 ± 0.41^{bA}	ND	46.92 ± 3.21^{aA}
	400	4.17 ± 0.39^{aAB}	7.81 ± 0.79^{bAB}	ND	50.46 ± 6.84^{aA}
	500	5.26 ± 0.56^{aBC}	12.01 ± 0.83^{bBC}	ND	63.26 ± 4.96^{aB}
	600	6.62 ± 0.62^{bC}	15.01 ± 1.75^{bC}	ND	65.89 ± 3.13^{aB}
$-V_a$ (cm^3 mol^{-1})		6.30 ± 0.24^{a}	9.11 ± 0.51^{b}	ND	
S. aureus	300	2.98 ± 0.16^{aA}	5.09 ± 0.59^{bA}	9.46 ± 1.13^{aA}	39.77 ± 1.93^{aC}
	400	3.57 ± 0.39^{aAB}	6.37 ± 0.48^{bA}	13.12 ± 1.42^{aA}	41.95 ± 2.76^{aAC}
	500	4.82 ± 0.87^{aBC}	10.64 ± 0.93^{bBC}	26.02 ± 2.18^{aB}	52.90 ± 4.45^{aD}
	600	6.15 ± 0.82^{aC}	12.23 ± 1.01^{bBC}	ND	63.12 ± 6.59^{aB}
$-V_a$ (cm^3 mol^{-1})		5.04 ± 0.87^{a}	7.81 ± 1.04^{b}	11.43 ± 2.16^{c}	

All values are means ± standard deviations of data from two independent experiments. Different superscripts (a, b, c) in the same row indicate significant difference ($P < 0.05$). Different superscripts (A, B, C, D) in the same column indicate significant difference ($P < 0.05$). ND not determined.

An increase in pressure resulted in a reduction in δ for all three pathogens. Similarly, at constant pressure, an increase in temperature led to a decrease in δ. The reduction in δ values with increasing pressure and temperature revealed that both parameters contributed additively or synergistically to the death of the bacteria. Increased pressure combined with increased temperature targets many factors in microbial cells rather a single factor, such as, membranes, ribosomes, nucleic acids, proteins (such as, enzymes), and so on. Hence, it is hard to separate the individual lethality of each processing parameter (Smelt et al., 2001).

The death rate constant (k, min^{-1}) for the three pathogens was dependent on both pressure and temperature. The pressure sensitivity of *E. coli, L. innocua*, and *S. aureus* were estimated using the activation volume, V_a. The V_a in microbial death kinetics represents the formation rates of an activated complex or the quasi-state equilibrium that is generally favored by compaction (Chakraborty et al., 2015). The decrease in V_a with increased temperature suggested an additive or synergistic effect of pressure and temperature on the microbial death rate. At all temperatures, the V_a values were negative, ranging between −5.65 and −8.11 cm^3 mol^{-1} for *E. coli,* −6.30 and −9.11 cm^3 mol^{-1} for *L. innocua*, and −5.04 and −11.43 cm^3 mol^{-1} for *S. aureus* (**Table 5**). This indicates that pressure has a lethal effect on the pathogens. In general, a large negative V_a value signifies a higher responsiveness to changes in pressure (Mussa et al., 1999). In the current study, the V_a values became more negative with increase in temperature, indicating that the pathogens became more responsive to pressure changes at increasing temperatures.

The temperature sensitivity of all three pathogens was estimated by computing the activation energy, E_a, at fixed pressures. The E_a values increased from 44.63 to 64.71 kJ mol^{-1}, 46.92 to 65.89 kJ mol^{-1}, and 39.77 to 63.12 kJ mol^{-1} for *E. coli*, *L. innocua*, and *S. aureus*, respectively, as the pressure was increased from 300 to 600 MPa (**Table 5**). This signified that the temperature sensitivity of the pathogens is higher at higher pressures. At lower pressures (300–400 MPa), the E_a values for the three pathogens were not significantly different ($P \geq 0.05$). Similarly, no significant difference in the E_a values ($P \geq 0.05$) was observed for pressure treatments of 500 and 600 MPa. The temperature sensitivity of the inactivation rate within a particular temperature domain varies considerably between species and even between bacterial strains. Previous studies have revealed that the temperature sensitivity (according to E_a values) of the inactivation rate of various microorganisms varied greatly at different pressures (Alpas et al., 1999; vanBoekel, 2002). The higher V_a and lower E_a values obtained in the present study might be due to the separation of the SPPE from the pressure-hold effect, which was not investigated in the earlier studies.

CONCLUSIONS

In this study, the effects of high pressure and temperature on the inactivation kinetics of *E. coli*, *L. innocua*, and *S. aureus* in black tiger shrimp were investigated. An additive or synergistic effect of pressure and temperature on the inactivation of the pathogens was noticed in the ranges of processing parameters studied. *S. aureus* was found to be the most baro-resistant species among the three pathogens. The minimum processing intensity required for complete destruction of *S. aureus* was 500 MPa/9 min/50°C. The study showed that the sensitivity of the microorganisms to the applied conditions was different during single-pulse and pressure-hold pressurization. The methodology used in this study could be used to develop a simplified Weibull models to

describe and predict non-linear survival curves of bacteria in other foods and of other microorganisms. Accurate prediction of survival curves at different pressures and temperatures would be beneficial to the food industry in terms of optimum selection of processing conditions to achieve the desired levels of bacterial inactivation, while also minimizing the production costs and maintaining a high degree of nutritional quality and good flavor and texture.

CHEMICALS USED IN THIS STUDY

Propylene glycol (PubChem CID: 1030).
Peptone (bacteriological) (PubChem CID: 9257).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Financial assistance was provided by the National Agricultural Innovation Project (NAIP) of the Indian Council of Agricultural Research (ICAR), which is supported by the World Bank (project code: NAIP/Comp-4/618001, grant number: F. No. NAIP/C4/C-30027/2008-09, dated 12/01/09).

REFERENCES

Ahn, J., Balasubramaniam, V. M., and Yousef, A. E. (2007). Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. *Int. J. Food Microbiol.* 113, 321–329. doi: 10.1016/j.ijfoodmicro.2006.08.012

Alpas, H., Kalchayanand, N., Bozoglu, F., and Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure sensitive strains of foodborne pathogens. *Int. J. Food Microbiol.* 60, 33–42. doi: 10.1016/S0168-1605(00)00324-X

Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C. P., and Ray, B. (1999). Variation inresistance to hydrostatic pressure among strains of food-borne pathogens. *Appl. Environ. Microbiol.* 65, 4248–4251.

APHA, (2001). "Enterobacteriaceae, coliforms, and *Escherichia coli* as quality and safety indicators *Staphylococcus aureus Listeria monocytogenes*," in *Compendium of Methods for the Microbiological Examination of Foods,* eds F. P. Downes and K. Ito (Washington, DC: American Public Health Association), 69–82, 105–115, 437–463.

Anang, D. M., Rusul, G., Bakar, J., and Ling, F. H. (2007). Effects of lactic acid and lauricidin on the survival of *Listeria monocytogenes*, *Salmonella Enteritidis* and *Escherichia coli* O157:H7 in chicken breast stored at 4°C. *Food Control* 18, 961–969. doi: 10.1016/j.foodcont.2006.05.015

Balasubramaniam, V. M., Farkas, D. F., and Turek, E. (2008). Preserving foods through high-pressure processing. *Food Technol.* 62, 32–38.

Basak, S., Ramaswamy, H. S., and Piette, J. P. G. (2002). High pressure destruction kinetics of *Leuconostocmesenteroides* and *Saccharomyces cerevisiae* in single strength and concentrated orange juice. *Innov. Food Sci. Emerg. Technol.* 3, 223–231. doi: 10.1016/S1466-8564(02)00008-5

Bayindirli, A., Alpas, H., Bozoglu, F., and Hizal, M. (2006). Efficiency of high pressure treatment on inactivation of pathogenic microorganisms and enzymes in apple, orange, apricot and sour cherry juices. *Food Control* 17, 52–58. doi: 10.1016/j.foodcont.2004.09.002

Bevilacqua, A., Speranza, B., Sinigaglia, M., and Corbo, M. R. (2015). A focus on the death kinetics in predictive microbiology: benefits and limits of the most important models and some tools dealing with their application in foods. *Foods* 4, 565–580. doi: 10.3390/foods4040565

Buzrul, S., and Alpas, H. (2004). Modeling the synergistic effect of high pressure and heat on inactivation kinetics of *Listeria innocua*: A preliminary study. *FEMS Microbiol. Lett.* 238, 29–36. doi: 10.1016/j.femsle.2004.07.011

Buzrul, S., Alpas, H., Largeteau, A., and Demazeau, G. (2008). Inactivation of *Escherichia coli* and *Listeria innocua* in kiwifruit and pineapple juices by high hydrostatic pressure. *Int. J. Food Microbiol.* 124, 275–278. doi: 10.1016/j.ijfoodmicro.2008.03.015

Cebrián, G., Ma-as, P., and Condón, S. (2016). Comparative resistance of bacterial foodborne pathogens to non-thermal technologies for food preservation. *Front. Microbiol.* 7:734. doi: 10.3389/fmicb.2016.00734

Chakraborty, S., Rao, P. S., and Mishra, H. N. (2014). Effect of pH on enzyme inactivation kinetics in high-pressure processed pineapple (*Ananascomosus*, L.) puree using response surface methodology. *Food Bioprocess. Technol.* 7, 3629–3645. doi: 10.1007/s11947-014-1380-0

Chakraborty, S., Rao, P. S., and Mishra, H. N. (2015). Empirical model based on Weibull distribution describing the destruction kinetics of natural microbiota in pineapple (*Ananascomosus*, L.) puree during high-pressure processing. *Int. J. Food Microbiol.* 211, 117–127. doi: 10.1016/j.ijfoodmicro.2015.06.017

Chen, H., and Hoover, D. G. (2003). Modeling the combined effect of high hydrostatic pressure and mild heat on the inactivation kinetics of *Listeria monocytogenes* Scott A in whole milk. *Innov. Food Sci. Emerg. Technol.* 4, 25–34. doi: 10.1016/S1466-8564(02)00083-8

Dogan, C., and Erkmen, O. (2004). High pressure inactivation kinetics of Listeria monocytogenesinactivation in broth, milk, and peach and orange juices. *J. Food Eng.* 62, 47–52. doi: 10.1016/S0260-8774(03)00170-5

Fernandez, A., Collado, J., Cunha, L. M., Ocio, M. J., and Mart nez, A. (2002). Empirical model building based on Weibull distribution to describe the joint effect of pH and temperature on the thermal resistance of *Bacillus cereus* in vegetable substrate.Int. *J. Food Microbiol.* 77, 147–153. doi: 10.1016/S0168-1605(02)00046-6

Gao, Y. L., and Jiang, H. H. (2005). Optimization of process conditions to inactivate *Bacillus subtilis* by high hydrostatic pressure and mild heat using response surface methodology. *Biochem. Eng. J.* 24, 43–48. doi: 10.1016/j.bej.2005.01.023

Garriga, M., Aymerich, M. T., Costa, S., Monfort, J. M., and Hugas, M. (2002). Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. *Food Microbiol.* 19, 509–518. doi: 10.1006/fmic.2002.0498

Guan, D., Chen, H., Ting, E. Y., and Hoover, D. G. (2006). Inactivation of *Staphylococcus aureus* and *Escherichia coli* O157:H7 under isothermal endpoint pressure conditions. *J. Food Eng.* 77, 620–627. doi: 10.1016/j.jfoodeng.2005.07.021

Hayakawa, I., Kanno, T., Tomita, M., and Figio, Y. (1994). Application of high pressure for spore inactivation and protein denaturation. *J. Food Sci.* 59, 159–163. doi: 10.1111/j.1365-2621.1994.tb06923.x

Hiremath, N. D., and Ramaswamy, H. S. (2011). High pressure destruction kinetics of spoilage and pathogenic microorganisms in mango juice. *J. Food Process. Preserv.* 36, 113–125. doi: 10.1111/j.1745-4549.2011.00559.x

Jofré, A., Aymerich, T., Grèbol, N., and Garriga, M. (2009). Efficiency of high hydrostatic pressure at 600 MPa against food-borne microorganisms by challenge tests on convenience meat products. *LWT Food Sci. Technol.* 42, 924–928. doi: 10.1016/j.lwt.2008.12.001

Kaur, B. P., and Rao, P. S. (2016). Process optimization for high pressure processing of black tiger shrimp (*Penaeus monodon*) using response surface methodology. *Food Sci. Technol. Int.* 23, 197–208. doi: 10.1177/1082013216673460

Mussa, D. M., Ramaswamy, H. S., and Smith, J. P. (1999). Ultra high pressure destruction kinetics of *Listeria monocytogenes* in pork. *J. Food Protoc.* 62, 165–170. doi: 10.4315/0362-028X-62.1.40

Nicolaides, L. (2009). "Crustacean shellfish," in *Microbiology Handbook Fish and Seafood*, ed R. Fernandes (Cambridge, UK: Leatherhead Publishing), 79–92.

Panagou, E. Z., Tassou, C. C., Manitsa, C., and Mallidis, G. (2007). Modelling the effect of high pressure on the inactivation kinetics of a

pressure-resistant strain of *Pediococcus damnosus* in phosphate buffer and gilt-head seabream (*Sparus aurata*). *J. Appl. Microbiol.* 102, 1499–1507. doi: 10.1111/j.1365-2672.2006.03201.x

Pavuluri, S. R., and Kaur, B. P. (2014). "High pressure inactivation kinetics of *Escherichia coli* in black tiger shrimp (*Penaeus monodon*)," in *International Conference on Biological, Civil and Environmental Engineering (BCEE-2014),* (Dubai), 167–169.

Pilavtepe-Çelik, M., Buzrul, S., Alpas, H., and Bozoglu, F. (2009). Development of a new mathematical model for inactivation of *Escherichia coli* O157:H7 and *Staphylococcus aureus* by high hydrostatic pressure in carrot juice and peptone water. *J. Food Eng.* 90, 388–394. doi: 10.1016/j.jfoodeng.2008.06.043

Pushparajan, N., and Soundarapandian, P. (2010). Recent farming of marine black tiger shrimp, Penaeus monodon (Fabricius) in South India. *Afr. J. Basic Appl. Sci.* 2, 33–36.

Rajan, S., Pandrangi, S., Balasubramaniam, V. M., and Yousef, A. E. (2006). Inactivation of *Bacillus stearothermophilus* spores in egg patties by pressure-assisted thermal processing. *LWT Food Sci. Technol.* 39, 844–851. doi: 10.1016/j.lwt.2005.06.008

Ramaswamy, H. S., Riahi, E., and Idziak, E. (2003). High pressure destruction kinetics of *E. coli* (29,055) in apple juice. *J. Food Sci.* 68, 1750–1756. doi: 10.1111/j.1365-2621.2003.tb12323.x

Ramaswamy, H. S., Zaman, S. U., and Smith, J. P. (2008). High pressure destruction kinetics of *Escherichia coli* (O157:H7) and *Listeria monocytogenes* (Scott A) in fish slurry. *J. Food Eng.* 87, 99–106. doi: 10.1016/j.jfoodeng.2007.11.017

Rendueles, E., Omar, M. K., Alvseike, O., Alonso-Calleja, C., Capita, R., and Prieto, M. (2011). Microbiological food safety assessment of high hydrostatic pressure processing: a review. *Food Sci. Technol.* 44, 1251–1260. doi: 10.1016/j.lwt.2010.11.001

Riahi, E., Ramaswamy, H. S., and Idziak, E. (2003). High pressure destruction kinetics of *Leuconostoc mesenteroides, Pichia membranafaciens* and *Zygosaccharomyces bailii* in apple juice. *Appl. Biotechnol Food Sci. Policy* 1, 1–8. doi: 10.1021/bp025667z

Ross, T. (1996). Indices for performance evaluation of predictive models in food microbiology. *J. Appl. Bacteriol.* 81, 501–508.

Ruiz-Espinosa, H., Amador-Espejo, G. G., Barcenas-Pozos, M. E., Angulo-Guerrero, J. O., Garcia, H., and Welti-Chanes, S. J. (2013). Multiple-pass high-pressure homogenization of milk for the development of pasteurization-like processing conditions. *Lett. Appl. Microbiol.* 56, 142–148. doi: 10.1111/lam.12027

Serment-Moreno, V., Barbosa-Ca'novas, G., Torres, J. A., and Welti-Chanes, J. (2014). High-pressure processing: kinetic models for microbial and enzyme inactivation. *Food Eng. Rev.* 6, 56–88. doi: 10.1007/s12393-014-9075-x

Serment-Moreno, V., Torres, J. A., Fuentes, C., Ríos-Alejandro, J. G., Barbosa-Ca'novas, G., and Welti-Chanes, J. (2016). Limitations of the log-logistic model for the analysis of sigmoidal microbial inactivation data for high-pressure processing (HPP). *Food Bioprocess. Technol.* 9, 904–916. doi: 10.1007/s11947-016-1677-2

Smelt, J. P., Hellemons, J. C., and Patterson, M. (2001). "Effects of high pressure on vegetative microorganism," in *Ultra High Pressure Treatments of Foods,* eds M. E. G. Hendrickx and D. Knorr (New York, NY: Kluwer Academic/Plenum), 55–76. doi: 10.1007/978-1-4615-0723-9_3

Smelt, J. P., Hellemons, J. C., Wouters, P. C., and Van, G. S. J. (2002). Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. *Int. J. Food Microbiol.* 78, 57–77. doi: 10.1016/S0168-1605(02)00242-8

Smelt, J. P. P. M. (1998). Recent advances in the microbiology of high pressure processing. *Trends Food Sci. Technol.* 9, 152–158. doi: 10.1016/S0924-2244(98)00030-2

Tassou, C. C., Panagou, E. Z., Samaras, F. J., Galiatsatou, P., and Mallidis, C. G. (2008). Temperature-assisted high hydrostatic pressure inactivation of *Staphylococcus aureus* in a ham model system: evaluation in selective and nonselective medium. *J. Appl. Microbiol.* 104, 1764–1773. doi: 10.1111/j.1365-2672.2007.03698.x

vanBoekel, M. A. J. S. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. Food Microbiol.* 749, 139–159. doi: 10.1016/S0168-1605(01)00742-5

Van Opstal, I., Vanmuysen, S. C. M., and Michiels, C. W. (2003). High sucrose concentration protects *E. coli* against high pressure inactivation but not against high pressure sensitization to the lactoperoxidase system. *Int. J. Food Microbiol.* 88, 1–9. doi: 10.1016/S0168-1605(03)00070-9

Wouters, P., Glaasker, E., and Smelt, J. P. P. M. (1998). Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* 64, 509–514.

Yuste, J., Capellas, M., Fung, D. Y. C., and Mor-Mur, M. (2004). Inactivation and sublethal injury of foodborne pathogens by high pressure processing: evaluation with conventional media and thin agar layer method. *Food Res. Int.* 37, 861–866. doi: 10.1016/j.foodres.2004.05.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Kaur and Rao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 July 2017
doi: 10.3389/fmicb.2017.01366

Colonisation of Meat by *Escherichia coli* O157:H7: Investigating Bacterial Tropism with Respect to the Different Types of Skeletal Muscles, Subtypes of Myofibres, and Postmortem Time

*Caroline Chagnot[1,2], Annie Venien[2], Sandra Renier[1], Nelly Caccia[1], Régine Talon[1], Thierry Astruc[2] and Mickaël Desvaux[1]**

[1] UMR454 MEDiS, INRA, Université Clermont Auvergne, Clermont-Ferrand, France, [2] INRA, UR370 Qualité des Produits Animaux, Saint-Genès Champanelle, France

Escherichia coli O157:H7 is an enterohaemorrhagic *E. coli* (EHEC) responsible for serious diseases, especially pediatric, and of great concern for the meat industry. Meat contamination by EHEC occurs at slaughtering, especially at dehiding stage, where bacteria can be transferred from hides to carcasses. The skeletal muscle tissues comprise four major types of myofibres, which differ in their contraction velocity and metabolism. Myofibres are surrounded by the extracellular matrix (ECM). Adhesion of *E. coli* O157:H7 to meat was investigated considering well-defined types of skeletal muscle and their constituent myofibres as well as postmortem changes in muscle, using fluorescence microscopy and immunohistochemical analyses. By analysing the adhesion of *E. coli* O157:H7 to model oxidative (soleus) and glycolytic [extensor digitorum longus (EDL)] skeletal muscles, it first appeared that differential adhesion occurred at the surface of these extreme skeletal muscle types. At a cellular level, bacterial adhesion appeared to occur essentially at the ECM. Considering the different constituent myofibres of types I, IIA, IIX and IIB, no significant differences were observed for adhering bacteria. However, bacterial adhesion to the ECM was significantly influenced by postmortem structural modifications of muscle tissues. By providing information on spatial localisation of *E. coli* O157:H7 on meat, this investigation clearly demonstrated their ability to adhere to skeletal muscle, especially at the ECM, which consequently resulted in their heterogeneous distribution in meat. As discussed, these new findings should help in reassessing and mitigating the risk of contamination of meat, the food chain and ultimately human infection by EHEC.

Keywords: bacterial adhesion, foodborne pathogens, extracellular matrix, meat products, food contamination

OPEN ACCESS

Edited by:
Pierina Visciano, University of Teramo, Italy

Reviewed by:
Catherine Maeve Burgess, Teagasc - The Irish Agriculture and Food Development Authority, Ireland
Alejandro Castillo, Texas A&M University, United States

****Correspondence:***
Mickaël Desvaux
mickael.desvaux@inra.fr

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *10 April 2017*
Accepted: *05 July 2017*
Published: *25 July 2017*

Citation:
Chagnot C, Venien A, Renier S, Caccia N, Talon R, Astruc T and Desvaux M (2017) Colonisation of Meat by Escherichia coli O157:H7: Investigating Bacterial Tropism with Respect to the Different Types of Skeletal Muscles, Subtypes of Myofibres, and Postmortem Time. Front. Microbiol. 8:1366. doi: 10.3389/fmicb.2017.01366

INTRODUCTION

In the meat industry, the significance of enterohemorrhagic *Escherichia coli* (EHEC) as a serious public health problem is undoubtedly recognised. EHEC are foodborne pathogens that produce Shiga toxins, which are responsible for serious diseases such as HUS (haemolytic uraemic syndrome) or TTP (thrombotic thrombocytopenic purpura) (Karch et al., 2005; Tarr et al., 2008; Gould et al., 2009). Worldwide, foodborne illness associated with the consumption of meat

products contaminated by EHEC has been reported and *E. coli* O157:H7 is the most frequently associated serotype. Ruminants (mainly cattle) are the natural reservoir for these bacteria and food infection occurs following direct or indirect faecal contamination (Chase-Topping et al., 2008). While the muscle masses of healthy animals are sterile (Gill, 1979), bacterial contamination of meat can occur at slaughtering, mainly upon transfer to carcasses at the dehiding stage and even when good practices are strictly respected, *i.e.* excluding evisceration accident (Giaouris et al., 2014). In fact, the respect of good hygienic practices in the beef industry during slaughtering reduces contamination of carcasses, but cannot guarantee the absence of *E. coli* O157:H7 from meat (Rhoades et al., 2009). Thus, the survival of bacteria in meat could also depend upon their ability to adhere to meat surfaces.

Whatever the mammalian species, the skeletal muscular tissue is highly structured into three main organisational levels, with the muscle cells (also called fibres) packed into fascicles, themselves regrouped to form a skeletal muscle (Schiaffino and Reggiani, 2011; Frontera and Ochala, 2015). While two extreme types of skeletal muscles can be considered, namely the white glycolytic and red oxidative muscles, a range of in-between skeletal muscles are found. These different skeletal muscles contain in the four main types of muscle fibres, namely the types I, II-A, II-X, and II-B, which are present in different proportions depending on the skeletal muscle function (Pette and Staron, 1990; Chagnot et al., 2015b). The various muscle fibres differ in their contraction velocity, i.e., slow twitch (types I) or fast twitch (types II) and in metabolism, i.e., oxidative (types I and II-A) or glycolytic (types II-X and II-B). Structural differences are also observed, such as variation of fibre cross-sectional area, depending on fibre specialisation and/or muscle type (Realini et al., 2013). Additionally, muscle fibres are surrounded by an extracellular matrix (ECM), which is divided into three types depending on its location with respect to the muscular organisational levels, namely (i) the endomysium, the deepest layer in contact with the muscle fibres, (ii) the perimysium surrounding the fascicles, and (iii) the epimysium, the outermost layer at the surface of the muscle. The three types of ECM are highly similar and are mainly composed of fibrillar collagens of types I and III. They differ slightly in the proportion of the other ECM proteins such as laminin or elastin (Gillies and Lieber, 2011; Nishimura, 2015).

According to the European Community (EC) regulation specifying the hygiene rules for foodstuffs (no. 853/2004), animal slaughter and cutting of carcasses into quarters can be carried out at room temperature before the meat is stored at low temperature. Prior to consumption, meat is deliberately stored for several days in these conditions to improve its texture and flavour. During this period, the muscles undergo postmortem modifications following a succession of significant metabolic, physical, structural and biochemical transformations resulting in meat (Bendall, 1973; Greaser, 1986). For instance, intracellular water diffuses into the extracellular space, which leads to a decrease in cell size and an increase in the intercellular spaces (Offer and Knight, 1988; Guignot et al., 1993; Huff Lonergan et al., 2010), whereas the action of endogenous proteolytic enzymes leads to the fragmentation of the muscle fibres (Taylor et al., 1995; Taylor and Koohmaraie, 1998; Waardenberg et al., 2008) and to the disintegration of the collagen fibres (Bailey and Light, 1989; Nishimura, 2010). The rate of these postmortem changes depends on the animal species, and mostly on the metabolic and contractile type of the muscle considered (Ouali, 1990; Lefaucheur, 2010).

While *E. coli* O157:H7 has the ability to attach to meat (Cabedo et al., 1997; Li and McLandsborough, 1999; Rivas et al., 2006; Chen et al., 2007), it also adheres specifically and non-specifically to some ECM fibrillar proteins (Chagnot et al., 2013a) such as some collagens (Medina, 2001; Auty et al., 2005; Chagnot et al., 2013a). Initial bacterial adhesion was shown to be strongly influenced by the bacterial physiology and environmental conditions such the temperature (Chagnot et al., 2013a). Considering that the interaction of *E. coli* O157:H7 with skeletal muscle tissue remains poorly characterised, we investigated the adhesion of *E. coli* O157:H7 to skeletal muscle fibres, considering the influence of metabolic and contractile fibre types as well as the postmortem muscle changes.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

For the purpose of this study, the detoxified strain (*stx*$^{-}$) of *E. coli* O157:H7 EDL933 was used (Perna et al., 2001), i.e., *E. coli* O157:H7 CM454 (Gobert et al., 2007; Chagnot et al., 2014). For direct visualisation, the strain was transformed with the vector pSaRe-Red1 expressing the fluorescent protein mRuby and carrying the erythromycine resistant gene (300 μg mL^{-1} final concentration) (Desvaux et al., 2005; Kredel et al., 2009; Chagnot et al., 2013a). Bacteria were cultured in two different growth media, i.e., (i) DMEM (Dulbecco's modified eagle medium, Gibco) and (ii) LB (lysogeny broth) adjusted with NaOH (0.1 M) to reach pH 7 at the time of sampling (Guedon et al., 2000; Chagnot et al., 2015a). A preculture was set up from one bacterial colony grown in DMEM or LB at 39°C (bovine temperature) in an orbital shaker at low speed (70 rpm) till the stationary phase.

For the adhesion tests, the preculture was diluted 1:100 and grown as described above. Sampling was performed during the exponential growth phase at an $OD_{600\ nm}$ of 0.5 (i.e., about 10^8 CFU ml^{-1}). Chloramphenicol (170 μg ml^{-1} final concentration) was added and mixed gently to prevent *de novo* protein synthesis and growth. Therefore, no growth is occurring during the time of contact of bacterial cells with muscle in the adhesion assay and only molecular determinants expressed during anterior growth conditions are involved in the bacterial adhesion to meat. Minimal mechanical treatment was used to preserve cell surface supramolecular structures potentially involved in adhesion (Chagnot et al., 2014).

Muscle Sampling and Maturation Processing

To control slaughter conditions, postmortem kinetics and the possibility of extracting the entire muscles without any lesion, rat muscles were used as well-recognised physiological models

(Pullen, 1977; Rogers and Evans, 1993; Lagord et al., 1998; Morey-Holton and Globus, 2002; Soukup et al., 2002; Schiaffino and Reggiani, 2011; Warren et al., 2014; Naili et al., 2016). Two muscle models were chosen based on their very divergent metabolic and contraction velocity features, namely the (i) soleus containing only oxidative fibres (types I and II-A), and the (ii) extensor digitorum Longus (EDL) containing essentially glycolytic fibres, i.e., II-X (24%), II-B (46%) (and, in lower proportion, fibres of types I (4%) and II-A (18%) (Chagnot et al., 2015b).

Rats from Janvier (St-Berthevin, France) were housed in the INRA animal facility until sacrifice ("Installation Expérimentale de Nutrition, Unité de Nutrition Humaine, INRA Auvergne-Rhône-Alpes, Site de Theix"; Agreement no. C63345.14). To respect animal welfare, the rats were euthanised under anaesthesia, without pain nor suffering, in strict accordance with the recommendations and with validation by the Regional Ethics Committee ("Comité d'Ethique pour l'Experimentation Animale Auvergne"; no. C2E2A-02), which takes into account the rule of the 3Rs (replacement, reduction, refinement). After an anaesthesia by isoflurane gas, Wistar male rats (5 months old, weighing about 500 g weights) were sacrificed by decapitation. Immediately after the slaughter, the lower limbs were carved and dissected under sterile conditions. From tendon to tendon, EDL and soleus muscles were sampled without lesion. Muscles were suspended in a sterile moisture chamber maintained at 20°C to prevent contamination from microorganisms and prevent muscle alteration by drying. The artificial tension of muscle imposed by the animal carcass was mimicked by using lead ballast (1.5 g) at the bottom muscle tendon. Based on previous studies (Chagnot et al., 2015a,b), two different postmortem times were considered, i.e., $t_{0\ h}$ (10 min after slaughtering as the minimum required for the dissection and extraction of muscles) and $t_{24\ h}$ (24 h after slaughtering).

Bacteria Adhesion to Whole Muscles and Muscle Cross-Sections

For bacterial adhesion to whole muscle, EDL and soleus muscles of at least two rats (i.e., four muscles of each type in total) were incubated statically for 30 min in 15 mL bacterial solution at 25°C. Muscles were washed by dipping three times in milliQ water to remove unattached cells. Muscles were then deposited in a sterile Petri dish adapted for inverted fluorescence microscopy (ibidi). Muscles were either incubated in LB bacterial growth conditions or in DMEM bacteria growth conditions.

The surface of muscles was observed in the fluorescence mode using an inverted microscope (Olympus IMT-2) coupled to a cooled CCD camera (Olympus DP30BW) optimised for high sensitivity fluorescence work and driven by the Cell A software v3.2 (Olympus France SAS, Rungis, France). The fluorescence light source was a mercury short arc lamp (HBO103W/2, OSRAM, Augsburg, Germany). Fluorescence acquisition was fitted with a cyanine-3 cube. Images were processed with the public domain image processing and analysis program ImageJ v1.43 (Schneider et al., 2012). The pixels corresponding to bacteria were extracted by thresholding segmentation of the light grey levels and the proportion of bacteria-overed surface was calculated.

To investigate bacterial adhesion to muscle cells, muscle sections of both right and left EDL and soleus muscles of rats were used. At $t_{0\ h}$ and $t_{24\ h}$, parts of EDL and soleus muscles were positioned on a cork plate with embedding medium (Tissue-Tek) and frozen to −160°C in isopentane with liquid nitrogen (−196°C). Based on a previously described protocol (Chagnot et al., 2015a,b), serial cross-sections (10 μm thick) were obtained using a cryostat (Microm, HM 560) and collected on glass slides. The sections were stored at −20°C under vacuum until use.

Muscle cross-sections were stained with picrosirius red, which revealed the intramuscular ECM (endomysium, perimysium and epimysium) in red and muscle fibres in yellow (Liu et al., 1994). Fibre typing was performed as previously described (Chagnot et al., 2015b). Briefly, mouse monoclonal antibodies specific to MyHC isoforms BA-D5, SC-71, BFF3 (AGRO-BIO France) were used in three different serial muscle cross sections to identify slow and fast myosin heavy chains isoforms (MyHC). The myofibre response to the different antibodies enabled us to identify the subtypes I, IIA, IIB and hybrid IIX-IIB; the types MyHC-IIX corresponded to the remaining unlabelled cells. The different primary MyHC antibodies were stained with Alexa Fluor 488-labelled goat anti-mouse IgG secondary antibody (A11001, Invitrogen). The ECM proteins, laminin, surrounding the muscle fibre, were stained using anti-laminin primary polyclonal antibody (L9393 Sigma) and a cyanine Cy3-labelled secondary antibody (111-165-008, Jackson). The cross-sections were incubated with primary antibodies. After washing, both labelled secondary antibodies were incubated to reveal the primary antibody binding. Controls were performed without primary antibody to validate the results.

Observations and image acquisitions were performed using a photonic microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software. For histological analysis, picrosirius red-stained sections were observed and images were acquired in bright field mode, whereas immunohistofluorescence images were acquired in the fluorescence mode (Cyanine 3: 550/570 nm; Alexa Fluor 488: 495/519 nm). Images of immunohistochemistry processing were recorded with the program FibTypFluo in Visilog v5.4 (Noesis, France) software to create a virtual image of the different fibre types composing the muscle section (Meunier et al., 2010).

On a histological section without any staining, the perimeter of a 1.5 cm^2 square was traced with hydrophobic gel (PAP pen for immunostaining, Sigma), to maintain the bacterial solution on the slide. Then, 500 μL of bacterial suspension was deposited with wide bore pipette tips. Muscle sections were incubated statically in a humid chamber at 25°C for 30 min. After incubation, bacterial suspension was removed by pipetting and washed by dipping two times, in milliQ water, to remove unattached cells. The experiment was repeated three times on serial sections of both muscles for each growth condition (DMEM or LB) and postmortem time ($t_{0\ h}$ and $t_{24\ h}$).

At least six fields of view (magnification X2000) were analysed on each muscle or muscle cross-section. On each field of view,

two images were systematically recorded in fluorescent and in bright field mode, respectively. Fluorescent recording allowed recording the adherent bacteria. For muscle cross-section, bright filed recording showed the muscle fibres previously identified by their fibre types using immunohistofluorescence on the other serial sections. Then, images were processed with Visilog v5.4 (Noesis, France) or ImageJ v1.43 (Schneider et al., 2012) software. The pixels corresponding to bacteria were extracted by thresholding segmentation of the light grey levels and the muscle fibres image (bright field mode) and bacteria (artificial grey scale images) were superimposed. The relative area of bacteria was assessed by quantifying the number of these pixels respective to the total number of pixels corresponding to the cell. With duplicate of serial cross-sections, ten cells of the same fibre type were compared for different conditions with respect to the postmortem time and growth medium.

Statistical Analysis

Data were analysed with XLSTAT software 2010 (Microsoft Office, Redmond, United States) using one-way analysis of variance (ANOVA) and the Student-Newman–Keuls test, or Student's *t*-test for date comparison of only two different populations. Given the large number of detected events (at least several 100s of pixels) used to generate the percentages of surface coverage, the binomial distribution can be approximated by a normal distribution and a classical ANOVA can be used. Results are expressed as mean ± standard error of the mean (SEM). Differences were considered as significant ($^{*}p < 0.05$), very significant ($^{**}p < 0.01$), highly significant ($^{***}p < 0.001$), or very highly significant ($^{****}p < 0.0001$).

RESULTS

Differential *E. coli* O157:H7 Adhesion Occurs at the Surface of Glycolytic and Oxidative Skeletal Muscle Types and Depends on Anterior Growth Conditions

Two extreme types of skeletal muscles were considered, i.e., glycolytic and oxidative muscles, in studying the adhesion ability of *E. coli* O157:H7 to the surface of skeletal muscles (fully extracted and without any lesion). As well-recognised physiological models with such distinct metabolic and contractile features, the rat EDL and soleus skeletal muscles, respectively, were used. Regarding the bacterial cells, *E. coli* O157:H7 CM454 were placed in physiological conditions where they exhibited differential attachment features, namely the induction of specific and non-specific adhesion to the ECM when grown in LB and DMEM, respectively (Chagnot et al., 2013a). These adhesion trends were typically observed *ex vivo* using bovine gastro-intestinal tract contents, and thus are likely displayed by some bacteria after shedding.

In LB, the surface of the muscles was covered unevenly by *E. coli* O157:H7 CM454, which appeared to adhere to specific structures of the epimysium by following the outline of the muscle fibres, which was especially apparent over the EDL (**Figure 1A**). In marked contrast, these muscles were covered all over by *E. coli* O157:H7 CM454 grown in DMEM, forming packs of bacterial cells in some areas (**Figure 1A**). As a result, bacterial surface coverage was very significantly higher at the surface of EDL or soleus when *E. coli* O157:H7 CM454 was previously grown in DMEM compared with LB (**Figure 1B**); with bacteria grown in LB, bacterial surface coverages on EDL were 0.3 and 0.4% on soleus, but reached 0.6 and 1.0% respectively, when *E. coli* O157:H7 CM454 was grown in DMEM. Besides, bacterial surface coverage appeared significantly higher at the surface of soleus than EDL whenever *E. coli* O157:H7 CM454 was grown in LB or DMEM (**Figure 1B**). Thus, it clearly appeared that some kind of tropism occurred for bacterial adhesion at the surface of these two extreme types of skeletal muscles, which could be related to variations in the composition and/or proportion of some of their components.

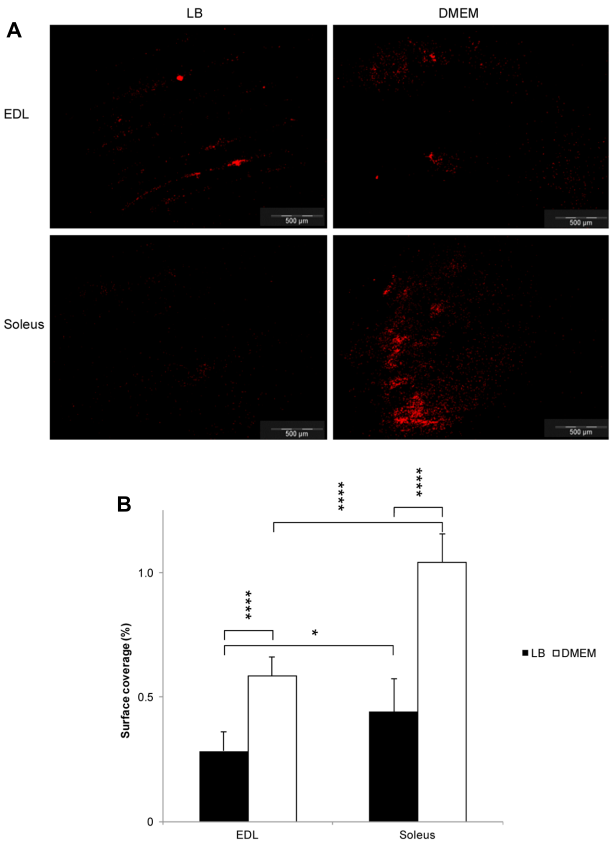


FIGURE 1 | Adhesion of *Escherichia coli* O157:H7 to the surface of glycolytic and oxidative muscles as observed by epifluorescence microscopy. **(A)** Observation in epifluorescence microscopy of *E. coli* O157:H7 CM454 pSaRe-Red1 first grown in LB or DMEM and adhering to the surface of EDL (glycolytic) and soleus (oxidative) muscles at pH 7 and 25°C. Fluorescent bacteria were stained virtually in red colour. **(B)** The level of adherent bacteria assessed using fluorescence intensity was expressed as the percentage of muscle surface covered by fluorescent bacterial cells.

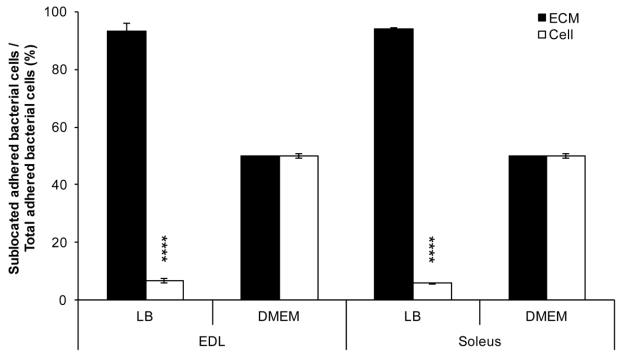


FIGURE 2 | Proportion of *E. coli* O157:H7 adhering to EDL and soleus muscles as a function of their sublocation in the muscle tissue. Bacterial cells at the ECM and on the muscle cells were discriminated. The proportion of each of those bacteria first grown in LB or DMEM was calculated and based on bacterial cell count from epifluorescence microscopic observations on EDL or soleus cross-sections (**Figure 3**).

E. coli O157:H7 Mainly Adheres to the ECM of Muscle Cells, But Similarly to the Different Myofibre Types

Considering that the EDL and soleus essentially vary in the composition and proportion of myofibres of types I, IIA, IIX, and IIB, differential bacterial adhesion to these muscle cells was further investigated. For this purpose, serial cross-sections of EDL and soleus were performed. It first appeared whenever grown in LB or DMEM, *E. coli* O157:H7 CM454 adhered to muscle cells cross-sections, but unevenly since bacterial cells essentially localised at the muscle cells periphery (**Figures 2**, **3**). With bacterial cells first grown in LB, more than 90% of adherent bacteria were essentially localised at the ECM of EDL and soleus muscle cells, respectively (**Figure 2**). In DMEM, half of the bacterial cells were found at the ECM (**Figure 2**) and the other half on the muscle fibres, especially on the edge of the cell (**Figure 3**).

Immunohistochemical analysis confirmed that, as a fast-twitch glycolytic skeletal muscle, the EDL contains the four main types of myofibres (I, IIA, IIX, IIX) but with the glycolytic fibres of types IIX and IIB in a much higher proportion, whereas, as an oxidative skeletal muscle, the soleus muscle was exclusively composed of oxidative fibres of types I and IIA (**Figure 3A**). Following myofibre typing, the cross-section muscle fibres present in EDL and soleus were further correlated with the adherent bacterial cells (**Figure 3B**). As expected from the results here above, it first appeared that the surface coverages of EDL or soleus muscle cross-sections was very significantly higher with *E. coli* O157:H7 CM454 grown in DMEM than in LB (**Figure 3C**); with respect to the different myofibre types and when grown in LB, bacterial surface coverages were about 0.3% on EDL and 0.4% on soleus, but reached 2.9 and 8.7% respectively when *E. coli* O157:H7 CM454 was grown in DMEM. However, we observed no difference in the bacterial surface coverages of type I, IIA, IIX, and IIB myofibres present in EDL or type I and IIA myofibres present in soleus (**Figures 3B,C**). Nonetheless, the surface coverage was still significantly higher for soleus than for EDL. This indicates that the different types of myofibres, with respect to their metabolic and/or contractile properties, did not influence the amount *E. coli* O157:H7 CM454 that adhered to meat. Altogether, differential *E. coli* O157:H7 tropism for oxidative over glycolytic skeletal muscle could not be explained by the differential proportion of contractile (types I vs. II) or metabolic (I and IIA vs. IIX and IIB) types of fibres.

Postmortem Changes in Muscle Influences *E. coli* O157:H7 Adhesion to Muscle Fibres

Considering that meat corresponds to the maturation of skeletal muscles after slaughtering, investigation of *E. coli* O157:H7 CM454 adhesion was further performed at two different postmortem times, namely t_{0h} and t_{24h}. As previously observed above (**Figure 3**) and whatever the postmortem time, bacterial cells mainly co-localised at the ECM (**Figures 4A,B**). For *E. coli* O157:H7 CM454 first grown in LB, the surface coverages of EDL and soleus cross-sections were quite low and did not exceed 0.5 and 0.3%, respectively and were significantly lower at $t_{24\ h}$ than at $t_{0\ h}$ (**Figure 4C**). When grown in DMEM, however, the surface coverage was similar at both postmortem times but the surface coverage was again very significantly higher compared with *E. coli* O157:H7 CM454 grown in LB and higher for soleus cross-sections compared with EDL (**Figure 4C**); when *E. coli* O157:H7 CM454 was grown in DMEM, the bacterial surface coverage was about 2.5% on EDL at $t_{0\ h}$ and t_{24h}, but reached 4.5% on soleus. Interestingly, both the EDL and soleus muscle tissues showed some structural changes at $t_{24\ h}$ postmortem, namely the size of intercellular spaces increased and the meshing of the ECM became loosen (**Figure 4B**).

DISCUSSION

Using the two well-characterised and extreme models of rat glycolytic and oxidative skeletal muscles, namely EDL and soleus, respectively, we show here that differential adhesion of *E. coli* O157:H7 occurred at the surface of skeletal muscles. The localisation trend observed at the surface of these whole skeletal muscles could be explained by variation in the organisation or thickness of the epimysium. Indeed, it is well known that the epimysium is a highly nonlinear ECM, which exhibits longitudinal periodicities (Gao et al., 2008; Gillies and Lieber, 2011). With respect to bovine muscle, and knowing that other skeletal muscles are in-between these two extremes, the white glycolytic EDL muscle (containing a high percentage of glycolytic myofibres but also some oxidative myofibres) is close to m. cutaneus trunci, biceps brachii, or latissimus dorsi, whereas the red oxidative soleus muscle (exclusively composed of oxidative myofibres) is close to the soleus and infraspinatus (Totland and Kryvi, 1991; Kirchofer et al., 2002). Rat EDL and soleus muscles are relevant models for simulating meat in lab experiments: (i) their muscular physiology is well-characterised, (ii) they show the same postmortem biochemical and structural changes as for farm animals, (iii) their maturation follows a faster evolution

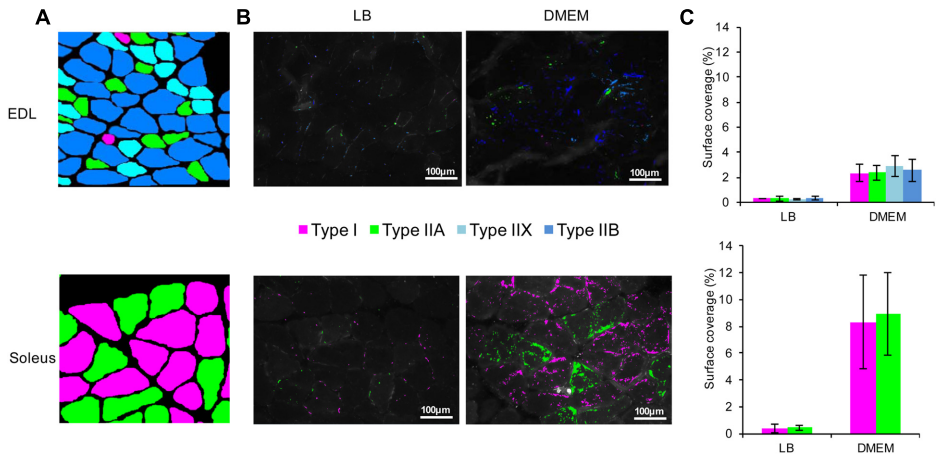


FIGURE 3 | Adhesion of *E. coli* O157:H7 to the different types of myofibres constituent of the EDL and soleus muscles. **(A)** Each myofibre type was identified according to its MyHC isoform as described in the Materials and Methods. From all immunohistofluorescence images and for EDL and soleus, a virtual image was reconstructed showing the precise fibre type for each muscle cell. **(B)** On serial cross-sections of the EDL and soleus, the adhered *E. coli* O157:H7 CM454 pSaRe-Red1 first grown in LB or DMEM was visualised by epifluorescence microscopy. Fluorescent bacteria were stained virtually in colours corresponding to the myofibre type. **(C)** The level of adherent bacteria was assessed by the fluorescence intensity and expressed as the percentage of muscle cross-section surface covered by fluorescent bacterial cells.

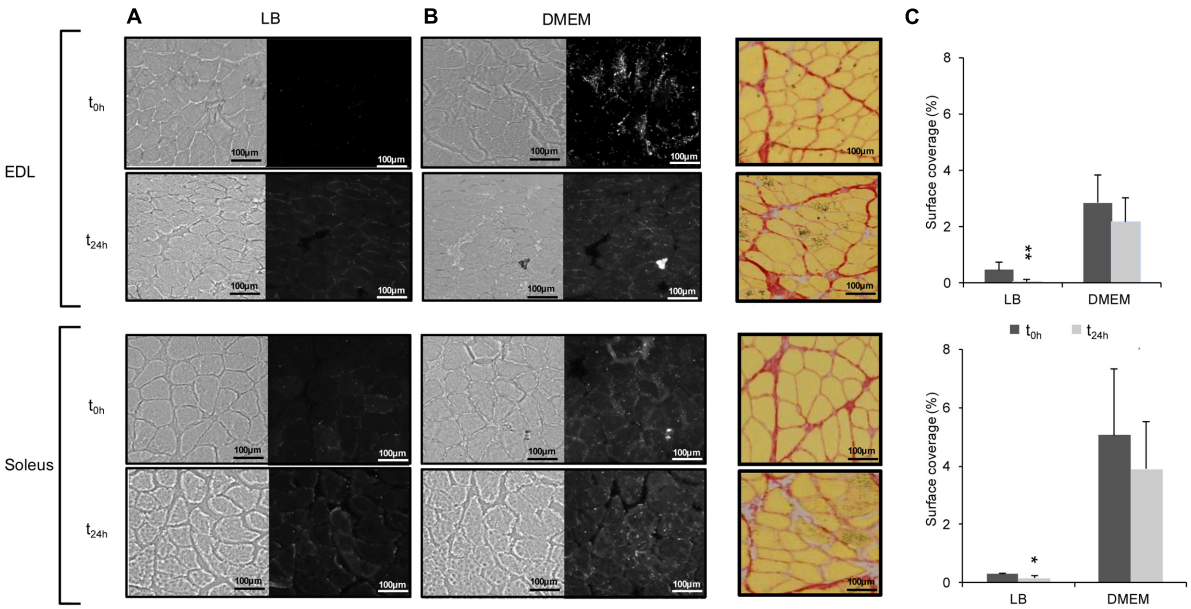


FIGURE 4 | Adhesion of *E. coli* O157:H7 to EDL and soleus muscle cross-sections as a function of postmortem time. **(A)** Bacterial adhesion to EDL and soleus cross-sections was assessed at two postmortem times, $t_{0\ h}$ (i.e., 10 min after slaugther) and $t_{24\ h}$ (24 h after slaughter). For each muscle cross-section, left images show muscle fibres in bright field mode and right images show adhering fluorescent *E. coli* O157:H7 CM454 pSaRe-Red1. **(B)** Structural changes in the ECM (coloured in red) at $t_{0\ h}$ and $t_{24\ h}$ postmortem on serial cross-sections of EDL and soleus muscles. **(C)** The level of adherent bacteria was assessed by the fluorescence intensity and expressed as the percentage of muscle cross-section surface covered by fluorescent bacterial cells.

kinetics, which can be rigorously monitored and controlled over time (Chagnot et al., 2015a,b; Filgueras et al., 2016). In fact, these muscles can be extracted entirely without lesions, shortly after death of the animal and their size is compatible with microscopic observations, which makes them models of choice to investigate bacterial interactions at the tissue and cellular levels.

Besides the type of skeletal muscle, differential adhesion depended on anterior growth conditions. Growth of *E. coli* O157:H7 EDL933/CM454 in LB was previously shown to induce specific adhesion to some ECM fibrillar proteins, especially collagens I and III, whereas DMEM was clearly shown to induce self-aggregation, resulting in non-specific bacterial adhesion

to ECM proteins (Chagnot et al., 2013a, 2014). Bacterial adhesion appeared to occur essentially around the muscles cells at the ECM and was significantly influenced by postmortem structural modifications. Recently, postmortem degradation of proteoglycans was suggested to destabilise the ECM and lead to the undocking of collagen fibres (Nishimura, 2010). Also, these postmortem structural modifications seems to affect the specific adhesion of bacteria to collagen as observed with bacterial cells grown in LB.

Whatever the different constituent myofibres of types I, IIA, IIX and IIB, however, no significant differences were observed for adhering bacteria. Muscle types, but not fibre types, affected the attachment of *E. coli* O157:H7, and this could be due to some variations in the ECM composition and/or organisation between the different muscles. The ECM at the periphery of the muscle fibres corresponds to the endomysium but the ECM at the surface of the muscle to the epimysium. Depending on the type of ECM at the different muscle structural organisation levels, the proportion of the different ECM proteins varies slightly (Gillies and Lieber, 2011; Hinds et al., 2011). From one muscle type to another, the composition of the ECM can also differ somewhat, as can the supramolecular organisation of the different components of the ECM, but elucidation is still needed.

By providing information on the spatial localisation of *E. coli* O157:H7 on meat, our results clearly demonstrate their ability to adhere to muscle tissue, especially at the ECM, which resulted in heterogeneous bacterial distribution in meat. *E. coli* O157:H7 exhibits a wide range of colonisation factors that could potentially participate in specific and non-specific adhesion to the ECM, e.g., several pili, flagella, and adhesins (Chagnot et al., 2012, 2013b; Monteiro et al., 2016). However, further studies are needed to determine the regulation and expression in different environmental conditions, as well as the exact contribution of each of these numerous molecular determinants of meat adhesion. For instance, the involvement of EhaB in binding to collagen or Cah in self-aggregation has been demonstrated, but the environmental conditions for their expression in *E. coli* O157:H7 have never been ascertained (Torres et al., 2002; Wells et al., 2009). In this regard, the present investigation opens the way to further characterisation of the molecular interactions between bacterial ECM-binding proteins and muscle ECM in different environmental conditions (Chagnot et al., 2012, 2013b).

This field of research has not attracted a lot of interest so far, but is promising and necessary. In the food industry and respective to HACCP (Hazard Analysis Critical Control Point), we could improve the preventive approach by designing measures to reduce the risks of ground beef meat contamination to an even safer level by considering differential *E. coli* O157:H7 adhesion and localisation to beef carcasses, with regards to different muscle types, bacterial behaviour in anterior and current environmental conditions, and heterogeneous bacterial distribution in meat with respect to ECM composition. Considering the meat ecosystem, understanding of the interactions of this foodborne pathogen with the food biotope and biocenosis, including the biotic and abiotic factors of the food matrix, could expedite development of novel preventive measures, such as the use of competitive microbial species for food biopreservation (Chaillou et al., 2015). Since the establishment of QMRA (quantitative microbial risk assessment) as the basis of food safety management, such information is required and is clearly highly relevant, but, as mentioned above, there are still major gaps in our basic knowledge. This also jeopardises the development of accurate predictive models incorporating the observations that food contamination with EHEC occurs at a low level, that the bacterial foodborne pathogen is physiologically diverse, and that the food matrix is heterogeneous. Besides its importance for risk assessment and for mitigation of EHEC contamination in the food chain and/or the food industry, knowledge of bacterial adhesion at molecular, cellular and tissue levels is also of great interest in the veterinary and/or medical sciences in relation to bacterial infection of muscle tissues.

AUTHOR CONTRIBUTIONS

CC, AV, TA, and MD conceived and designed the experiments. CC, AV, SR, and NC performed the experiments. CC, RT, TA, and MD analysed and interpreted the data. CC, AV, TA, and MD contributed reagents, materials, and analysis tools. CC, RT, TA, and MD wrote the paper.

ACKNOWLEDGMENTS

This work was supported in part by INRA ("Institut national de la Recherche Agronomique") with the MICel ("Mécanismes moléculaires impliqués dans l'interaction entre bactéries et cellules musculaires") project funded by the inter-department CEPIA/MICA ("Caractérisation et Elaboration des Produits Issus de l'Agriculture/Microbiologie et Chaîne Alimentaire") AIP ("Action Incitative Programmée"). COST (European Cooperation in Science and Technology) Action 1202 BacFoodNet (A European Network for Mitigating Bacterial Colonisation and Persistence On Foods and Food Processing Environments), and the "Conseil Régional d'Auvergne" CPER ("Contrat de Plan État-Région") ASTERisk ("Auvergne STEC Risque") is also acknowledged. Dr. Caroline Chagnot was a Ph.D. Research Fellow granted by the "Conseil Région Auvergne – FEDER (Fonds Européen de Développement Régional)". The authors are very grateful to David Marsh (djmarsh@wanadoo.fr) for correcting the European English of the manuscript.

REFERENCES

Auty, M., Duffy, G., O'beirne, D., Mcgovern, A., Gleeson, E., and Jordan, K. (2005). In situ localization of *Escherichia coli* O157:H7 in food by confocal scanning laser microscopy. *J. Food Prot.* 68, 482–486. doi: 10.4315/0362-028X-68.3.482

Bailey, A. J., and Light, N. D. (1989). *Connective Tissue in Meat and Meat Products*. London: Elsevier Applied Science.

Bendall, J. R. (1973). *Post Mortem Changes in Muscle: Structure and Function of Muscle*. New York, NY: Academic Press.

Cabedo, L., Sofos, J. N., Schmidt, G. R., and Smith, G. C. (1997). Attachment of *Escherichia coli* O157:H7 and other bacterial cells grown in two media to beef adipose and muscle tissues. *J. Food Prot.* 60, 102–106. doi: 10.4315/0362-028X-60.2.102

Chagnot, C., Agus, A., Renier, S., Peyrin, F., Talon, R., Astruc, T., et al. (2013a). In vitro colonization of the muscle extracellular matrix components by *Escherichia coli* O157:H7: the influence of growth medium, temperature and pH on initial adhesion and induction of biofilm formation by collagens I and III. *PLoS ONE* 8:e59386. doi: 10.1371/journal.pone.0059386

Chagnot, C., Caccia, N., Loukiadis, E., Ganet, S., Durand, A., Bertin, Y., et al. (2014). Colonization of the meat extracellular matrix proteins by O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Int. J. Food Microbiol.* 188, 92–98. doi: 10.1016/j.ijfoodmicro.2014.07.016

Chagnot, C., Listrat, A., Astruc, T., and Desvaux, M. (2012). Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. *Cell Microbiol.* 14, 1687–1696. doi: 10.1111/cmi.12002

Chagnot, C., Venien, A., Jamme, F., Refregiers, M., Desvaux, M., and Astruc, T. (2015a). Hyperspectral deep ultraviolet autofluorescence of muscle fibers is affected by postmortem changes. *J. Agric. Food Chem.* 63, 4782–4789. doi: 10.1021/acs.jafc.5b00668

Chagnot, C., Venien, A., Peyrin, F., Jamme, F., Refregiers, M., Desvaux, M., et al. (2015b). Deep UV excited muscle cell autofluorescence varies with the fibre type. *Analyst* 140, 4189–4196. doi: 10.1039/c5an00172b

Chagnot, C., Zorgani, M. A., Astruc, T., and Desvaux, M. (2013b). Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. *Front. Microbiol.* 4:303. doi: 10.3389/fmicb.2013.00303

Chaillou, S., Chaulot-Talmon, A., Caekebeke, H., Cardinal, M., Christieans, S., Denis, C., et al. (2015). Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. *ISME J.* 9, 1105–1118. doi: 10.1038/ismej.2014.202

Chase-Topping, M., Gally, D., Low, C., Matthews, L., and Woolhouse, M. (2008). Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat. Rev. Microbiol.* 6, 904–912. doi: 10.1038/nrmicro2029

Chen, J. R., Rossman, M. L., and Pawar, D. M. (2007). Attachment of enterohemorrhagic *Escherichia coli* to the surface of beef and a culture medium. *Lwt-Food Sci. Technol.* 40, 249–254. doi: 10.1016/j.lwt.2005.10.011

Desvaux, M., Khan, A., Beatson, S., Scott-Tucker, A., and Henderson, I. R. (2005). Protein secretion systems in *Fusobacterium nucleatum*: genomic identification of Type 4 piliation and complete Type V pathways brings new insight in mechanisms of pathogenesis. *Biochim. Biophys. Acta-Biomembr.* 1713, 92–112. doi: 10.1016/j.bbamem.2005.05.002

Filgueras, R., Peyrin, F., Venien, A., Henot, J. M., and Astruc, T. (2016). Sodium chloride diffusion during muscle salting evidenced by energy-dispersive X-ray spectroscopy imaging. *J. Agric. Food Chem.* 64, 699–705. doi: 10.1021/acs.jafc.5b04058

Frontera, W. R., and Ochala, J. (2015). Skeletal muscle: a brief review of structure and function. *Calcif. Tissue Int.* 96, 183–195. doi: 10.1007/s00223-014-9915-y

Gao, Y., Waas, A. M., Faulkner, J. A., Kostrominova, T. Y., and Wineman, A. S. (2008). Micromechanical modeling of the epimysium of the skeletal muscles. *J. Biomech.* 41, 1–10. doi: 10.1016/j.jbiomech.2007.08.008

Giaouris, E., Heir, E., Hébraud, M., Chorianopoulos, N., Langsrud, S., Moretro, T., et al. (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Sci.* 97, 298–309. doi: 10.1016/j.meatsci.2013.05.023

Gill, C. O. (1979). A review: intrinsic bacteria in meat. *J. Appl. Bacteriol.* 47, 367–378. doi: 10.1111/j.1365-2672.1979.tb01196.x

Gillies, A. R., and Lieber, R. L. (2011). Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve* 44, 318–331. doi: 10.1002/mus.22094

Gobert, A. P., Vareille, M., Glasser, A. L., Hindre, T., De Sablet, T., and Martin, C. (2007). Shiga toxin produced by enterohemorrhagic *Escherichia coli* inhibits PI3K/NF-kappaB signaling pathway in globotriaosylceramide-3-negative human intestinal epithelial cells. *J. Immunol.* 178, 8168–8174. doi: 10.4049/jimmunol.178.12.8168

Gould, L. H., Demma, L., Jones, T. F., Hurd, S., Vugia, D. J., Smith, K., et al. (2009). Hemolytic uremic syndrome and death in persons with *Escherichia coli* O157:H7 infection, foodborne diseases active surveillance network sites, 2000-2006. *Clin. Infect. Dis.* 49, 1480–1485. doi: 10.1086/644621

Greaser, M. L. (1986). *Conversion of Muscle to Meat: Muscle as Food*. Cambridge, MA: Academic Press. doi: 10.1016/b978-0-12-084190-5.50007-2

Guedon, E., Payot, S., Desvaux, M., and Petitdemange, H. (2000). Relationships between cellobiose catabolism, enzyme levels, and metabolic intermediates in *Clostridium cellulolyticum* grown in a synthetic medium. *Biotechnol. Bioeng.* 67, 327–335. doi: 10.1002/(SICI)1097-0290(20000205)67:3<327::AID-BIT9>3.0.CO;2-U

Guignot, F., Vignon, X., and Monin, G. (1993). Post mortem evolution of myofilament spacing and extracellular space in veal muscle. *Meat Sci.* 33, 333–347. doi: 10.1016/0309-1740(93)90005-3

Hinds, S., Bian, W., Dennis, R. G., and Bursac, N. (2011). The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials* 32, 3575–3583. doi: 10.1016/j.biomaterials.2011.01.062

Huff Lonergan, E., Zhang, W., and Lonergan, S. M. (2010). Biochemistry of postmortem muscle - lessons on mechanisms of meat tenderization. *Meat Sci.* 86, 184–195. doi: 10.1016/j.meatsci.2010.05.004

Karch, H., Tarr, P. I., and Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *Int. J. Med. Microbiol.* 295, 405–418. doi: 10.1016/j.ijmm.2005.06.009

Kirchofer, K. S., Calkins, C. B., and Gwartney, B. L. (2002). Fiber-type composition of muscles of the beef chuck and round. *J. Anim. Sci.* 80, 2872–2878. doi: 10.2527/2002.80112872x

Kredel, S., Oswald, F., Nienhaus, K., Deuschle, K., Rocker, C., Wolff, M., et al. (2009). mRuby, a bright monomeric red fluorescent protein for labeling of subcellular structures. *PLoS ONE* 4:e4391. doi: 10.1371/journal.pone.0004391

Lagord, C., Soulet, L., Bonavaud, S., Bassaglia, Y., Rey, C., Barlovatz-Meimon, G., et al. (1998). Differential myogenicity of satellite cells isolated from extensor digitorum longus (EDL) and soleus rat muscles revealed in vitro. *Cell Tissue Res.* 291, 455–468. doi: 10.1007/s004410051015

Lefaucheur, L. (2010). A second look into fibre typing–relation to meat quality. *Meat Sci.* 84, 257–270. doi: 10.1016/j.meatsci.2009.05.004

Li, J., and McLandsborough, L. A. (1999). The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* 53, 185–193. doi: 10.1016/S0168-1605(99)00159-2

Liu, A., Nishimura, T., and Takahashi, K. (1994). Structural changes in endomysium and perimysium during post-mortem aging of chicken semitendinosus muscle-contribution of structural weakening of intramuscular connective tissue to meat tenderization. *Meat Sci.* 38, 315–328. doi: 10.1016/0309-1740(94)90120-1

Medina, M. B. (2001). Binding of collagen I to *Escherichia coli* O157:H7 and inhibition by carrageenans. *Int. J. Food Microbiol.* 69, 199–208. doi: 10.1016/S0168-1605(01)00497-4

Meunier, B., Picard, B., Astruc, T., and Labas, R. (2010). Development of image analysis tool for the classification of muscle fibre type using immunohistochemical staining. *Histochem. Cell Biol.* 134, 307–317. doi: 10.1007/s00418-010-0733-7

Monteiro, R., Ageorges, V., Rojas-Lopez, M., Schmidt, H., Weiss, A., Bertin, Y., et al. (2016). A secretome view of colonisation factors in Shiga toxin-encoding *Escherichia coli* (STEC): from enterohaemorrhagic *E. coli* (EHEC) to related enteropathotypes. *FEMS Microbiol. Lett.* 363, doi: 10.1093/femsle/fnw179

Morey-Holton, E. R., and Globus, R. K. (2002). Hindlimb unloading rodent model: technical aspects. *J. Appl. Physiol.* 92, 1367–1377. doi: 10.1152/japplphysiol.00969.2001

Naili, I., Buonsanti, C., Soriani, M., Desvaux, M., Jubelin, G., Vieru, M., et al. (2016). In vivo characterization of the immune response towards the pathogenic *Escherichia coli* antigen SsIE and modulation of the intestinal microbiota. *J. Immunol.* 196, 215–219.

Nishimura, T. (2010). The role of intramuscular connective tissue in meat texture. *Anim. Sci. J.* 81, 21–27. doi: 10.1111/j.1740-0929.2009.00696.x

Nishimura, T. (2015). Role of extracellular matrix in development of skeletal muscle and postmortem aging of meat. *Meat Sci.* 109, 48–55. doi: 10.1016/j.meatsci.2015.05.015

Offer, G., and Knight, P. (1988). "The structural basis of water holding in meat. Part 2: drip losses," in *Developments in Meat Science*, 4th Edn, ed. R. Lawrie (London: Elsevier Science Publication).

Ouali, A. (1990). Meat tenderization: possible causes and mechanisms, A review. *J. Muscle Foods* 1, 129–165. doi: 10.1111/j.1745-4573.1990.tb00360.x

Perna, N. T., Plunkett, G. III, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., et al. (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533. doi: 10.1038/35054089

Pette, D., and Staron, R. S. (1990). Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev. Physiol. Biochem. Pharmacol.* 116, 1–76. doi: 10.1007/3540528806_3

Pullen, A. H. (1977). The distribution and relative sized of fibre types in the extensor digitorum longus and soleus muscles of the adult rat. *J. Anat.* 123, 467–486.

Realini, C. E., Perez-Juan, M., Gou, P., Diaz, I., Sarraga, C., Gatellier, P., et al. (2013). Characterization of *Longissimus thoracis*, semitendinosus and masseter muscles and relationships with technological quality in pigs. 2. Composition of muscles. *Meat Sci.* 94, 417–423. doi: 10.1016/j.meatsci.2013.03.007

Rhoades, J. R., Duffy, G., and Koutsoumanis, K. (2009). Prevalence and concentration of verocytotoxigenic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: a review. *Food Microbiol.* 26, 357–376. doi: 10.1016/j.fm.2008.10.012

Rivas, L., Dykes, G. A., and Fegan, N. (2006). Attachment of shiga toxigenic *Escherichia coli* to beef muscle and adipose tissue. *J. Food Prot.* 69, 999–1006. doi: 10.4315/0362-028X-69.5.999

Rogers, M. A., and Evans, W. J. (1993). Changes in skeletal muscle with aging: effects of exercise training. *Exerc. Sport Sci. Rev.* 21, 65–102. doi: 10.1249/00003677-199301000-00003

Schiaffino, S., and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol. Rev.* 91, 1447–1531. doi: 10.1152/physrev.00031.2010

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Soukup, T., Zacharova, G., and Smerdu, V. (2002). Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem.* 104, 399–405. doi: 10.1078/0065-1281-00660

Tarr, C. L., Nelson, A. M., Beutin, L., Olsen, K. E., and Whittam, T. S. (2008). Molecular characterization reveals similar virulence gene content in unrelated clonal groups of *Escherichia coli* of serogroup O174 (OX3). *J. Bacteriol.* 190, 1344–1349. doi: 10.1128/JB.01317-07

Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M., and Goll, D. E. (1995). Is Z-disk degradation responsible for postmortem tenderization? *J. Anim. Sci.* 73, 1351–1367. doi: 10.2527/1995.7351351x

Taylor, R. G., and Koohmaraie, M. (1998). Effects of postmortem storage on the ultrastructure of the endomysium and myofibrils in normal and callipyge longissimus. *J. Anim. Sci.* 76, 2811–2817. doi: 10.2527/1998.76112811x

Torres, A. G., Perna, N. T., Burland, V., Ruknudin, A., Blattner, F. R., and Kaper, J. B. (2002). Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. *Mol. Microbiol.* 45, 951–966. doi: 10.1046/j.1365-2958.2002.03094.x

Totland, G. K., and Kryvi, H. (1991). Distribution patterns of muscle fibre types in major muscles of the bull (*Bos taurus*). *Anat. Embryol. (Berl.)* 184, 441–450. doi: 10.1007/BF01236050

Waardenberg, A. J., Reverter, A., Wells, C. A., and Dalrymple, B. P. (2008). Using a 3D virtual muscle model to link gene expression changes during myogenesis to protein spatial location in muscle. *BMC Syst. Biol.* 2:88. doi: 10.1186/1752-0509-2-88

Warren, B. E., Lou, P. H., Lucchinetti, E., Zhang, L., Clanachan, A. S., Affolter, A., et al. (2014). Early mitochondrial dysfunction in glycolytic muscle, but not oxidative muscle, of the fructose-fed insulin-resistant rat. *Am. J. Physiol. Endocrinol. Metab.* 306, E658–E667. doi: 10.1152/ajpendo.00511.2013

Wells, T. J., Mcneilly, T. N., Totsika, M., Mahajan, A., Gally, D. L., and Schembri, M. A. (2009). The *Escherichia coli* O157:H7 EhaB autotransporter protein binds to laminin and collagen I and induces a serum IgA response in O157:H7 challenged cattle. *Environ. Microbiol.* 11, 1803–1814. doi: 10.1111/j.1462-2920.2009.01905.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Chagnot, Venien, Renier, Caccia, Talon, Astruc and Desvaux. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 July 2017
doi: 10.3389/fmicb.2017.01433

Comparison of the Microbiological Quality and Safety between Conventional and Organic Vegetables Sold in Malaysia

Chee-Hao Kuan[1], Yaya Rukayadi[1], Siti H. Ahmad[2], Che W. J. Wan Mohamed Radzi[3], Tze-Young Thung[1], Jayasekara M. K. J. K. Premarathne[1], Wei-San Chang[1], Yuet-Ying Loo[1], Chia-Wanq Tan[1], Othman B. Ramzi[1], Siti N. Mohd Fadzil[1], Chee-Sian Kuan[4], Siok-Koon Yeo[5], Mitsuaki Nishibuchi[6] and Son Radu[1,7]*

[1] Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Malaysia, [2] Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Malaysia, [3] Department of Science and Technology Studies, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, [4] Neogenix Laboratoire Sdn Bhd, Petaling Jaya, Malaysia, [5] School of Biosciences, Taylor's University Lakeside, Subang Jaya, Malaysia, [6] Center for Southeast Asian Studies, Kyoto University, Kyoto, Japan, [7] Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Seri Kembangan, Malaysia

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Bernadette Dora Gombossy de Melo Franco, University of São Paulo, Brazil
Alejandro Castillo, Texas A&M University, United States

***Correspondence:**
Chee-Hao Kuan
raymondkuan87@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 May 2017*
Accepted: *14 July 2017*
Published: *31 July 2017*

Citation:
Kuan C-H, Rukayadi Y, Ahmad SH, Wan Mohamed Radzi CWJ, Thung T-Y, Premarathne JMKJK, Chang W-S, Loo Y-Y, Tan C-W, Ramzi OB, Mohd Fadzil SN, Kuan C-S, Yeo S-K, Nishibuchi M and Radu S (2017) Comparison of the Microbiological Quality and Safety between Conventional and Organic Vegetables Sold in Malaysia. Front. Microbiol. 8:1433. doi: 10.3389/fmicb.2017.01433

Given the remarkable increase of public interest in organic food products, it is indeed critical to evaluate the microbiological risk associated with consumption of fresh organic produce. Organic farming practices including the use of animal manures may increase the risk of microbiological contamination as manure can act as a vehicle for transmission of foodborne pathogens. This study aimed to determine and compare the microbiological status between organic and conventional fresh produce at the retail level in Malaysia. A total of 152 organic and conventional vegetables were purchased at retail markets in Malaysia. Samples were analyzed for mesophilic aerobic bacteria, yeasts and molds, and total coliforms using conventional microbiological methods. Combination methods of most probable number-multiplex polymerase chain reaction (MPN-mPCR) were used to detect and quantify foodborne pathogens, including *Escherichia coli* O157:H7, Shiga toxin-producing *E. coli* (STEC), *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Salmonella* Enteritidis. Results indicated that most types of organic and conventional vegetables possessed similar microbial count ($P > 0.05$) of mesophilic aerobic bacteria, yeasts and molds, and total coliforms. *E. coli* O157:H7 and *S.* Typhimurium were not detected in any sample analyzed in this study. Among the 152 samples tested, only the conventional lettuce and organic carrot were tested positive for STEC and *S.* Enteritidis, respectively. *L. monocytogenes* were more frequently detected in both organic (9.1%) and conventional vegetables (2.7%) as compared to *E. coli* O157:H7, *S.* Typhimurium, and *S.* Enteritidis. Overall, no trend was shown that either organically or conventionally grown vegetables have posed greater microbiological risks. These findings indicated that one particular type of farming practices would not affect the microbiological profiles of fresh produce. Therefore, regardless of farming methods, all vegetables should be subjected to appropriate post-harvest handling practices from farm to fork to ensure the quality and safety of the fresh produce.

Keywords: *Escherichia coli* O157:H7, salmonella, *Listeria monocytogenes*, fresh produce, organic farming

INTRODUCTION

Public awareness of healthy eating habits have been intensified in recent years and prompted an increased demand for fresh fruits and vegetables (Olaimat and Holley, 2012). Despite the health benefits derived from consuming fresh produce, the risk of microbiological contamination in vegetables is of concern as the contamination can possibly occur through the food chain, from farm to fork. Over the past decade, numerous foodborne disease outbreaks caused by *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp. were related to the consumption of contaminated fresh vegetables (Beuchat, 2002; Centers for Disease Control and Prevention, 2011, 2012; Maffei et al., 2013).

Given people's growing awareness of health and environmental sustainability, organic farming systems have become more well-received because conventional farming uses large amounts of synthetic pesticides and chemical fertilizers. Organic foods are perceived as safer and more healthful foods due to the chemical-free farming techniques used for their production as compared to conventionally produced foods (Somasundram et al., 2016). In Europe, the consumption of fresh organic produce has increased annually (Willer and Kilcher, 2009). In Malaysia, although the organic food remains a niche market and comprises only a small fraction of the food market, the demand for organic food has grown steadily. There were about 131 ha organic farms in Malaysia in 2001. However, the land area for organic farms was increased by 18-fold–2,367 ha, of which 962 ha are certified organic within a 5-year period. In Malaysia, the production of organic food is limited to vegetables and fruits only and most of the fresh organic produce are sold in domestic markets (Mohamad et al., 2014; Tiraieyari et al., 2014; Somasundram et al., 2016).

Despite the widespread consumers' belief that organic foods are "safer" and "more healthful" than conventional foods, evidence to support this concept is difficult to determine. Microbiological quality and safety of organic produce remain to be controversial and debated (Magkos et al., 2006). This issue emerged due to the lack of research and limited scientific data to reveal the actual scenario. The view that fresh organic produce is "safer" than conventionally grown food appears to be constructed on the perception that organic fruits and vegetables are free from chemical fertilizers and synthetic pesticides (Institute of Food Technologists, 2000; Somasundram et al., 2016). Conversely, previous studies have suggested that organic production practices, such as the use of manure may increase the risk of microbiological contamination. Manure may harbor foodborne pathogens, such as *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157:H7 (Stephenson, 1997; McMahon and Wilson, 2001; Williams, 2002; Johannessen et al., 2004). Also, manure may introduce various pathogenic microorganisms that can persist for a long duration in the soil (Pell, 1997). However, it is difficult to conclude that the consumption of fresh organic produce would confer greater microbiological risk to consumers than conventional food. Other than cultivation method, microbial contamination can occur during harvesting, post-harvest handling or at any point along the food supply chain (Beuchat and Ryu, 1997).

The present study aimed to investigate and compare the microbiological status of different organic and conventional vegetables sold in the retail markets in Malaysia. To the best of our knowledge, this is the first comprehensive study on the comparison of microbiological quality and safety level between organically and conventionally grown vegetables in Southeast Asia.

MATERIALS AND METHODS

Sample Collection

A total of 152 organic and conventional vegetables, comprising of 77 organic (certified by competent national and overseas authorities) and 75 conventional, were randomly purchased from hypermarkets and wet markets in Kuala Lumpur, Selangor, and Putrajaya. The samples collected included: cabbage (*Brassica oleracea*), carrot (*Daucus carota* subsp. *sativus*), calamondin (× *Citrofortunella microcarpa)*, cherry tomato (*Solanum lycopersicum* var. *cerasiforme*), Bird's eye chili (*Capsicum annum*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), winged bean (*Psophocarpus tetragonolobus*), Romaine lettuce (*Lactuca sativa* var. *longifolia*), Iceberg lettuce (*Lactuca sativa* var. *capitata*), *Looseleaf lettuce* (*Lactuca sativa* var. *crispa*), *Butterhead lettuce* (*Lactuca sativa* var. *capitata*), sweet potato (*Ipomoea batatas*), tomato (*Solanum lycopersicum*), and white radish (*Raphanus sativus*). Sampling was carried out over a 1-year period (November 2015 to October 2016). All samples (250–300 g each) were randomly collected from bulk quantities of vegetables, placed in sterile bags (BagMixer® 400 mL, Interscience, Saint-Nom-la-Bretèche, France), kept in an insulated box with ice packs and transported immediately to the Food Safety and Quality Laboratory, Universiti Putra Malaysia for microbiological analyses.

Microbiological Analysis

Twenty-five grams of each sample was cut into small pieces, weighed, placed in a sterile stomacher bag, and followed by homogenization using a stomacher machine (BagMixer® 400P, Interscience, Saint-Nom-la-Bretèche, France) with 225 mL of 0.1% (v/v) peptone water (Oxoid™, Basingstoke, Hampshire, UK) for 1 min. The pH of the bacterial culture broth was neutralized to pH 7.0 with 0.5 M NaOH solution. Mesophilic aerobic bacteria, total coliforms, and yeasts and molds were enumerated using conventional methods (Beuchat and Cousin, 2001; Kornacki and Johnson, 2001; Morton, 2001). Each sample was analyzed in triplicate and all the results were expressed as colony-forming units per gram (CFU/g).

Detection and Enumeration of Foodborne Pathogens by MPN-PCR Method

Most-Probable-Number (MPN)

Each sample was cut into small pieces, and then a total of 10 g of sample was mixed with 90 mL of Tryptic Soy Broth (TSB; Merck, Darmstadt, Hesse, Germany), Listeria Enrichment Broth (LEB; Merck, Darmstadt, Hesse, Germany), and Buffered Peptone Water (BPW; Merck, Darmstadt, Hesse, Germany) for detection of *E. coli* O157:H7, *Listeria* spp., and *Salmonella* spp.,

respectively, in sterile stomacher bag and homogenized using a stomacher machine (BagMixer® 400P, Interscience, Saint-Nom-la-Bretèche, France) for 1 min. The pH of the enrichment broths was adjusted to pH 7.0 with 0.5 M NaOH solution before incubation. For the three-tube MPN analysis, 1 mL of the 10-, 100-, and 1,000-fold dilutions of the enriched bacteria culture were incubated in MPN tubes for 24 h at 37°C for detection and enumeration of *E. coli* O157:H7 and *Salmonella* spp., and 48 h at 30°C for detection and enumeration of *Listeria* species.

Genomic DNA Extraction and Multiplex-PCR

All the incubated MPN tubes were subjected to DNA extraction using the boiled-cell method as described in **Table 1**. Multiplex-PCR assays and gel electrophoresis for the detection of Shiga toxin-producing *E. coli* (STEC), *E. coli* O157:H7, *Listeria* spp., *L. monocytogenes*, *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium were performed based on the methods as summarized in **Table 2**.

Statistical Analysis

Colony counts were converted into $\log_{10}$ CFU/g. The data were subjected to a one-way analysis of variance (ANOVA) analysis using Minitab 16.0 software (Minitab Inc., State College, Pennsylvania, U.S.A.) to evaluate if there were differences between the organic and conventional vegetables at $P \leq 0.05$ level of significance.

RESULTS

Microbiological Quality of Conventional and Organic Vegetables

Table 3 shows the microbial counts of mesophilic aerobic bacteria, yeasts and molds, and total coliforms in 12 types of organic and conventional vegetables. Among the 12 types of vegetables analyzed, no trend was shown that either organic or conventional vegetable has a greater microbial count of mesophilic aerobic bacteria, yeasts and molds, and total coliforms. However, cabbage, carrot, and winged bean showed significant differences ($P < 0.05$) in mesophilic aerobic population between organic and conventional samples. For mesophilic aerobic bacteria, the results varied from 3 to >7 $\log_{10}$ CFU/g for organic vegetables and 3 to $>$ 8 $\log_{10}$ CFU/g for conventional vegetables. Most of the samples had a mesophilic aerobic bacteria count that ranged from 5 to 7 $\log_{10}$ CFU/g.

For total coliforms counts, results varied from 1 to 7 $\log_{10}$ CFU/g for organic and conventional vegetables. In this study, no coliform bacterium was detected in calamondin samples and carrot was the only sample that showed significant differences ($P < 0.05$) in coliform populations between organic and conventional samples. Overall, greater microbial counts (mesophilic aerobic bacteria, yeasts and molds, and total coliforms) were detected in chili samples whereas lower microbial counts were found in cherry tomato and tomato samples.

The yeasts and molds counts in the vegetables were lower compared to mesophilic aerobic bacteria, ranged from 1 to $>$ 6 $\log_{10}$ CFU/g for organic vegetables and 0.5 to $>$ 6 $\log_{10}$ CFU/g for conventional vegetables. The yeasts and molds counts of most samples varied from 3 to 6 $\log_{10}$ CFU/g. In this study, all conventionally and organically grown vegetable samples showed comparable yeast and mold counts except for conventional winged bean which showed a higher count compared to the organic counterpart.

Microbiological Safety of Conventional and Organic Vegetables

As shown in **Tables 4–6**, there was obviously not much difference in the prevalence of foodborne pathogens between conventional and organic vegetables. Therefore, statistical analysis for comparison of the positive-negative data for foodborne pathogens between conventional and organic vegetables was not conducted in this study. In this study, *E. coli* O157:H7 and *S.* Typhimurium were not detected in 152 vegetable samples (**Tables 4, 5**). Of the 152 samples analyzed, only the conventional lettuce (**Table 4**) and organic carrot (**Table 5**) were contaminated with STEC and *S.* Enteritidis, respectively. *L. monocytogenes* were more frequently detected in both organic and conventional vegetables as compared to *E. coli* O157:H7, *S.* Typhimurium, and *S.* Enteritidis (**Tables 4–6**). The prevalence of *L. monocytogenes* in conventional and organic vegetables was 9.1% (seven positive samples out of 77 samples) and 2.7% (two positive samples

TABLE 1 | Procedures for extraction of pathogens genomic DNA from conventional and organic vegetables.

Target pathogens	Genomic DNA extraction procedures	References
STEC O157:H7 and STEC non-O157	Each MPN tube was centrifuged at 13,400 × *g* for 1 min. The supernatants were discarded and the pellets were resuspended with 200 μL of Tris-EDTA buffer. The mixture was then boiled for 10 min and cooled at −20°C immediately for another 10 min before it was centrifuged at 13,400 × *g* for 2 min. The supernatant was used as a template DNA in PCR assays.	Loo et al., 2013
Listeria spp. and *L. monocytogenes*	Each MPN tube was centrifuged at 13,400 × *g* for 1 min. The supernatants were discarded and the pellets were resuspended in 200 μL of Tris-EDTA buffer. The mixture was then boiled for 10 min and cooled at −20°C immediately for another 10 min before it was subjected to centrifugation at 13,400 × *g* for 3 min. The supernatant was used as a template DNA in PCR assays.	Kuan et al., 2013
Salmonella spp., *S.* Enteritidis, and *S.* Typhimurium	Each MPN tube was centrifuged at 15,000 × *g* for 3 min. The supernatants were discarded and the pellet was resuspended in 200 μL Tris-EDTA buffer and boiled for 10 min. The mixture was then cooled at −20°C immediately for another 10 min, followed by centrifugation at 15,000 × *g* for 3 min. The supernatant was used as a template DNA in PCR assays.	Pui et al., 2011

TABLE 2 | Target genes, primer sequences, PCR preparations, thermocycling conditions, and gel electrophoresis conditions used in this study.

Target genes	Primer sequences	PCR preparations*	Thermocycling conditions	Gel electrophoresis conditions	Amplicon size	Reference(s)
Shiga-toxin genes (*stx1* and *stx2*) were targeted for the detection of STEC	stx1-F: 5′-ATA AAT CGC CAT TCG TTG ACT AC-3′ stx1-R: 5′-AGA ACG CCC ACT GAG ATC ATC-3′ stx2-F: 5′-GGC ACT GTC TGA AAC TGC TCC-3′ stx2-R: 5′-TCG CCA GTT ATC TGA CAT TCT G-3′	5 μL 5 × PCR buffer, 2 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTP mix, 1 U Taq DNA Polymerase, 0.5 μM for each primer, 10.3 μL of sterile distilled water and 2 μL of extracted DNA.	Initialisation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation for 72°C for 45 s, and followed by final elongation at 72°C for 7 min.	An aliquot (5 μL) of the amplified PCR product was loaded and electrophoresed on 1.5% (w/v) agarose gel at 100 V for 28 min.	180 bp (*stx1*) and 255 bp (*stx2*)	Çadlrc et al., 2010; Loo et al., 2013
fliCh7 and *rfbO157* genes were targeted for the detection of O antigen and flagellar antigen in *E. coli* O157:H7	rfbO157-F: 5′-CGG ACA TCC ATG TGA TAT GG-3′ rfbO157-R: 5′-TTG CCT ATG TAC AGC TAA TCC-3′ fliCh7-F: 5′-GCG CTG TCG AGT TCT ATC GAG-3′ fliCh7-R: 5′-CAA CGG TGA CTT TAT CGC CAT TCC-3′	5 μL 5 × PCR buffer, 2 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTP mix, 1 U Taq DNA Polymerase, 0.5 μM for each primer, 10.3 μL of sterile distilled water and 2 μL of extracted DNA.	Initialisation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 1 min and followed by a final elongation at 72°C for 10 min.	An aliquot (5 μL) of the amplified PCR product was loaded and electrophoresed on 1.5% (w/v) agarose gel at 100 V for 28 min.	259 bp (*rfbO157*) and 625 bp (*fliCh7*)	Hashemi et al., 2010; Loo et al., 2013
16S rRNA gene was targeted for the detection of *Listeria* spp. *hlyA* gene was targeted for the detection of *L. monocytogenes*	U1: 5′-CTC CAT AAA GGT GAC CCT-3′ LI1: 5′-CAG CMG CCG CGG TAA TWC-3′ LM1: 5′-CCT AAG ACG CCA ATC GAA-3′ LM2: 5′-AAG CGC TTG CAA CTG CTC-3′	5 μL 5 × PCR buffer, 1.5 μL 25 mM $MgCl_2$, 0.2 μL 10 mM deoxynucleoside triphosphate (dNTP) mix, 1.5 U Taq DNA Polymerase, 0.5 μM for each primer LM1 and LM2, 1.0 μM of each primer U1 and LI1, 14 μL of sterile distilled water and 2 μL of extracted DNA.	Initialisation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min, and elongation at 72°C for 2 min, and followed by a final elongation at 72°C for 7 min.	An aliquot (5 μL) of the amplified PCR product was loaded and electrophoresed on 1.0% (w/v) agarose gel at 100 V for 28 min.	938 bp 702 bp	Border et al., 1990; Kuan et al., 2013
Random fragment was targeted for the detection of *Salmonella* spp. *SdfI* gene was targeted for the detection of *S.* Enteritidis *fliC* gene was targeted for the detection of *S.* Typhimurium	ST11: 5′-GCC AAC CAT TGC TAA ATT GGC GCA-3′ ST15: 5′-GGT AGA AAT TCC CAG CGG GTA CTG G-3′ ENTF: 5′-TGT GTT TTA TCT GAT GCA AGA GG-3′ ENTR: 5′-TGA ACT ACG TTC GTT CTT CTG G-3′ Fli15: 5′-CGG TGT TGC CCA GGT TGG TAA T-3′ Typ04: 5′-ACT GGT AAA GAT GGC T-3′	5 μL 5 × PCR buffer, 2.5 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTP mix, 1.5 U Taq DNA polymerase, 0.5 μL primer mix, (0.2 μM for ST11 and ST15, 1.2 μM for Fli15, Typ04, ENTF, and ENTR) 14.2 μL sterile distilled water, and 2 μL of extracted DNA.	Initialisation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, elongation at 72°C for 1 min, and followed by a final elongation at 72°C for 7 min.	An aliquot (5 μL) of the amplified PCR product was loaded and electrophoresed on 1.5% (w/v) agarose gel at 100 V for 40 min.	429 bp 304 bp 620 bp	Soumet et al., 1999; Alvarez et al., 2004; Pui et al., 2011; Thung et al., 2016

**Amplification of DNA was performed in 25 μL reaction mixtures.*

TABLE 3 | Microbial counts (log_{10} CFU/g) of mesophilic aerobic bacteria, yeasts and molds, and total coliforms in different organic and conventional vegetables purchased at retail markets in Malaysia.

Samples	Mesophilic aerobic bacteria		Total coliforms		Yeasts and molds	
	Conventional	Organic	Conventional	Organic	Conventional	Organic
Cabbage	6.97 ± 0.51	5.82 ± 0.71*	4.83 ± 2.89	2.28 ± 2.57	6.36 ± 1.07	5.40 ± 0.74
Calamondin	5.10 ± 0.61	4.56 ± 1.62	ND[a]	ND[a]	4.86 ± 0.79	4.84 ± 1.43
Carrot	5.57 ± 0.35	6.29 ± 0.41*	4.22 ± 0.89	5.62 ± 0.45*	5.59 ± 0.70	6.03 ± 0.51
Cherry tomato	3.90 ± 2.17	3.17 ± 2.46	2.09 ± 2.73	1.89 ± 2.10	1.51 ± 1.89	3.15 ± 2.50
Chili	8.06 ± 0.38	7.91 ± 0.55	7.04 ± 0.85	6.94 ± 0.61	5.95 ± 0.55	6.48 ± 1.18
Cucumber	5.55 ± 0.60	6.24 ± 0.53	2.48 ± 2.36	2.67 ± 3.07	3.35 ± 1.58	4.58 ± 0.91
Eggplant	6.89 ± 0.74	6.23 ± 0.49	5.18 ± 2.33	4.13 ± 2.09	5.53 ± 0.81	4.78 ± 0.76
Winged bean	7.41 ± 0.35	6.91 ± 0.17*	4.73 ± 2.22	3.49 ± 1.75	6.05 ± 0.30	5.61 ± 0.08*
Lettuce	6.70 ± 0.58	7.14 ± 0.60	4.61 ± 0.88	4.06 ± 2.14	5.77 ± 0.66	6.30 ± 0.70
Sweet potato	7.09 ± 0.28	6.50 ± 1.42	5.70 ± 0.69	4.05 ± 2.34	5.48 ± 0.35	4.84 ± 0.92
Tomato	4.90 ± 1.50	3.63 ± 3.02	1.00 ± 1.71	1.70 ± 2.77	0.48 ± 1.26	1.37 ± 2.14
White radish	7.76 ± 0.77	7.13 ± 0.39	6.11 ± 1.01	5.01 ± 1.35	6.90 ± 0.76	6.28 ± 0.60

Mean comparisons between conventional and organic within mesophilic aerobic bacteria, total coliforms and yeasts and molds by one-way ANOVA at $P \leq 0.05$. The results were expressed as mean ± SD (log_{10} CFU/g) of three measurements.

**Significantly different at $P \leq 0.05$.*

[a]ND: Not detectable in 25 g.

TABLE 4 | Prevalence of STEC O157:H7 and STEC non-O157 in conventional and organic vegetables purchased at retail markets in Malaysia using the MPN-PCR method.

Types of samples	STEC O157:H7						STEC non-O157					
	Conventional		Organic		Total		Conventional		Organic		Total	
	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%
Cabbage	(0/5)	0	(0/6)	0	(0/11)	0	(0/5)	0	(0/6)	0	(0/11)	0
Calamondin	(0/6)	0	(0/7)	0	(0/13)	0	(0/6)	0	(0/7)	0	(0/13)	0
Carrot	(0/6)	0	(0/7)	0	(0/13)	0	(0/6)	0	(0/7)	0	(0/13)	0
Cherry tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Chili	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Cucumber	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Eggplant	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Winged bean	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Lettuce	(0/6)	0	(0/7)	0	(0/13)	0	(1/6)	16.7	(0/7)	0	(1/13)	7.7
Sweet potato	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0
Tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
White radish	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0
Total	(0/77)	0	(0/75)	0	(0/152)	0	(1/77)	1.3	(0/75)	0	(1/152)	0.7

n_p, Total number of positive samples confirmed by MPN-PCR; n_t, Total number of samples; %, Percentage of positive sample.

out of 75 samples), respectively. The microbial load of *L. monocytogenes* in vegetable samples ranged between < 3 and 6.0 MPN/g (**Table 7**). Overall, the microbial load for most positive samples was ranged from < 3 to 3.0 MPN/g.

DISCUSSION

Since vegetables are grown in soil and exposed to different kind of environmental conditions and hazards, these conditions would be reflected in the mesophilic aerobic count. Therefore, the mesophilic aerobic count can be used as an indicator to access the microbiological quality of foods (Pianetti et al., 2008). Brackett and Splittstoesser (1992) found that mesophilic aerobic counts in vegetables can be as high as 7 log_{10} CFU/g. A previous study suggested that fruits and vegetables grown without or under low level of pesticides can be contaminated with larger microbial population since some pesticides have been found to inhibit the growth of some microorganisms (Guan et al., 2001). Oliveira et al. (2010) also reported that

TABLE 5 | Prevalence of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium in conventional and organic vegetables purchased at retail markets in Malaysia using the MPN-PCR method.

Types of samples	*Salmonella* spp.						*S.* Enteritidis						*S.* Typhimurium					
	Conventional		Organic		Total		Conventional		Organic		Total		Conventional		Organic		Total	
	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%
Cabbage	(0/5)	0	(0/6)	0	(0/11)	0	(0/5)	0	(0/6)	0	(0/11)	0	(0/5)	0	(0/6)	0	(0/11)	0
Calamondin	(0/6)	0	(1/7)	14.3	(1/13)	7.7	(0/6)	0	(0/7)	0	(0/13)	0	(0/6)	0	(0/7)	0	(0/13)	0
Carrot	(0/6)	0	(1/7)	14.3	(1/13)	7.7	(0/6)	0	(1/7)	14.3	(1/13)	7.7	(0/6)	0	(0/7)	0	(0/13)	0
Cherry tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Chili	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Cucumber	(0/7)	0	(1/6)	16.7	(1/13)	7.7	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Eggplant	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Winged bean	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Lettuce	(0/6)	0	(0/7)	0	(0/13)	0	(0/6)	0	(0/7)	0	(0/13)	0	(0/6)	0	(0/7)	0	(0/13)	0
Sweet potato	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0
Tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
White radish	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0
Total	(0/77)	0	(3/75)	4.0	(3/152)	2.0	(0/77)	0	(1/75)	1.3	(1/152)	0.7	(0/77)	0	(0/75)	0	(0/152)	0

n_p, Total number of positive samples confirmed by MPN-PCR; n_t, Total number of samples; %, Percentage of positive sample.

TABLE 6 | Prevalence of *Listeria* spp. and *L. monocytogenes* in conventional and organic vegetables purchased at retail markets in Malaysia using the MPN-PCR method.

Types of samples	*Listeria* spp.						*L. monocytogenes*					
	Conventional		Organic		Total		Conventional		Organic		Total	
	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%	(n_p/n_t)	%
Cabbage	(1/5)	20.0	(1/6)	16.7	(2/11)	18.2	(1/5)	20.0	(1/6)	16.7	(2/11)	18.2
Calamondin	(1/6)	16.7	(0/7)	0	(1/13)	7.7	(1/6)	16.7	(0/7)	0	(1/13)	7.7
Carrot	(1/6)	16.7	(0/7)	0	(1/13)	7.7	(1/6)	16.7	(0/7)	0	(1/13)	7.7
Cherry tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Chili	(3/7)	42.9	(0/6)	0	(3/13)	23.1	(1/7)	14.3	(0/6)	0	(1/13)	7.7
Cucumber	(1/7)	14.3	(0/6)	0	(1/13)	7.7	(1/7)	14.3	(0/6)	0	(1/13)	7.7
Eggplant	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
Winged bean	(1/7)	14.3	(0/6)	0	(1/13)	7.7	(1/7)	14.3	(0/6)	0	(1/13)	7.7
Lettuce	(1/6)	16.7	(2/7)	28.6	(3/13)	23.1	(1/6)	16.7	(0/7)	0	(1/13)	7.7
Sweet potato	(0/6)	0	(0/6)	0	(0/12)	0	(0/6)	0	(0/6)	0	(0/12)	0
Tomato	(0/7)	0	(0/6)	0	(0/13)	0	(0/7)	0	(0/6)	0	(0/13)	0
White radish	(0/6)	0	(2/6)	33.3	(2/12)	16.7	(0/6)	0	(1/6)	16.7	(1/12)	8.3
Total	(9/77)	11.7	(5/75)	6.7	(14/152)	9.2	(7/77)	9.1	(2/75)	2.7	(9/152)	5.9

n_p, Total number of positive samples confirmed by MPN-PCR; n_t, Total number of samples; %, Percentage of positive sample.

organic lettuce contained larger mesophilic aerobic population than conventional lettuce. Surprisingly, nine out of 12 types of organically and conventionally grown vegetables in this study showed comparable and no significant difference ($P > 0.05$) in mesophilic aerobic population.

Our findings were in tandem with the data obtained from previous studies (Oliveira et al., 2010; Maffei et al., 2013) in which yeasts and molds counts were lower than mesophilic aerobic bacteria counts. Yeasts and molds, depending on genus and species, are the main culprit in most fresh produce spoilage and can be pathogenic. These microbial groups can invade fresh produce in the field prior to harvest and during storage. The presence of yeasts and molds not only link to food spoilage problems in vegetable, they can also pose health risks due to mycotoxins production (Tournas, 2005; Tournas and Katsoudas, 2005). Diseases caused by exposure to mycotoxins include allergic reactions, immunosuppressive diseases, and possibly cancers (Kovács, 2004; Buyukunal et al., 2015).

TABLE 7 | Prevalence and microbial load (MPN/g) of STEC non-O157:H7, *L. monocytogenes* and S. Enteritidis in organic and conventional vegetables purchased at retail markets in Malaysia.

Types of vegetables	STEC non-O157:H7				*L. monocytogenes*				S. Enteritidis			
	Conventional		Organic		Conventional		Organic		Conventional		Organic	
	Prevalence (%)	Microbial load (MPN/g)	Prevalence (%)	Microbial load (MPN/g)	Prevalence (%)	Microbial load (MPN/g)	Prevalence (%)	Microbial load (MPN/g)	Prevalence (%)	Microbial load (MPN/g)	Prevalence (%)	Microbial load (MPN/g)
Cabbage	0	< 3.0	0	< 3.0	20.0	< 3.0–3.0	16.7	< 3.0–3.0	0	< 3.0	0	< 3.0
Calamondin	0	< 3.0	0	< 3.0	16.7	< 3.0–6.0	0	< 3.0	0	< 3.0	0	< 3.0
Carrot	0	< 3.0	0	< 3.0	16.7	< 3.0–3.0	0	< 3.0	0	< 3.0	14.3	< 3.0–3.0
Cherry tomato	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0
Chili	0	< 3.0	0	<3.0	14.3	< 3.0–3.0	0	< 3.0	0	< 3.0	0	< 3.0
Cucumber	0	< 3.0	0	< 3.0	14.3	< 3.0 -3.0	0	< 3.0	0	< 3.0	0	< 3.0
Eggplant	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0
Winged bean	0	< 3.0	0	< 3.0	14.3	< 3.0–3.0	0	< 3.0	0	< 3.0	0	< 3.0
Lettuce	16.7	< 3.0–> 1100	0	< 3.0	16.7	< 3.0–3.0	0	< 3.0	0	< 3.0	0	< 3.0
Sweet potato	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0
Tomato	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0	0	< 3.0
White radish	0	< 3.0	0	< 3.0	0	< 3.0	16.7	< 3.0–3.0	0	< 3.0	0	< 3.0
Total	1.3	< 3.0–> 1100	0	< 3.0	9.1	< 3.0–6.0	2.7	< 3.0–3.0	0	< 3.0	1.3	< 3.0–3.0

Coliform bacteria are commonly used as an indicator of sanitary quality of foods or to check potential contamination of pathogenic microorganisms (Kornacki and Johnson, 2001). In this study, the presence of coliform bacteria in vegetable samples suggested the deterioration of the quality of vegetable due to fecal contamination. It may be caused by different contamination sources, such as the use of polluted irrigation water during pre-harvest, transportation, improper storage conditions, or poor handling practices along the entire food chain (National Advisory Committee on Microbiological Criteria for Foods, 1999). Despite most of the coliform bacteria do not cause disease, uncommon strain, such as *E. coli* O157:H7 is pathogenic to human which contributed toward many foodborne disease outbreaks (Delaquis et al., 2007; Cieslik and Bartoszcze, 2011; Chang et al., 2013). Interestingly, no coliform bacterium was found in both organic and conventional calamondin samples. This might due to the bioactive compounds on the peel of calamondin that protect it from microbial deterioration (Jeong et al., 2004; Rubiatul et al., 2015). Also, the acidic internal environment of calamondin does not favor the growth of coliform bacteria. Although most of the pathogens are distributed on the surface of fresh produce, contamination might occur through the internalization of opportunistic pathogens or spoilage bacteria into fresh produce. Montville and Matthews (2008) and Ryser et al. (2009) pointed out that microorganisms can gain access to the internal tissues of fresh produce via stomata, lenticels, trichomes, lesions caused by plant pathogens, and stem scars. Internalization of pathogens, such as *Salmonella* spp. and *E. coli* O157:H7 in fresh produce also have been widely reported (Bordini et al., 2007; Deering et al., 2012; Ge et al., 2012; Najwa et al., 2015; Nicholson et al., 2015).

In this study, emphasis was given to the detection of *S.* Typhimurium and *S.* Enteritidis among 2,463 serovars of *Salmonella* species. This was mainly due to *S.* Typhimurium and *S.* Enteritidis have been reported to be the most prevalent serovars and common causes of human of salmonellosis (Herikstad et al., 2002; Bangtrakulnonth et al., 2004; Rabsch et al., 2013; Najwa et al., 2015). Hence, it is our interest to investigate the occurrence of these two serovars in Malaysia. Also, based on the previous prevalence studies and epidemiological data, *S.* Typhimurium and *S.* Enteritidis are the common foodborne pathogens detected in Malaysia (Modarressi and Thong, 2010; Nillian et al., 2011; Pui et al., 2011; Adzitey et al., 2012; Najwa et al., 2015; Thung et al., 2016). It is worth noting that *E. coli* O157:H7 and *S.* Typhimurium were not detected in any samples. According to the study by Ryu et al. (2014), neither *E. coli* O157:H7 nor *Salmonella* spp. were detected in conventional and fresh organic produce. In another study conducted by Mukherjee et al. (2004), organic and conventional fresh produce in Minnesota, United States were found to be negative for *Salmonella* but positive for *E. coli* O157:H7. Although *E. coli* O157:H7 was not detected in all the samples, one of the conventional lettuce samples was contaminated with STEC. STEC are well-known as important pathogenic bacteria causing many foodborne illness outbreaks that are linked to consumption of raw vegetables (Loo et al., 2013). STEC can produce Shiga toxin which causes severe bloody diarrhea and results in life-threatening complications, such as

haemolytic-uremic syndrome (HUS) (Mead and Griffin, 1998; Sarimehmetoglu et al., 2009).

The overall prevalence of foodborne pathogens in fresh produce (including conventional and fresh organic produce) were 0.7, 9.2, 5.9, 2.0, and 0.7% for STEC non-O157, *Listeria* spp., *L. monocytogenes*, *Salmonella* spp., and *S.* Enteritidis, respectively, which were comparatively lower as compare to previous local studies (Arumugaswamy et al., 1994; Jeyaletchumi et al., 2010; Chang et al., 2013; Loo et al., 2013; Najwa et al., 2015). These findings are also contrary to the findings by Oliveira et al. (2010) that no pathogen was found in 72 organically and conventionally grown lettuces. In this study, contaminations by *Listeria* spp. and *L. monocytogenes* in both conventional and organic vegetables were obviously observed, and being slightly higher in the conventional vegetables than in the organic vegetables. Although there was a low microbial load of *L. monocytogenes* in the fresh produce (ranging between < 3 and 6.0 MPN/g) and listeriosis cases are also rarely reported in Malaysia, it may pose a safety risk to consumers as a warm and humid environment may encourage proliferation of *L. monocytogenes* to a dangerous level in vegetables (Steinbruegge et al., 1988).

In this study, the comparison of microbiological quality and safety of organic and conventional vegetables showed no trend whether conventional fresh produce is more or less safe than organic ones. Regardless of the cultivation methods, fresh produce can be contaminated starting from the pre-harvest stage, for example, through the use of fresh or non-composted animal manure, irrigation water, wild animals, pests, and insects (Beuchat and Ryu, 1997; Mandrell, 2009; Talley et al., 2009; Mishra et al., 2017). Post-harvest handling activities, such as selection, trimming, precooling, washing, grading, packaging, storage, and transportation can exacerbate the situation (Mandrell, 2009; Buchholz et al., 2012; Maffei et al., 2013).

Additionally, the differences in the contamination levels of vegetables can be affected by farm location, weather or climatic conditions, and types of vegetable crops (e.g., leafy and salad, bulb and stem, root and tuber, flower and flower buds, seed and fruit) (Ryu et al., 2014). In Malaysia, the great difference in price between organic and conventional fresh produce by as much as 100–300%, indirectly may affect the microbiological quality of vegetables. Since fresh organic produce is sold at higher prices compared to those of conventional produce, farmers or retailers tend to use better post-harvest handling practices and higher quality packaging materials for organic produce. As a result, quality and safety of organic vegetables can be preserved.

CONCLUSIONS

Findings in this study indicated that regardless of farming methods, either organic or conventional, raw vegetables can act as a potential vehicle for transmission of *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 and thus, pose a health risk to consumers. Although the use of composted manure as a nutrient source for fresh produce in organic farming is believed to pose a greater risk of microbial contamination, this research found that one particular type of cultivation practices would not affect the microbiological status of fresh produce. However, more works are required to verify the observations in this study. Environmental factors, such as weather conditions and the post-harvest handling practices along the entire food chain should also be taken into account in future studies, since they may also affect the microbial level of organic and conventional fresh produce. The present study provides baseline information on the microbial profiles of organically and conventionally grown vegetables in Malaysia. Meanwhile, the data obtained in this study also serves as useful information in future risk assessment.

AUTHOR CONTRIBUTIONS

CHK, YR, SA, CW, and SR developed the study design. CHK, CSK, and SY co-ordinated the collection of samples in retail markets required for this study. CHK, TT, JP, WC, YL, CT, OR, and SM conducted the microbiological analysis of food samples and carried out the PCR confirmation of specific foodborne pathogens, for example, *Listeria* spp. and *L. monocytogenes*, STEC, *E. coli* O157:H7, *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium. MN provided culture media, PCR reagents, and technical advice on the study. CHK interpreted the data, drafted the manuscript, and revised the manuscript. YR, SA, CW, CSK, SY, and SR vetted the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This research was funded by Research University Grant Scheme Initiative Six (RUGS 6) of Universiti Putra Malaysia (GP-IPS 9438703) and Fundamental Research Grant Scheme (FRGS) of Ministry of Higher Education (MOHE), Malaysia (02-01-14-1475FR) and, in part, by the Kakenhi Grant-in-Aid for Scientific Research (KAKENHI 24249038), Japan Society for the Promotion of Sciences and grant-in-aid of Ministry of Health, Labor and Welfare, Japan.

REFERENCES

Adzitey, F., Rusul, G., and Huda, N. (2012). Prevalence and antibiotic resistance of *Salmonella* serovars in ducks, duck rearing and processing environments in Penang, Malaysia. *Food Res. Int.* 45, 947–952. doi: 10.1016/j.foodres.2011.02.051

Alvarez, J., Sota, M., Vivanco, A. B., Perales, I., Cisterna, R., Rementeria, A., et al. (2004). Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. *J. Clin. Microbiol.* 42, 1734–1738. doi: 10.1128/JCM.42.4.1734-1738.2004

Arumugaswamy, R. K., Ali, G. R., and Abd Hamid, S. N. (1994). Prevalence of *Listeria monocytogenes* in foods in Malaysia. *Int. J. Food Microbiol.* 23, 117–121. doi: 10.1016/0168-1605(94)90227-5

Bangtrakulnonth, A., Pornreongwong, S., Pulsrikarn, C., Sawanpanyalert, P., Hendriksen, R. S., Wong, D. M., et al. (2004). *Salmonella* serovars from humans and other sources in Thailand, 1993–2002. *Emerg. Infect. Dis.* 10, 131–136. doi: 10.3201/eid1001.02-0781

Beuchat, L. R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microb. Infect.* 4, 413–423. doi: 10.1016/S1286-4579(02)01555-1

Beuchat, L. R., and Cousin, M. A. (2001). "Yeasts and molds," in *Compendium of Methods for the Microbiological Examination of Foods,* 4th Edn., eds F. P. Downes and K. Ito (Washington, DC: American Public Health Association), 209–215.

Beuchat, L. R., and Ryu, J. H. (1997). Produce handling and processing practices. *Emerg. Infect. Dis.* 3, 459–465. doi: 10.3201/eid0304.970407

Border, P. M., Howard, J. J., Plastow, G. S., and Siggens, K. W. (1990). Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. *Lett. Appl. Microbiol.* 11, 158–162. doi: 10.1111/j.1472-765X.1990.tb00149.x

Bordini, M. E. B., Ristori, C. A., Jakabi, M., and Gelli, D. S. (2007). Incidence, internalization and behavior of *Salmonella* in mangoes, var. *Tommy Atkins. Food Control.* 18, 1002–1007. doi: 10.1016/j.foodcont.2006.06.003

Brackett, R. E., and Splittstoesser, D. F. (1992). "Fruits and vegetables," in *Compendium of Methods for the Microbiological Examination of Food,* 3rd Edn., eds C. Vanderzant and D. F. Splittstoesser (Washington, DC: A*merican Public Health Association)*, 919–927.

Buchholz, A. L., Davidson, G. R., Marks, B. P., Todd, E. C., and Ryser, E. T. (2012). Quantitative transfer of *Escherichia coli* O157:H7 to equipment during small scale production of fresh-cut leafy greens. *J. Food Prot.* 75, 1184–1197. doi: 10.4315/0362-028X.JFP-11-489

Buyukunal, S. K., Issa, G., Aksu, F., and Vural, A. (2015). Microbiological quality of fresh vegetables and fruits collected from supermarkets in Istanbul, Turkey. *J. Food Nutr. Sci.* 3, 152–159. doi: 10.11648/j.jfns.20150304.13

ÇadIrc, I. O., SirIken, B., Inat, G., and Kevenk, T. O. (2010). The prevalence of *Escherichia coli* O157 and O157:H7 in ground beef and raw meatball by immunomagnetic separation and the detection of virulence genes using multiplex PCR. *Meat Sci.* 84, 553–556. doi: 10.1016/j.meatsci.2009.10.011

Centers for Disease Control and Prevention (2011). *Multistate Outbreak of Human Salmonella Enteritidis Infections Linked to Alfalfa Sprouts and Spicy Sprouts (Final Update)*. Available Online at: http://www.cdc.gov/salmonella/2011/alfalfa-spicy-sprouts-7-6-2011.html (Accessed December 11, 2016).

Centers for Disease Control and Prevention (2012). *Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update)*. Available Online at: http://www.cdc.gov/ecoli/2011/romaine-lettace-3-23-12.html (Accessed December 11, 2016).

Chang, W. S., Afsah-Hejri, L., Rukayadi, Y., Khatib, A., Lye, Y. L., Loo, Y. Y., et al. (2013). Quantification of *Escherichia coli* O157:H7 in organic vegetables and chickens. *Int. Food Res. J.* 20, 1023–1029.

Cieslik, P., and Bartoszcze, M. (2011). Enterohaemorrhagic *Escherichia coli* (EHEC) infections: a threat to public health. *Med. Weter* 67, 571–578.

Deering, A. J., Mauer, L. J., and Pruitt, R. E. (2012). Internalization of *E. coli* O157: H7 and *Salmonella* spp. in plants: a review. *Food Res. Int*. 45, 567–575. doi: 10.1016/j.foodres.2011.06.058

Delaquis, P., Bach, S., and Dinu, L. D. (2007). Behavior of *Escherichia coli* O157:H7 in leafy vegetables. *J. Food Prot*. 70, 1966–1974. doi: 10.4315/0362-028X-70.8.1966

Ge, C., Lee, C., and Lee, J. (2012). The impact of extreme weather events on *Salmonella* internalization in lettuce and green onion. *Food Res. Int*. 45, 1118–1122. doi: 10.1016/j.foodres.2011.06.054

Guan, T. Y., Blank, G., Ismond, A., and Van Acker, R. (2001). Fate of foodborne bacterial pathogens in pesticide products. *J. Sci. Food Agr.* 81, 503–512. doi: 10.1002/jsfa.835

Hashemi, M., Khanzadi, S., and Jamshidi, A. (2010). Identification of *Escherichia coli* O157:H7 isolated from Cattle Carcasses in Mashhad Abattoir by multiplex PCR. *World Appl. Sci. J.* 10, 703–708.

Herikstad, H., Motarjemi, Y., and Tauxe, R. V. (2002). *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiol. Infect.* 129, 1–8. doi: 10.1017/S0950268802006842

Institute of Food Technologists. (2000). *IFT Expert Report on Emerging Microbiological Food Safety Issues-Implications for Control in the 21st Century*. Available Online at: http://www.ift.org/~/media/Knowledge%20Center/Science%20Reports/Expert%20Reports/Emerging%20Microbiological/Emerging%20Micro.pdf (Accessed April 27 2017).

Jeong, S. M., Kim, S. Y., Kim, D. R., Jo, S. C., Nam, K. C., Ahn, D. U., et al. (2004). Effect of heat treatment on the antioxidant activity of extracts from citrus peels. *J. Agric. Food Chem.* 52, 3389–3393. doi: 10.1021/jf049899k

Jeyaletchumi, P., Tunung, R., Margaret, S. P., Son, R., Ghazali, F. M., Cheah, Y. K., et al. (2010). *Listeria monocytogenes* in raw salad vegetables sold at retail level in malaysia. *Food Control* 21, 774–778. doi: 10.1016/j.foodcont.2009.09.008

Johannessen, G. S., Froseth, R. B., Solemdal, L., Jarp, J., Wasteson, Y., and Rorvik, L. M. (2004). Influence of bovine manure as fertilizer on the bacteriological quality of organic Iceberg lettuce. *J. Appl. Microbiol.* 96, 787–794. doi: 10.1111/j.1365-2672.2004.02208.x

Kornacki, J. L., and Johnson, J. L. (2001). "Enterobacteriaceae, coliforms, and Escherichia coli as quality and safety indicators," in *Compendium of Methods for the Microbiological Examination of Foods*, 4th Edn., eds F. P. Downes and K. Ito (Washington, DC: American Public Health Association), 69–82.

Kovács, M. (2004). Nutritional health aspects of mycotoxins. *Orv. Hetil.* 145, 1739–1746.

Kuan, C. H., Goh, S. G., Loo, Y. Y., Chang, W. S., Lye, Y. L., Soopna, P., et al. (2013). Prevalence and quantification of *Listeria monocytogenes* in chicken offal at the retail level in Malaysia. *Poult. Sci.* 92, 1664–1669. doi: 10.3382/ps.2012-02974

Loo, Y. Y., Puspanadan, S., Goh, S. G., Kuan, C. H., Chang, W. S., Lye, Y. L., et al. (2013). Quantitative detection and characterization of Shiga toxin-producing *Escherichia coli* O157 and non-O157 in raw vegetables by MPN-PCR in Malaysia. *Int. Food Res. J.* 20, 3313–3317.

Maffei, D. F., Silveira, N. F. A., and Catanozi, M. P. L. M. (2013). Microbiological quality of organic and conventional vegetables sold in Brazil. *Food Control.* 29, 226–230. doi: 10.1016/j.foodcont.2012.06.013

Magkos, F., Arvaniti, F., and Zampelas, A. (2006). Organic food: buying more safety or just peace of mind? a critical review of the literature. *Crit. Rev. Food Sci. Nutr.* 46, 23–56. doi: 10.1080/10408690490911846

Mandrell, R. E. (2009). "Enteric human pathogens associated with fresh produce: sources, transport, and ecology," in *Microbial Safety of Fresh Produce*, eds X. Fan, B. Niemira, C. J. Doona, F. Feeherry, and R. B. Gravani (Ames, IA: IFT Press/Wiley-Blackwell Publishing), 5–41.

McMahon, M. A. S., and Wilson, I. G. (2001). The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables. *Int. J. Food Microbiol*. 70, 155–162. doi: 10.1016/S0168-1605(01)00535-9

Mead, P. S., and Griffin, P. M. (1998). *Escherichia coli* O157: H7. *Lancet* 352, 1207–1212. doi: 10.1016/S0140-6736(98)01267-7

Mishra, A., Guo, M., Buchanan, R. L., Schaffner, D. W., and Pradhan, A. K. (2017). Development of growth and survival models for *Salmonella* and *Listeria monocytogenes* during non-isothermal time-temperature profiles in leafy greens. *Food Control* 71, 32–41. doi: 10.1016/j.foodcont.2016.06.009

Modarressi, S., and Thong, K. L. (2010). Isolation and molecular sub typing of *Salmonella enterica* from chicken, beef and street foods in Malaysia. *Sci. Res. Essays* 5, 2713–2720.

Mohamad, S. S., Rusdi, S. D., and Hashim, N. H. (2014). Organic food consumption among urban consumers: preliminary results. *Procedia. Soc. Behav. Sci.* 130, 509–514. doi: 10.1016/j.sbspro.2014.04.059

Montville, T. J., and Matthews, K. R. (2008). "Enterohemorrhagic Escherichia coli," in *Food Microbiology: an Introduction, 2nd Edn.,* eds T. J. Montville and K. R. Matthews (Washington, DC: ASM Press), 123–140.

Morton, R. D. (2001). "Aerobic plate count," in *Compendium of Methods for the Microbiological Examination of Foods*, *4th Edn.,* eds F. P. Downes and K. Ito (Washington, DC: American Public Health Association)*,* 63–67.

Mukherjee, A., Speh, D., Dyck, E., and Diez-Gonzalez, F. (2004). Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. *J. Food Prot.* 67, 894–900. doi: 10.4315/0362-028X-67.5.894

Najwa, M. S., Rukayadi, Y., Ubong, A., Loo, Y. Y., Chang, W. S., Lye, Y. L., et al. (2015). Quantification and antibiotic susceptibility of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw vegetables(ulam). *Int. Food Res. J.* 22, 1761–1769.

National Advisory Committee on Microbiological Criteria for Foods (1999). Microbiological safety evaluations and recommendations on fresh produce. *Food Control* 10, 117–143. doi: 10.1016/S0956-7135(99)00026-2

Nicholson, A. M., Gurtler, J. B., Bailey, R. B., Niemira, B. A., and Douds, D. D. (2015). Influence of mycorrhizal fungi on fate of *E. coli* O157: H7 and *Salmonella* in soil and internalization into Romaine lettuce plants. *Int. J. Food Microbiol*. 192, 95–102. doi: 10.1016/j.ijfoodmicro.2014.10.001

Nillian, E., Chai, L. C., Pui, C. F., Tunung, R., Ubong, A., Tuan Zainazor, T. C., et al. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella* Enteritidis and

Salmonella Typhimurium in raw salad vegetables and vegetarian burger patties. *Food Nutr. Sci.* 2, 1077–1081. doi: 10.4236/fns.2011.210144

Olaimat, A. N., and Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: a review. *Food Microbiol.* 32, 1–19. doi: 10.1016/j.fm.2012.04.016

Oliveira, M., Usall, J., Vinas, I., Anguera, M., Gatius, F., and Abadias, M. (2010). Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiol.* 27, 679–684. doi: 10.1016/j.fm.2010.03.008

Pell, A. N. (1997). Manure and microbes: public and animal health problem? *J. Dairy Sci.* 80, 2673–2681. doi: 10.3168/jds.S0022-0302(97)76227-1

Pianetti, A., Sabatini, L., Citterio, B., Pierfelici, L., Ninfali, P., and Bruscolini, F. (2008). Changes in microbial populations in ready-to-eat vegetable salads during shelf-life. *Ital. J. Food Sci.* 20, 245–254.

Pui, C. F., Wong, W. C., Chai, L. C., Nillian, E., Mohamad Ghazali, F., Cheah, Y. K., et al. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. *Food Control* 22, 337–342. doi: 10.1016/j.foodcont.2010.05.021

Rabsch, W., Simon, S., and Humphrey, T. (2013). "Public health aspects of *Salmonella* infections," in *Salmonella in Domestic Animals*, *2nd Edn.*, ed P. A. Barrow and U. Methner (Wallingsford: CABI), 351–376.

Rubiatul, A. S., Nor Helya, I. K., Zarina, Z., Dachyar, A., and Nurul Ain, H. A. (2015). Antibacterial properties of limau kasturi (*C. microcarpa*) peels extract. *Adv. Environ. Biol.* 9, 5–9.

Ryser, E. T., Jao, J., and Yan, Z. (2009). *Internalization of Pathogens in Produce in Microbial Safety of Fresh Produce*, ed X. Fan, B. A. Brendan, C. J. Doona, F. E. Feeherry and R. B. Gravani (Ames, IA: John Wiley & Sons Inc.), 55–80.

Ryu, J. H., Kim, M., Kim, E. G., Beuchat, L. R., and Kim, H. (2014). Comparison of the microbiological quality of environmentally friendly and conventionally grown vegetables sold at retail markets in Korea. *J. Food Sci.* 79, 1739–1744. doi: 10.1111/1750-3841.12531

Sarimehmetoglu, B., Aksoy, M. H., Ayaz, N. D., Ayaz, Y., Kuplulu, O., and Kaplan, Y. Z. (2009). Detection of *Escherichia coli* O157:H7 in ground beef using immunomagnetic separation and multiplex PCR. *Food Control* 20, 357–361. doi: 10.1016/j.foodcont.2008.06.002

Somasundram, C., Razali, Z., and Santhirasegaram, V. (2016). A Review on organic food production in Malaysia. *Hort* 2, 1–5. doi: 10.3390/horticulturae2030012

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G., et al. (1999). Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. *Lett. Appl. Microbiol.* 28, 113–117. doi: 10.1046/j.1365-2672.1999.00488.x

Steinbruegge, E. G., Maxcy, R. B., and Liewen, M. B. (1988). Fate of *Listeria monocytogenes* on ready-to-serve lettuce. *J. Food Prot.* 51, 596–599. doi: 10.4315/0362-028X-51.8.596

Stephenson, J. (1997). Public health experts take aim at a moving target: foodborne infections. *J. Am. Med. Assoc.* 277, 97–98. doi: 10.1001/jama.1997.03540260011004

Talley, J. L., Wayadande, A. C., Wasala, L. P., Gerry, A. C., Fletcher, J., DeSilva, U., et al. (2009). Association of *Escherichia coli* O157:H7 with filth flies (*Muscidae* and *Calliphoridae*) captured in leafy greens fields and experimental transmission of *E. coli* O157:H7 to spinach leaves by house flies. *J. Food Prot.* 72, 1547–1552. doi: 10.4315/0362-028X-72.7.1547

Thung, T. Y., Mahyudin, N. A., Basri, D. F., Wan Mohamed Radzi, C. W. J., Nakaguchi, Y., Nishibuchi, M., et al. (2016). Prevalence and antibiotic resistance of *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw chicken meat at retail markets in Malaysia. *Poult. Sci.* 95, 1888–1893. doi: 10.3382/ps/pew144

Tiraieyari, N., Hamzah, A., and Samah, B. A. (2014). Organic farming and sustainable agriculture in Malaysia: organic farmers' challenges towards adoption. *Asian Soc. Sci.* 10, 1–7. doi: 10.5539/ass.v10n4p1

Tournas, V. H. (2005). Molds and yeasts in fresh and minimally processed vegetables, fruits, and sprouts. *Int. J. Food Microbiol.* 99, 71–77. doi: 10.1016/j.ijfoodmicro.2004.08.009

Tournas, V. H., and Katsoudas, E. (2005). Mould and yeast flora in fresh berries, grapes and citrus fruits. *Int. J. Food Microbiol.* 105, 11–17. doi: 10.1016/j.ijfoodmicro.2005.05.002

Willer, H., and Kilcher, L. (2009). *The World of Organic Agriculture-Statistics and Emerging Trends 2009*. FIBL-IFOAM Report. Geneva; Bonn; Frick: IFOAM; FiBL; ITC.

Williams, C. M. (2002). Nutritional quality of organic food: shades of grey or shades of green? *Proc. Nutr. Soc.* 61, 19–24. doi: 10.1079/PNS2001126

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Kuan, Rukayadi, Ahmad, Wan Mohamed Radzi, Thung, Premarathne, Chang, Loo, Tan, Ramzi, Mohd Fadzil, Kuan, Yeo, Nishibuchi and Radu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 August 2017
doi: 10.3389/fmicb.2017.01493

Rapid Flow Cytometry Detection of a Single Viable *Escherichia coli* O157:H7 Cell in Raw Spinach Using a Simplified Sample Preparation Technique

Anna J. Williams[1], Willie M. Cooper[1], Shawn Ramsaroop[2], Pierre Alusta[1], Dan A. Buzatu[1] and Jon G. Wilkes[1]*

[1] Division of Systems Biology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR, United States, [2] Vivione Biosciences, LLC, Pine Bluff, AR, United States

Very low cell count detection of *Escherichia coli* O157:H7 in foods is critical, since an infective dose for this pathogen may be only 10 cells, and fewer still for vulnerable populations. A flow cytometer is able to detect and count individual cells of a target bacterium, in this case *E. coli* O157:H7. The challenge is to find the single cell in a complex matrix like raw spinach. To find that cell requires growing it as quickly as possible to a number sufficiently in excess of matrix background that identification is certain. The experimental design for this work was that of a U.S. Food and Drug Administration (FDA) In-House Level 3 validation executed in the technology's originating laboratory. Using non-selective enrichment broth, 6.5 h incubation at 42°C, centrifugation for target cell concentration, and a highly selective *E. coli* O157 fluorescent antibody tag, the cytometry method proved more sensitive than a reference regulatory method ($p = 0.01$) for detecting a single target cell, one *E. coli* O157:H7 cell, in 25 g of spinach. It counted that cell's daughters with at least 38× signal-to-noise ratio, analyzing 25 samples in total-time-to-results of 9 h.

Keywords: sample preparation, food pathogens, bacterial detection in food, bacterial quantification, public health

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Satoshi Ishii, University of Minnesota, United States
Lucia Vannini, Università di Bologna, Italy

****Correspondence:***
Anna J. Williams
anna.williams@fda.hhs.gov

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 May 2017*
Accepted: *25 July 2017*
Published: *14 August 2017*

Citation:
Williams AJ, Cooper WM, Ramsaroop S, Alusta P, Buzatu DA and Wilkes JG (2017) Rapid Flow Cytometry Detection of a Single Viable Escherichia coli O157:H7 Cell in Raw Spinach Using a Simplified Sample Preparation Technique. Front. Microbiol. 8:1493. doi: 10.3389/fmicb.2017.01493

INTRODUCTION

Many foodborne outbreaks caused by *Escherichia coli* O157:H7 are associated with vegetables and fruits as a result of fecal contamination from domestic or wild animals at some phase during cultivation or handling (World Health Organization, 2016). Transmission to humans can occur through contacting or consuming contaminated raw foods, milk, or water (World Health Organization, 2016). To address this problem, regulators, food producers, retailers, and distributors need effective microbiological testing for quality control purposes (American Type Culture Collection [ATCC], 2012). Major requirements are for sensitive, specific, and rapid results. Most *E. coli* strains are harmless to the host (Souza et al., 1999), but those that produce Shiga-like toxins cause diarrheal and other significant diseases in humans (Paton et al., 1996). *E. coli* O157:H7, almost all strains of which produce Shiga-like toxins, is the serotype most often associated with pathogenicity and is implicated in many cases of foodborne illness in the United States (Doyle et al., 2006; Gehring et al., 2006).

This organism can cause as high as 50% mortality in the elderly (The Center for Food Security and Public Health, 2009) and kidney failure in children (Reilly, 1998). It is estimated that ingesting only 10–100 cells of *E. coli* O157:H7 can cause foodborne illness (*Escherichia coli* O157:H7, 2017), but many people eat more than 25 g at a sitting and individuals with immature or compromised immune systems may be more sensitive to low level contamination. Thus detection of very low level contaminants is important.

A variety of rapid methods for detecting it in food have been developed to augment or replace plate count techniques (López-Campos et al., 2012). These include the enzyme-linked immunosorbent assay, pulsed-field gel electrophoresis, PCR, microarrays, and flow cytometry (López-Campos et al., 2012).

Objectives for rapid food pathogen detection include decreasing the time to results (TTR) and increasing surveillance throughput. Traditional methods typically take several days to detect and confirm the presence of a pathogen or toxin in a particular food (López-Campos et al., 2012). Since a constraint in food analysis involves existence of indigenous microorganisms, that are not necessarily harmful, however, their being there often hinders the selective identification and isolation of certain pathogens, which are usually present in low numbers (Mandal et al., 2011), it is important for the detection method to have the ability to remove the microorganisms from the food to the detection system (Hardin, 2011). Various strategies, using antibody-based as well as chemical and physical methods, have been developed to isolate pathogens from a variety of food sample matrices (Stevens and Jaykus, 2004; Bhunia, 2008). In the case of flow cytometry detection, isolation from the food is not necessary if physical occlusion of the flow channel can be eliminated and optical interference can be reduced. Occlusion in this work is eliminated by using a flow cell with a wide channel and by filtering sample suspension. Our previous publications have reported a number of sample preparation techniques that reduce optical interference (Wilkes et al., 2012; Buzatu et al., 2013, 2014; Williams et al., 2015).

Here we propose flow cytometry as an alternative instrumental platform. Its use produces results the same day as sample arrival, in the presence of the food matrix, and without the necessity of plate-based strain isolation before determination. That is, *E. coli* O157 can be detected and distinguished from non-pathogenic *E. coli* in perishable foods with fewer days TTR than other technologies allow.

The U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) chapter for detection of Diarrheagenic *E. coli* O157:H7 in food, BAM 4a, specifies the use of PCR, although earlier plate-based methods based on selective media and assessment of colony morphology are also allowed when PCR instruments are not available (Feng et al., 2011). The BAM is the current regulatory or reference method used to detect *E. coli* O157:H7 in foods. The rationale for developing new microbiological methods is to detect the offending pathogen more quickly, with greater specificity, and with greater sensitivity.

The FDA has established protocols for new and rapid validation methods—FDA Methods Validation Guidelines for Microbial Pathogens (FDA Foods Program, Science, Research Steering Committee, 2011, p. 8). These exist in several levels, corresponding to the number of samples run and the number of laboratories involved in the validation exercise. For example, in a Level 1 validation, the originating laboratory characterizes the method with respect to linearity, specificity, inclusivity, and exclusivity. In a Level 2 validation, 20 samples are required to be analyzed by the originating laboratory: 10 non-inoculated and 10 inoculated with the target pathogen and refrigerated overnight (aged) to stress the cells. In a Level 3 validation, 25 samples must be analyzed by the originating laboratory and at least one collaborating laboratory. Refrigeration after inoculation before recovery, enrichment, and analysis must last 48–72 h. Preparatory to a multi-lab validation the agency describes an in-house variant executed by the originating laboratory. This is to fully prove all aspects of the process before potentially wasting time and money on a multi-lab test.

In this study, the rapid and regulatory methods were being analyzed based on parallel samples rather than samples split after enrichment because the two used different enrichment media, incubation temperatures, and non-selective enrichment periods. The different media and enrichment temperatures resulted from extensive optimization of the flow cytometry method intended to reduce TTR as much as possible, to obtain results within the same day rather than after overnight enrichment. Consequently, comparison of sensitivity was only possible based on recoveries—that is, by comparing the percentage of positive recoveries by the two methods for the nominally positive samples. The required sensitivity for the rapid method is equal or better recovery than an accepted regulatory method.

Comparison was made to a reference method (BAM Chapter 4a) for detection of *E. coli* O157:H7 in spinach. These experiments used a non-PCR reference method (Feng et al., 2011) because the laboratory originating the flow cytometry method did not have the equipment or expertise to practice the reference DNA amplification method. The DNA amplification method and the alternative plate method have equivalent sensitivity and either can be used. The non-PCR method is more tedious but can be used whenever a laboratory is not fitted out for PCR. The PCR and non-PCR approved regulatory methods both include a 5 h enrichment step after which selective inhibitors are added to suppress growth of non-target incurred background microflora.

The goal of this work was to demonstrate consistent detection of a single cell of *E. coli* O157:H7 in raw spinach. The results of these experiments are detailed herein.

MATERIALS AND METHODS

Methods of Analysis

A disease outbreak isolate (*E. coli* serotype O157:H7, ATCC 43895), which produces both Shiga-like toxins I and II (Feng et al., 2001), was used as the target strain in this study. The food source was raw spinach obtained in two pound bags (West Creek, Richmond, VA, United States). The stock culture of *E. coli*, was grown 24 h to stationary phase at 37°C in Tryptic Soy Broth (Becton Dickinson and Company, DIFCO™). This

stock was diluted using sterile 1× PBS from 10^{-1} to 10^{-8}. In preparing low level inoculum with a desired concentration of 1 cell/100 μL, the sample custodian first used the flow cytometer with *E. coli* O157 antibodies to determine the target cell concentration in a 10^{-7} dilution grown to stationary phase overnight. Triplicate measurements counted 18, 18, and 23 cells or an average of 19.7 ± 2.4 per 100 μL. A 1:19 dilution of this suspension was estimated to have an average concentration of 0.74 cells per 100 μL. TSA plates were inoculated with 100 μL of this stock suspension and incubated at 37°C for 24 h. The resulting colony counts from three plates (0,0,1 or 0.33 ± 0.47) were used for retrospective confirmation of the estimate but the 1:19 dilution was immediately used for inoculation of all positive samples, both reference BAM and the flow cytometry alternative.

Other aspects of the experimental process are detailed below including more specifics of sample preparation (see Experimental Design and Sample Preparation), the flow cytometer's unusual features (see The Flow Cytometer and RAPID-B Reagents), an approved modification of the BAM reference method to assure fair comparison of sensitivity [see Bacteriological Analytical Manual Processing Method (Modified)], and an abbreviated protocol for sample processing before flow cytometric analysis (see Abbreviated Flow Cytometry Processing Protocol).

Experimental Design and Sample Preparation

Recently we published successful completion of an FDA Level 2 validation study in raw spinach using the flow cytometry system and reagents specific for *E. coli* O157:H7 (Williams et al., 2015). The flow cytometer combined with the appropriately selective tagging reagent detects a few cells of *E. coli* O157:H7, but is insensitive to other *E. coli* serotypes and non-*E. coli* bacteria (Buzatu et al., 2015). Analysis takes 1 min with approximately two additional minutes required for an automated rinse procedure and reloading of the next sample. However, to achieve required sensitivity, eliminate flow cell occlusion, and reduce background interference from the spinach matrix required 18 sample preparation steps including photobleaching, centrifugation, filtration, and gradient centrifugation.

In a single case during the published Level 2 validation, when only a 5-h enrichment was specified, the rapid method failed to detect a single cell (Williams et al., 2015). To address this observation, the enrichment period was lengthened. Simultaneously many other details of the preparation method were simplified. Testing these modifications was another purpose of the work presented here. In addition, the design of an FDA Level 3 validation was used to assure that the method as modified could stand up to a rigorous comparison challenge and qualify for a multi-lab validation.

During further method optimization after the FDA Level 2 validation (Williams et al., 2015), we realized that large scale centrifugation, phloxine B photobleaching, and gradient centrifugation were not necessary to prevent confusion of spinach matrix particles with target cells that grow as rapidly as *E. coli*. In a preliminary experiment, growing the cells an hour and a half longer eliminated the need for preprocessing, particularly the concentration steps, and yielded results comparable to or better than the Level 2 experiments (preliminary data not shown—see Williams et al., 2015). Thus, this work used greatly abbreviated processing for the flow cytometry samples.

For this raw spinach test matrix, addition of a competitor strain was deemed unnecessary by the FDA expert advisor because up to 10^7 non-pathogenic bacteria were already present per gram of spinach as normal flora (email sent to author from agency official on February 27, 2013[1]). The low inoculation level was intended to assess the detection limit for the novel flow cytometry method. Range-finding established its nominal failure at inoculations so low that the most likely explanation was failure to introduce even a single cell of the target pathogen.

Twenty-five samples of 25 g spinach each were processed and analyzed for each method (50 total). The two methods, the rapid and regulatory, were based on flow cytometer event counts and plate counts, respectively. For each method, five blank samples (i.e., containing no *E. coli* O157 cells) as well as 20 samples inoculated with target *E. coli* O157. The target cell inoculations were at a low number such that, for the experimental method, 25–75% fractional recovery (the percentage of nominally false negative results) would be obtained. In setting up the method comparison, 40 of the spinach samples were each inoculated with approximately one cell of *E. coli* O157:H7 per 100 μL as described in Section "Methods of Analysis." Ten spinach samples representing negatives were each inoculated with 100 μL of sterile 1× PBS. Similarly, 40 spinach samples representing positives were each inoculated with 100 μL of the dilution from stock described in Section "Methods of Analysis." In this study, the samples were refrigerator aged for 48 h, as described in Section "Sample Setup," then incubated at 42°C in BHI broth for 6.5 h before analysis. We had determined from several years' experimentation that these incubation conditions were optimal for injured *E. coli* cell recovery and early transition out of lag phase. These experimental designs, criteria, and procedures were based on requirements for an FDA Level 3 validation.

In this study, the reference method was adapted for a smaller 25 g sample as detailed in Section "Bacteriological Analytical Manual Processing Method (Modified)." This method modification was approved by Thomas Hammack, the FDA expert advisor in charge of new rapid method evaluation. Failure to modify the method and consequent addition of the larger volume of PBS appropriate for a 150–200 g sample led to catastrophic failure of the reference method because the one to two cells were much less likely to appear in the aliquot tested.

The rapid and alternative regulatory methods were being analyzed based on parallel samples rather than samples split after enrichment because the two used different enrichment media, different incubation temperatures, and different periods of non-selective enrichment. Therefore, comparison of sensitivity was only possible based on recoveries—that is, by comparing the number positive by the two methods for 20 nominally

[1]Thomas Hammack, email message sent to authors, February 27, 2013.

positive samples each. The required sensitivity was that the rapid alternative method should achieve equivalent or better recovery than the regulatory standard method.

The Flow Cytometer and RAPID-B Reagents

The flow cytometer is a model A40 (Apogee, Hemel Hempstead, England, United Kingdom). Using an unusual flow cell design, the A40 achieves 130 nm optical resolution in low angle and high angle light scattering channels, performance particularly useful for detecting particles the size of bacteria (Wilkes et al., 2012). Its excitation source is a solid state 20 mW 488 nm (blue) laser. Fluorescence emission is detected at flow cytometry standard wavelengths: FL1 = 525 λ, FL2 = 575 λ, and FL3 = >610 λ. To maximize sensitivity for detecting small particle events, a photomultiplier tube is used for each light scatter and fluorescence emission channel. The electronic gains and voltages are factory calibrated so that, using a data acquisition protocol developed for the designated target (here, *E. coli* O157), the transmitted and excluded events are optimal and consistent. This enables sharing of method gate definitions among model A40 instruments (Wilkes et al., 2012).

In RAPID-B, specificity for detection of *E. coli* O157 is obtained by adding, 5 min before cytometric analysis, two reagents the composition of which is detailed in the Level 2 validation study (Williams et al., 2015). Briefly, Reagent A contains *E. coli* O157-specific purified polyclonal antibodies tagged with an FL1-emitting (green) fluorophore (Vivione Biosciences, LLC, Pine Bluff, AR, United States). Reagent B includes a mixture of components that prepare the bacterial cell surfaces freeing epitopes for easy access by the antibody and a membrane-impermeable DNA-intercalating dye then emits in the Fl3 (red) channel. When a bacterium dies, its cell membrane becomes porous so that the dye penetrates in to the DNA and the cell glows red, a signal that it is no longer viable even if it is tagged with the target-specific antibody. Enumeration of target cells is usually counted as events that scatter the incident blue light in expected intensities and that glow green but not red.

Bacteriological Analytical Manual Processing Method (Modified)

As stated above, parallel samples were prepared for BAM 4a analysis using the same inoculum and procedures as for the flow cytometry procedure. The samples were then processed, using a modification of the standard regulatory procedure. Since we only used a 25 g sample of spinach per bag, instead of a 200 g sample amount (typically specified for composite samples), a proportionally lower amount of sterile PBS was added (i.e., 25 mL) to each sample before they were placed on a shaker-incubator for 5 min. 20 mL of 2× modified buffered peptone water pyruvate (mBPWP, Remel, Labsource, Romeoville, IL, United States) was added to each before placing them back into the incubator at 37°C for 5 h. At this point the BAM4a specifies addition of inhibitors to which background microflora are typically more sensitive than the target *E. coli* O157:H7. We added 333 μL of an ACV cocktail (Acriflavine, Cefsulodin, Vancomycin) containing Acriflavine and Cefsulodin at 7.5×10^{-4} g/mL each and Vancomycin at 6.0×10^{-4} g/mL, all from MP Biomedicals, LLC, Solon, OH, United States. The volume added was adjusted proportionally for the smaller 45 mL suspension volume for single 25 g spinach samples. All subsequent processing for the regulatory samples was the same as outlined in the BAM manual (Feng et al., 2011).

Abbreviated Flow Cytometry Processing Protocol

As mentioned above, this work used greatly abbreviated processing for the flow cytometry samples, which simplified the sample handling protocol. Compared to 18 previously used, five sample setup, pre-analysis, and analysis steps are required in this abbreviated method to determine *E. coli* O157:H7. (Only the first of the six steps listed below under Setup would be used when analyzing real-world unknowns for incurred contamination.) The TTR for 25 samples was equal to that in the Level 2 work (Williams et al., 2015), even with the extra 1.5 h enrichment, because of the smaller number of preparation steps.

Sample Setup

1. Twenty-five grams of spinach were weighed into each of a specified number of individual sterile Whirl-Pak filter bags.

2. A 100 μL volume of either *E. coli* O157:H7 ATCC 43895 or sterile 1× PBS was inoculated into each sample bag.

3. The inoculum was massaged into the spinach leaves and placed in a refrigerator set at 3–5°C for 48 h.

4. To confirm cell counts, 100 μL of the stock dilution used to inoculate each bag of spinach, was plated onto triplicate TSA plates and incubated at 37°C overnight (17 h).

5. Forty-eight hours later a 75 mL aliquot of sterile, preheated BHI (stored at 42°C overnight) broth was added to each sample to be analyzed on the flow cytometer.

6. Each sample to be analyzed using flow cytometry was then massaged by hand, 10× each, before being placed in the 42°C incubator for 6.5 h to allow for growth of the bacteria.

Pre-analysis

7. After the 6.5 h enrichment, each sample was massaged by hand one final time to ensure homogeneous suspension of the bacteria.

8. A 1 mL aliquot from each suspension was filtered into a sterile 2 mL microcentrifuge tube using a 5-μm pore size 25 mm diameter PVDF syringe filter (Millipore Corporation, Billerica, MA, United States; Becton Dickinson and Company, Sparks, MD, United States).

9. A 100-μL aliquot of the filtrate was mixed with 650 μL of sterile 1× PBS, 240 μL of Reagent B, and 10 μL of Reagent A. This was gently vortexed at a setting of 2 on a Vortex-Genie 2 fitted with a 48 hole foam rack (Daigger, Wheaton, IL, United States) at lab ambient temperature for 5 min before analysis.

Analysis

10. Samples were analyzed every 3 min on the flow cytometer (1 h and 15 min for 25 samples). Details of instrumental setup, operation, and cleanup between samples can be found at Williams

TABLE 1 | Results using the In-House Level 3 Validation experimental design.

Sample Number	Cytometry Cell Counts (Cts.; Re-Cts.)	Cytometry I.D.*	BAM I.D.*	"Positive" Samples	Negative Samples
1	0	–	–		–
6	0	–	–		–
9	0	–	–		–
13	1	–	–		–
21	0	–	–		–
2	13;13↑	+	+	+	
3	0	–	–	+	
4	0	–	–	+	
5	86	+	–	+	
7	0	–	–	+	
8	0	–	–	+	
10	40	+	–	+	
11	157	+	+	+	
12	6;31↑	+	–	+	
14	0	–	–	+	
15	1	–	–	+	
16	12220	+	–	+	
17	3201	+	–	+	
18	312	+	–	+	
19	0	–	+	+	
20	413	+	+	+	
22	1713	+	–	+	
23	520	+	–	+	
24	1591	+	–	+	
25	2780	+	–	+	

**Bold red "–" symbols indicate inconsistency when compared to "Positive" Sample I.D.*[†]*For the rapid method, the second number in a pair represents the event count from a reprocessed aliquot of sample that was left at ambient laboratory temperature overnight. Reprocessing was done in the two cases when initial screens were ambiguous. Counts were as 476 measured after the 6.5 h enrichment. Re-counts were measured after refrigeration overnight.*

et al. (2015, section 2.4). No events appeared in the final counting gate for non-inoculated samples.

RESULTS

In these studies, neither method reported false positives. The flow cytometry method TTR for 25 samples was 9 h, including the 6.5 h incubation time; if a sample reported an ambiguous result it was possible to repeat analysis the next day. A result was considered ambiguous if the counted number of events greatly exceeded typical negatives (0 or 1) and was far fewer than typical positives (20s, hundreds, thousands). For the BAM, TTR was much longer, 51–60 h. The plate-based and PCR regulatory method reported a much higher percentage of negative results for nominally positive samples, 16 of 20 (80%), compared to the rapid method's 7 of 20 (35%). An explanation of results in **Table 1** is included in the paragraphs that follow.

The Sample Numbers for Cytometry and BAM analysis samples correspond with respect to the nominal "Positive" Sample I.D., but individual samples are not the same for BAM and Cytometry nor do they necessarily correspond for positive samples with respect to actual I.D. because an inoculated, nominally positive sample might or might not have actually contained any *E. coli* O157 cells. Lack of correspondence between nominally positive Cytometry and BAM is possible because they were parallel, not split after enrichment. Experimental design causing this potential discrepancy was necessary because, unlike the BAM, the Cytometry enrichment conditions were fully optimized for consistent recovery of very low level contaminations measurable within the same day of sample arrival and without strain isolation.

Initial flow cytometry results were ambiguous in two cases, Sample 2 and Sample 12. These samples were reanalyzed the next day after overnight refrigeration. Flow cytometer Samples 2 and 12 were ambiguous because, respectively, the 13 or 6 counted events were so few in comparison to other positive samples, but the observed events appeared to cluster in the middle of the final counting gate like real target cells (See **Figure 1**). After reprocessing, they were confirmed as true positives. Because of the characteristic location of the signals and the absence of nearby matrix signals, these 13 or 31 counts, respectively, were no longer ambiguous.

A substantial portion of the BAM TTR involved overnight plate confirmation of presumed positives by re-culturing isolates on Tellurite Cefixime Sorbitol MacConkey Agar plates (TC-SMAC). The regulatory method did not report questionable results in these tests so BAM repeat analyses were not warranted.

DISCUSSION

For these experiments, the cytometry-estimated and plate-confirmed inoculation levels were 0.74 ± 0.43 and 0.25 ± 0.43 cfu/100 μL, respectively. That is, the majority of the nominally positive samples were actually inoculated with only one or zero viable *E. coli* O157:H7 cells. Two viable cells, appearing more than two standard deviations above the average, would occur in approximately 5% of the cases, 1 of 20 samples. The flow cytometry method achieved positive results in 65% of the nominally positive inoculations, which met an FDA method validation criterion that inoculation levels be chosen such that the rapid method achieves 25–75% positive recovery. The 65% observed recovery by the experimental rapid method is consistent with an explanation that it failed only in samples where zero recoverable cells were inoculated. The BAM 4a regulatory method yielded 4/20 (20%) positive samples. These results represent a statistically significant performance difference between the flow cytometry method and the reference method ($p = 0.01$ in a two-sided Fisher's exact test). That is, the flow cytometry method was more sensitive than the regulatory method in these experiments is confirmed with statistically significant certainty.

The lack of ambiguity implied that reduced BAM sensitivity might be explained by factors influencing recovery that preceded selective plate confirmation steps (i.e., no observable colonies to recover for more specific plating). A potential explanation for

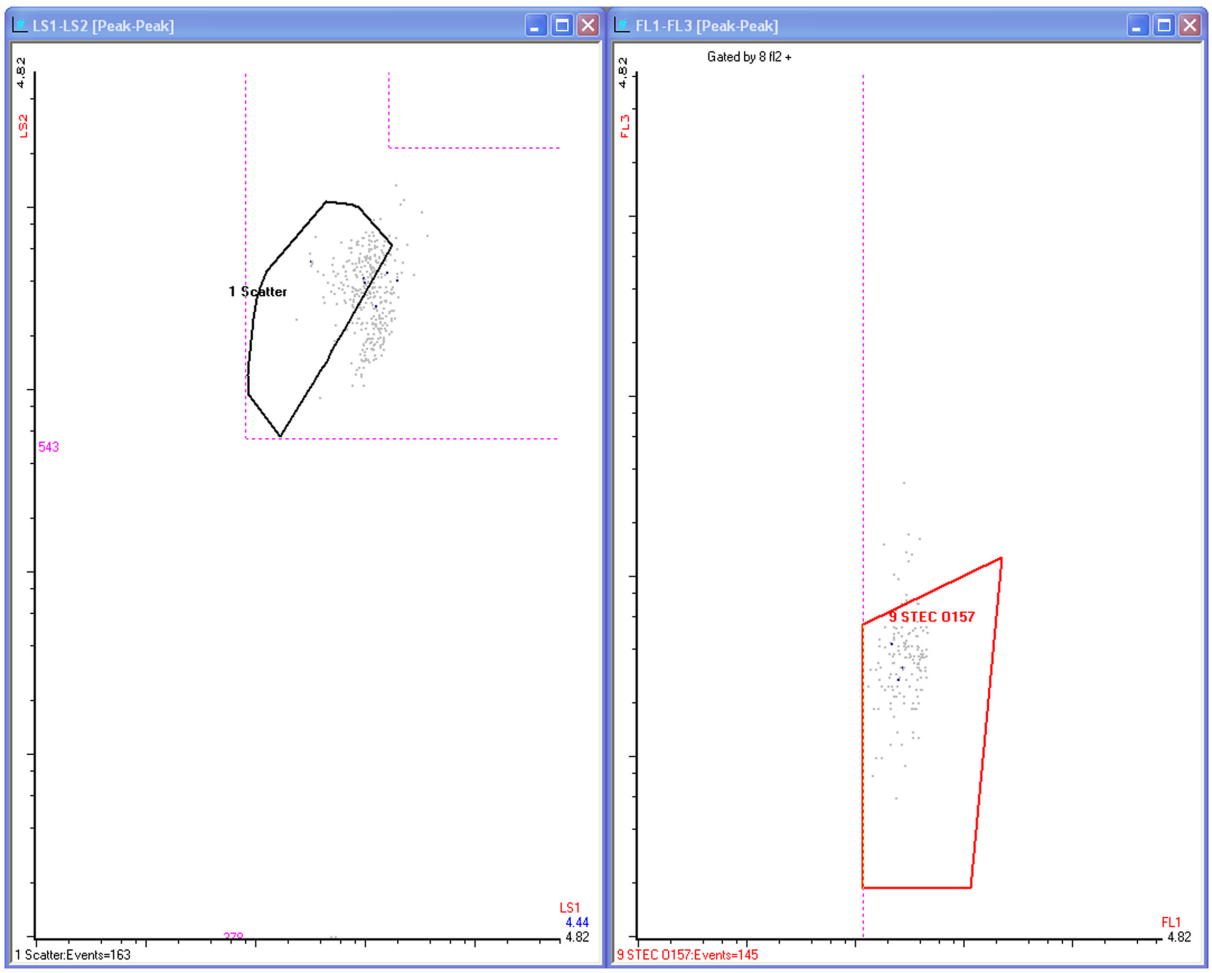


FIGURE 1 | This shows two of the flow cytometer screens, dot plots for light scatter and fluorescence emission for a typical positive sample in this work. The cluster of dots appearing within the FL1 (green) vs FL3 (red) fluorescence gate are events transmitted through multiple gates and each dot represents detection of a viable *E. coli* O157 cell. They number 145, seen at the left bottom of the plot.

BAM 4a reduced sensitivity is that its enrichment period prior to introduction of growth inhibitors that depress competitive microflora may be too short to allow consistent recovery of a single, stressed target cell. The BAM PCR-based and plate-based regulatory methods both add selective growth inhibitors at five h post-inoculation. The lag phase is known to lengthen when bacteria are isolated and/or stressed (Feng et al., 2011). These results suggest the detection limit for the current BAM 4a official method may be greater than one cell and that the flow cytometry method with a 6.5 h non-competitive enrichment may be more sensitive. BAM 4a might become as reliably sensitive as the flow cytometry method by postponing addition of growth inhibitors an extra hour and a half, a strategy that assumes such postponement would not lead to interference at a later stage of the assay from the increased abundance of competitive microflora. The flow cytometer method appears to be less vulnerable to the effects of competitive microflora, perhaps because its analysis is completed after 6.5–9 h, responses are measured for each cell, and unlike the BAM PCR method there is no minimum number of cells (required by PCR prior to DNA amplification) so that signal is sufficient to record a positive.

For any sample that the flow cytometer determined to be positive, there were at least six counts although typically there were many more (1920 ± 3280). Each sample defined as blank by the key showed counts of only 0 or 1. Based on the sample key, such samples were correctly classified as negatives. There were no confirmed false positives by either method. Of seven nominally positive samples deemed negative by the flow cytometry method, six reported zero counts and one reported a single count. The nominal positive/actual negatives and true negatives averaged 0.16 ± 0.37 counts. The minimum signal-to-noise ratio for the smallest count eventually deemed positive was 6/0.16 = 38. The average signal-to-noise ratio was 1920/0.16 = 12,000.

The four orders of magnitude range of cells counted after the 6.5 h enrichment were estimated to have arisen in 95% of the cases from a single viable cell inoculated. The fact that up to

2.5 orders of magnitude difference can result from apparently similar circumstances underscores the critical contribution of cell stress in determining how quickly it is feasible to enrich the cell numbers during such experiments. Evidence that bacterial cell isolation is a major contributor to stress (and thus that a lengthened lag phase might occur) can be inferred by comparison to results from our other publications in this series (Buzatu et al., 2013; Williams et al., 2015). In those experiments when inoculation levels were only a little higher (14 cells and two to four cells, respectively) it was possible to observe clusters of counts estimated to arise from low single digit inoculations. That is, when a particular number of cells was inoculated, the resulting cell count after short-term enrichment was fairly consistent among samples and the different clusters of similar cell counts appeared to be a linear function of a hypothesized low-single-digit number inoculated. In other words, when a cell was not completely alone, its lag phase duration and consequent post-enrichment cell counts were more predictable. The hypothesized solitary cell phenomenon causing a lengthened lag phase is consistent with the concept of "quorum sensing" amongst bacterial cells (Miller and Bassler, 2001).

CONCLUSION

The flow cytometry method for determining *E. coli* O157:H7 contamination in raw spinach provided results in 9 h for 25 samples compared to 60 h required by the reference regulatory method. Comparison of results for nominally positive samples showed that the flow cytometry method is significantly more sensitive for detecting *E. coli* O157:H7 in raw spinach than the BAM method. Using only four steps of sample preparation and analysis, the cytometry method detected single cell contamination with a more than 38× signal-to-noise ratio. In summary, compared to BAM 4a, the flow cytometry method (1) takes much less time, (2) is much less labor intensive, (3) is as accurate, and (4) is more sensitive. For these reasons, the abbreviated cytometry protocol with flow cytometry detection has potential utility as a screening tool to detect *E. coli* O157:H7 in foods.

AUTHOR CONTRIBUTIONS

DB and JW are principal investigators of the project. AW and WC are co-PI's who performed the experiments. AW and JW wrote the manuscript, with assistance from DB in editing. SR set up inoculations, validated the inoculation levels by plate count, and served as sample custodian. PA assisted in the experiments and helped with the editing.

ACKNOWLEDGMENTS

We thank Gwendolyn Anderson, Joanna Deck, and Deborah Bass for providing supplies and advice. We thank Beth Juliar and Stephen Harris for statistical analysis.

REFERENCES

American Type Culture Collection [ATCC] (2012). *Setting the Standard with "Big Six" Non-O157 STEC Reference Materials.* Food Safety Magazine. Available at: www.foodsafetymagazine.com/magazine-archive1/octobernovember-2012/food-safety-insider-rapid-micro-solutions/setting-the-standard-with-e2809cbig-sixe2809d-non-o157-stec-reference-materials (accessed August 1, 2017).

Bhunia, A. K. (2008). Biosensors and bio-based methods for the separation and detection of foodborne pathogens. *Adv. Food Nutr. Res.* 54, 1–44. doi: 10.1016/S1043-4526(07)00001-1

Buzatu, D., Cooper, W. M., Summage-West, C., Sutherland, J. B., Williams, A. J., Bass, D. A., et al. (2013). Photobleaching with phloxine B sensitizer to reduce food matrix interference for detection of *Escherichia coli* serotype O157:H7 in fresh spinach by flow cytometry. *Food Microbiol.* 36, 416–425. doi: 10.1016/j.fm.2013.07.007

Buzatu, D. A., Moskal, T. J., Williams, A. J., Cooper, W. M., Mattes, W. B., and Wilkes, J. G. (2014). An integrated flow cytometry-based system for real time, high sensitivity bacterial detection and identification. *PLoS ONE* 9:e94254. doi: 10.1371/journal.pone.0094254

Buzatu, D. A., Wilkes, J. G., Moskal, T. A., Nevius, B., Taylor, J. T., Tucker, R. K., et al. (2015). Flow cytometry-based systems and methods for detecting microbes. U.S. Patent No. 9,194,868. Washington, DC: U.S. Patent and Trademark Office.

Doyle, M. E., Archer, J., Kaspar, C. W., and Weiss, R. (2006). *Human Illness Caused by E. coli O157:H7 from Food and Non-Food Sources*. Available at: https://fri.wisc.edu/files/Briefs_File/FRIBrief_EcoliO157H7humanillness.pdf (accessed August 1, 2017).

Escherichia coli O157:H7 (2017). *See under Heading Transmission*. Available at: https://en.wikipedia.org/wiki/Escherichia_coli_O157:H7

FDA Foods Program, Science, Research Steering Committee (2011). *Foods and Veterinary Medicine Science and Research Steering Committee.* Availble at: www.fda.gov/downloads/ScienceResearch/ucm273918.pdf (accessed August 2, 2017).

Feng, P., Dey, M., Abe, A., and Takeda, T. (2001). Isogenic strain of *Escherichia coli* O157:H7 that has lost both shiga toxin 1 and 2 genes. *Clin. Diagn. Lab. Immunol.* 8, 711–717. doi: 10.1128/CDLL8.4.711-717.2001

Feng, P., Weagent, S. D., and Jinneman, K. (2011). *BAM: Diarrheagenic Escherichia coli.* Available at: https://wayback.archive-it.org/7993/20170722103534/https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm (accessed August 1, 2017).

Gehring, A., Albin, D. M., Irwin, P. L., Reed, S. A., and Tu, S. I. (2006). Comparison of enzyme-linked immunomagnetic chemiluminescence with U.S. food and drug administration's bacteriological analytical manual method for the detection of *Escherichia coli* O157:H7. *J. Microbiol. Methods* 67, 527–533. doi: 10.1016/j.mimet.2006.04.025

Hardin, M. (2011). *Microbial Detection – Taking It to the Limits.* Food Safety Magazine. Available at: www.foodsafetymagazine.com/magazine-archive1/junejuly-2011/microbial-detection-taking-it-to-the-limits (accessed August 2, 2017).

López-Campos, G., Martínez-Suárez, J. V., Aguado-Urda, M., and López-Alonso, V. (2012). *Microarray Detection and Characterization of Bacterial Foodborne Pathogens: Springer Briefs in Food, Health, and Nutrition*. New York, NY: Springer Science and Business Media, LLC.

Mandal, P. K., Biswas, A. K., Choi, K., and Pal, U. K. (2011). Methods for rapid detection of foodborne pathogens: an overview. *Am. J. Food Technol.* 2, 87–102. doi: 10.3923/ajft.2011.87.102

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Paton, A. W., Ratcliff, R. M., Doyle, R. M., Seymour-Murray, J., Davos, D., Lanser, J. A., et al. (1996). Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* 34, 1622–1627.

Reilly, A. (1998). "Prevention and control of enterohaemorrhagic *Escherichia coli* (EHEC) infections: Memorandum from a WHO meeting." *Bull. World Health Organ.* 76, 245–255.

Souza, V., Rocha, M., Valera, A., and Equiarte, L. E. (1999). Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Appl. Environ. Microbiol.* 65, 3373–3385.

Stevens, K. A., and Jaykus, L. A. (2004). Bacterial separation and concentration from complex sample matrices: a review. *Crit. Rev. Microbiol.* 30, 7–24. doi: 10.1080/10408410490266410

The Center for Food Security and Public Health (2009). *Enterohemorrhagic Escherichia coli and other E. coli Causing Hemolytic Uremic Syndrome*. Ames, IA: Iowa State University.

Wilkes, J. G., Tucker, R. K., Montgomery, J. A., Cooper, W. M., Sutherland, J. B., and Buzatu, D. A. (2012). Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. *Food Microbiol.* 30, 281–288. doi: 10.1016/j.fm.2011.11.002

Williams, A. J., Cooper, W. M., Summage-West, C. V., Sims, L. M., Woodruff, R., Christman, J., et al. (2015). Level 2 validation of a flow cytometric method for detection of *Escherichia coli* O157:H7 in raw spinach. *Int. J. Food Microbiol.* 215, 1–6. doi: 10.1016/j.ijfoodmicro.2015.08.011

World Health Organization (2016). *E. coli Fact Sheet*. Available at: http://www.who.int/mediacentre/factsheets/fs125/en/

Disclaimer: The views presented do not necessarily reflect opinions or official policy of the U.S. Food and Drug Administration.

Conflict of Interest Statement: Some of the authors are inventors of technologies, which have been patented by the US FDA, related to this paper, and for which royalties can be received.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Williams, Cooper, Ramsaroop, Alusta, Buzatu and Wilkes. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 September 2017
doi: 10.3389/fmicb.2017.01828

Gene Expression Response of *Salmonella enterica* Serotype Enteritidis Phage Type 8 to Subinhibitory Concentrations of the Plant-Derived Compounds *Trans*-Cinnamaldehyde and Eugenol

Anup Kollanoor Johny[1], Jonathan G. Frye[2], Annie Donoghue[3], Dan J. Donoghue[4], Steffen Porwollik[5], Michael McClelland[5] and Kumar Venkitanarayanan[6]*

[1] *Department of Animal Science, University of Minnesota, Saint Paul, MN, United States,* [2] *Bacterial Epidemiology and Antimicrobial Resistance Research Unit, USDA-ARS, Richard B. Russell Research Center, Athens, GA, United States,* [3] *Poultry Production and Product Safety Research Unit, USDA, Fayetteville, AR, United States,* [4] *Department of Poultry Science, University of Arkansas, Fayetteville, AR, United States,* [5] *Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA, United States,* [6] *Department of Animal Science, University of Connecticut, Storrs, CT, United States*

OPEN ACCESS

Edited by:
Maria Schirone, Università di Teramo, Italy

Reviewed by:
Alberto Quesada, University of Extremadura, Spain
Héctor Argüello, University of Córdoba, Colombia

***Correspondence:**
Anup Kollanoor Johny
anupjohn@umn.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *16 May 2017*
Accepted: *06 September 2017*
Published: *26 September 2017*

Citation:
Kollanoor Johny A, Frye JG, Donoghue A, Donoghue DJ, Porwollik S, McClelland M and Venkitanarayanan K (2017) Gene Expression Response of Salmonella enterica Serotype Enteritidis Phage Type 8 to Subinhibitory Concentrations of the Plant-Derived Compounds Trans-Cinnamaldehyde and Eugenol. Front. Microbiol. 8:1828. doi: 10.3389/fmicb.2017.01828

Background: *Salmonella* Enteritidis phage type 8 (PT8) is a major poultry-associated *Salmonella* strain implicated in foodborne outbreaks in the United States. We previously reported that two plant-derived compounds generally recognized as safe (GRAS), *trans*-cinnamaldehyde (TC), and eugenol (EG), significantly reduced *S*. Enteritidis colonization in broiler and layer chickens. To elucidate potential PT8 genes affected by TC and EG during colonization, a whole-genome microarray analysis of the bacterium treated with TC and EG was conducted.

Results: *S*. Enteritidis PT8 was grown in Luria-Bertani broth at 37°C to an OD_{600} of ∼0.5. Subinhibitory concentrations (SICs; concentration that does not inhibit bacterial growth) of TC (0.01%; 0.75 mM) or EG (0.04%; 2.46 mM) were then added to the culture. *S*. Enteritidis PT8 RNA was extracted before and 30 min after TC or EG addition. Labeled cDNA from three replicate experiments was subsequently hybridized to a microarray of over 99% of *S.* Enteritidis PT4 genes, and the hybridization signals were quantified. The plant-derived compounds down-regulated ($P < 0.005$) expression of *S*. Enteritidis PT8 genes involved in flagellar motility, regulation of the *Salmonella* Pathogenicity Island 1, and invasion of intestinal epithelial cells. TC and EG also suppressed transcription of genes encoding multiple transport systems and outer membrane proteins. Moreover, several metabolic and biosynthetic pathways in the pathogen were down-regulated during exposure to the plant-derived compounds. Both TC and EG stimulated the transcription of heat shock genes, such as *dnaK*, *dnaJ*, *ibpB*, and *ibpA* in *S*. Enteritidis

PT8 ($P < 0.005$). The results obtained from microarray were validated using a quantitative real-time PCR.

Conclusion: The plant-derived compounds TC and EG exert antimicrobial effects on *S*. Enteritidis PT8 by affecting multiple genes, including those associated with virulence, colonization, cell membrane composition, and transport systems.

Keywords: plant-derived, *trans*-cinnamaldehyde, eugenol, *Salmonella* Enteritidis PT8, microarray, antibacterial

BACKGROUND

Salmonella enterica serovar Enteritidis (*S*. Enteritidis) is one of the most commonly isolated *Salmonella* serotypes from poultry (Centers for Disease Control and Prevention, 2010; Campioni et al., 2013; Gould et al., 2013), and is responsible for about one third of the reported human salmonellosis outbreaks in the United States (Gould et al., 2013). The pathogen poses a significant health concern for human health due to the contamination of poultry meat and eggs, which constitute the most common food products linked to human salmonellosis (Guard-Petter, 2001; Marcus et al., 2007). In chickens, the cecum is the most common site for *Salmonella* residency (Gantois et al., 2009; Kollanoor Johny et al., 2012a,b,c). The cecal colonization by the pathogen results in fecal shedding, invasion of reproductive organs, contamination of egg shells and yolks, and carcass contamination during slaughter (Keller et al., 1995; Gantois et al., 2009). Therefore, reducing *S*. Enteritidis in the chicken intestinal tract would reduce contamination of poultry products, minimizing human health risk (Altekruse et al., 1993). Thus, intervention strategies for controlling *S*. Enteritidis colonization in chickens are critical for improving the microbiological safety of poultry-derived foods. Since a multitude of sources can transmit *S*. Enteritidis to chickens at farms, a variety of pre-harvest approaches, especially in-feed supplementation of antimicrobials, has been explored for reducing the pathogen persistence in poultry (reviewed in Kollanoor Johny et al., 2012a,b,c).

We previously reported that two phytophenolics, namely *trans*-cinnamaldehyde (TC) and eugenol (EG), were effective in reducing *S*. Enteritidis *in vitro* and in broiler chickens (Kollanoor Johny et al., 2008, 2010, 2012a,b,c). Further, we observed that in-feed supplementation of TC in layers significantly reduced *S*. Enteritidis colonization in the internal organs of birds and egg-borne transmission of the bacterium (Upadhyaya et al., 2015). *Trans*-cinnamaldehyde, an aromatic aldehyde extracted from the bark of cinnamon (*Cinnamomum zeylandicum*), has well-known antimicrobial properties (Friedman et al., 2002, reviewed by Burt, 2004). Eugenol (EG), a compound obtained from clove (*Eugenia caryophillis*) oil, is also reported to be effective in killing pathogenic microorganisms (Suhr and Nielsen, 2003, reviewed by Burt, 2004). Both these compounds are classified as generally recognized as safe (GRAS) for use in foods by the United States Food and Drug Administration (USFDA) (TC–21CFR182.60; EG–21CFR582.60).

In light of our previous findings, the objective of this study was to analyze the genome-wide response of *S*. Enteritidis Phage Type 8 (or PT8) to a sub-inhibitory concentration of TC or EG using DNA microarrays. We sought to delineate the arsenal of genes affected by the two phytochemicals.

Abbreviations: TC, *trans*-cinnamaldehyde; EG, Eugenol; SIC, subinhibitory concentration; cDNA, complementary DNA; CDC, Centers for Disease Control and Prevention; GRAS, Generally Recognized as Safe; USFDA, United States Food and Drug Administration; RT-qPCR, Real-Time Quantitative Polymerase Chain Reaction; mRNA, messenger RNA; M, Molar.

RESULTS

The DNA microarray analysis revealed that about 10% of genes in the *S*. Enteritidis PT8 genome were significantly modulated after exposure to TC and EG. The major genes predicted to be differentially regulated in PT8 exposed to TC and EG based on the microarray data are provided in **Table 1**. The analysis identified 566 genes (9.7% genes on the array) with differentially expressed transcripts due to exposure to TC, and 483 genes (8.4% on the array) with differential expression as a result of EG exposure ($M \pm 1.5$, $P < 0.005$; **Tables 2**, **3**). TC significantly down-regulated 275 genes, whereas EG did so for 289 genes ($P < 0.005$). Many of the genes modulated are known to play a critical role in *S*. Enteritidis virulence *in vivo* based on previously published reports (Dhawi et al., 2011; Harvey et al., 2011) and are discussed here. Additional data on genes that are not discussed in depth in the manuscript can be found in the Supplementary Tables S1–S19.

Salmonella Pathogenicity Island-1 (SPI-1) and Type Three Secretion System Genes (T3SS)

The virulence genes associated with the major pathogenicity island, SPI-1, were affected in response to TC exposure, including major regulators, such as *hilC* and *hilD* (**Table 1**). Transcription of these genes was down-regulated 2.0- and 2.2-fold, respectively, by TC ($P < 0.005$). In addition, genes associated with cell invasion (*sipABCD*) and outer membrane protein syntheses (*sopB*) were significantly down-regulated by TC (**Table 2**).

Motility, Chemotaxis, and Adherence

Several genes associated with *Salmonella* motility, chemotaxis and adherence to host cells were down-regulated by both plant-derived compounds, although EG was significantly more effective than TC (**Tables 1**, **3**). For example, EG significantly down-regulated the transcription of genes responsible for bacterial

TABLE 1 | Major genes predicted to be differentially regulated in PT8 exposed to SICs of both TC and EG ($P < 0.005$, M ± 1.5), based on the microarray data.

Gene	Definition/Function/Gene product	TC	EG
***Salmonella* PATHOGENICITY ISLAND-1 (SPI-1)**			
hilC	Invasion genes transcription activator	−2.2	−3.0
hilD	Regulatory helix-turn-helix proteins, araC family	−2.0	−1.6
sicA	Surface presentation of antigens; secretory proteins	−1.4	−1.5
sprB	Possible AraC-family transcriptional regulator	−1.6	−1.5
MOTILITY AND CHEMOTAXIS			
flhD	Regulator of flagellar biosynthesis, acts on class 2 operons	−1.5	−1.5
fliC	Flagellar biosynthesis; flagellin, filament structural protein	−1.4	−2.2
fliZ	Putative regulator of FliA	−1.3	−2.4
OUTER MEMBRANE PROTEINS			
ompW	Outer membrane protein W; colicin S4 receptor	−4.3	−2.7
ompC	Outer membrane protein 1b (ib;c), porin	−2.8	−2.8
ompS1	Putative porin	−2.3	−1.7
nmpC	New outer membrane protein; predicted bacterial porin	−4.6	−3.5
tsx	Nucleoside channel receptor of phage T6 and colicin K	−1.8	−1.8
CARBOHYDRATE METABOLISM AND TRANSPORT			
agp	Glucose-1-phosphatase	−1.8	−1.8
mglB	ABC superfamily (peri_perm), galactose transport protein	−1.9	−3.4
cydA	Cytochrome d terminal oxidase, polypeptide subunit I	−3.1	−3.0
frdA	Fumarate reductase, anaerobic, flavoprotein subunit	−2.0	−2.6
frdB	fumarate reductase, anaerobic, Fe-S protein subunit	−2.0	−2.0
mglA	ABC superfamily, galactose transport protein	−1.7	−2.8
mglC	ABC superfamily, methyl-galactoside transport protein	−1.5	−1.8
AMINOACID TRANSPORT			
tdcB	Threonine dehydratase, catabolic	−2.4	−3.7
tdcA	Transcriptional activator of tdc operon (LysR family)	−2.2	−3.0
tdcG	L-serine deaminase	−2.8	−3.0
pepT	Putative peptidase T (aminotripeptidase)	−2.5	−1.6
speF	Ornithine decarboxylase isozyme, inducible	−5.2	−4.3
potE	APC family, putrescine/ornithine antiporter	−4.2	−3.2
TETRATHIONATE REDUCTION			
ttrS	Tetrathionate reductase complex: sensory kinase	−2.2	−2.3
PROPANEDIOL UTILIZATION			
pocR	Propanediol utilization: transcriptional regulation, AraC family	−2.5	−2.2
pduA	Propanediol utilization: polyhedral bodies	−2.5	−1.9
pduB	Propanediol utilization: polyhedral bodies	−4.0	−3.5
pduC	Propanediol utilization: dehydratase, large subunit	−4.2	−3.8
pduD	Propanediol utilization: dehydratase, medium subunit	−4.2	−4.6
pduE	Propanediol utilization: dehydratase, small subunit	−2.1	−1.6
pduG	Propanediol utilization: diol dehydratase reactivation	−3.2	−3.0
pduK	Propanediol utilization: polyhedral bodies	−2.5	−2.6
pduL	Propanediol utilization	−2.5	−3.3
pduM	Propanediol utilization	−2.4	−2.3
pduO	Propanediol utilization: B12 related	−1.9	−1.8
pduP	Propanediol utilization: CoA-dependent dehydrogenase	−2.6	−3.1
pduQ	Propanediol utilization: propanol dehydrogenase	−1.8	−2.6
pduS	Propanediol utilization: polyhedral bodies	−1.7	−2.0
pduT	Propanediol utilization: polyhedral bodies	−2.0	−2.6
pduU	Propanediol utilization: polyhedral bodies	−1.6	−2.5
pduW	Propanediol utilization: propionate kinase	−1.9	−3.1
ETHANOLAMINE UTILIZATION			
eutE	Putative aldehyde oxidoreductase in ethanolamine utilization	−2.6	−1.6
eutT	Putative cobalamin adenosyltransferase, ethanolamine utilization	−2.8	−1.6
eutQ	Putative ethanolamine utilization protein	−3.1	−1.9
eutS	Putative carboxysome structural protein, ethanol utilization	−1.5	−1.6
VITAMIN B12 SYNTHESIS			
cbiP	Putative carboxysome structural protein, ethanol utilization	−1.8	−2.0
cboQ	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.2	−2.6
cbiN	Synthesis of vitamin B12 adenosyl cobalamide precursor	−1.7	−2.1
cbiM	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.8	−2.6
cbiL	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.7	−2.6
cbiH	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.0	−2.4
cbiG	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.3	−2.5
cbiF	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.3	−2.4
cbiC	Synthesis of vitamin B12 adenosyl cobalamide precursor	−1.7	−1.8
cbiA	Synthesis of vitamin B12 adenosyl cobalamide precursor	−2.2	−1.8
HEAT SHOCK			
dnaK	Chaperone Hsp70 in DNA biosynthesis/cell division	2.9	3.3
dnaJ	Heat shock protein, DnaJ and GrpE stimulates DnaK	2.7	3.3
ibpA	Small heat shock protein	4.1	4.3
ibpB	Small heat shock protein	5.8	3.5
mopA	Chaperone Hsp60 with peptide-dependent ATPase activity	2.8	3.3
mopB	Chaperone Hsp10, affects cell division	1.7	2.3
htpG	Chaperone Hsp9	3.2	4.0
ANTIBIOTIC RESISTANCE			
marA	Transcriptional Activator	5.5	4.5
marR	Transcriptional repressor of *marRAB* operon	3.7	5.9

motility, including *motA*, *motB*, and *flhC*, chemotaxis genes, *cheA, cheY* and *cheZ*, and the major flagellin *fliC* (**Table 3**).

Outer Membrane Proteins (OMPs)

Both plant-derived compounds significantly down-regulated genes encoding OMPs, namely *ompW*, *ompC*, *ompS1*, and *nmpC* (**Table 1**). The *nmpC* gene, encoding a predicted bacterial porin, was down-regulated by 4.6- and 3.5-fold by TC and EG, respectively. Similarly, *ompW*, a colicin S4 receptor gene, was also reduced in transcription by 4.3- and 2.7-fold, respectively, by TC and EG. However, some of the major OMP genes, such as *ompA*, *ompR*, and *ompX,* were not affected by both compounds.

Metabolism and Biosynthetic Pathway Genes

As revealed by microarray analysis, several genes responsible for energy production and conversion in *Salmonella* were down-regulated by TC and EG. For example, the majority of the genes regulating the carbohydrate metabolism and transport, amino

TABLE 2 | Select genes predicted to be differentially regulated in PT8 exposed to SICs of TC but not EG ($P < 0.005$, M ± 1.5), based on the microarray data.

Gene	Function	*M*-value
INVASION		
sipA	Cell invasion protein	−1.8
sipD	Cell invasion protein	−1.8
sipC	Cell invasion protein	−1.8
sipB	Cell invasion protein	−2.1
sopB	*Salmonella* outer protein: homologous to *ipgD* of *Shigella*	−1.9
TETRATHIONATE, ETHANOLAMINE, AND DIMETHYLSUPHIDE REDUCTION		
pduF	Propanediol utilization: propanediol diffusion facilitator	−2.4
pduN	Propanediol utilization: polyhedral bodies	−1.6
eutC	Ethanolamine ammonia-lyase, light chain	−1.5
eutB	Ethanolamine ammonia-lyase, heavy chain	−2.1
eutH	Putative transport protein, ethanolamine utilization	−2.1
eutJ	Paral Putative heatshock protein (Hsp70)	−2.1
eutN	Putative detox protein in ethanolamine utilization	−2.3
dmsB	Anaerobic dimethyl sulfoxide reductase subunit B	−4.5
dmsA	Putative anaerobic dimethyl sulfoxide reductase, subunit A pseudogene	−5.3
dmsC	Putative dimethyl sulfoxide reductase subunit C	−3.0
STM1498	Putative dimethyl sulfoxide reductase	−2.9
STM1499	Putative dimethyl sulfoxide reductase, chain A1	−2.9
dmsB	Anaerobic dimethyl sulfoxide reductase subunit B	−4.5
dmsA	Putative anaerobic dimethyl sulfoxide reductase, subunit A pseudogene	−5.3
HYDROGEN SULFIDE PRODUCTION		
phsC	Hydrogen sulfide production: membrane anchoring protein	−2.6
phsB	Hydrogen sulfide production: iron- sulfur subunit; electron transfer	−3.0
phsA	Hydrogen sulfide production: membrane anchoring protein	−2.8
phsC	Hydrogen sulfide production: membrane anchoring protein	−2.6

TABLE 3 | Select genes predicted to be differentially regulated in PT8 exposed to SICs of EG, but not TC ($P < 0.005$, M ± 1.5), based on the microarray data.

Gene	Function	*M*-value
MOTILITY		
flhC	Flagellar transcriptional activator	−2.5
motA	Proton conductor component of motor, torque generator	−3.3
motB	Enables flagellar motor rotation, linking torque machinery to cell wall	−3.8
cheA	Sensory histidine protein kinase, transduces signal between chemo- signal receptors and CheB and CheY	−4.4
cheB	cheA sensor	−2.8
cheM	Chemotaxis protein II	−4.2
cheR	Response regulator for chemotaxis	−2.7
cheW	Purine-binding chemotaxis protein; regulation	−1.6
cheY	Chemotaxis regulator, transmits chemoreceptor signals to flagellar motor components	−2.8
cheZ	Chemotactic response; CheY protein phophatase	−2.4
fliA	Sigma F (sigma 28) factor of RNA polymerase, transcription of late flagellar genes (class 3a and 3b operons)	−3.2
fliD	Flagellar biosynthesis; filament capping protein; enables filament assembly	−1.9
fliC	Flagellar biosynthesis; flagellin, filament structural protein	−2.2
fliT	Flagellar biosynthesis; possible export chaperone for FliD	−2.9
flgK	Flagellar biosynthesis, hook-filament junction protein 1	−4.4
flgL	Flagellar biosynthesis; hook-filament junction protein	−3.5
flgM	Anti-FliA (anti-sigma) factor; also known as RflB protein	−3.2
flgN	Flagellar biosynthesis: belived to be export chaperone for FlgK and FlgL	−1.7
TETRATHIONATE AND DIMETHYLSUPHIDE REDUCTION		
ttrA	Tetrathionate reductase complex, subunit A	−2.1
ttrB	Tetrathionate reductase complex, subunit B	−1.6
ttrR	Tetrathionate reductase complex: response regulator	−2.0
dmsB	Anaerobic dimethyl sulfoxide reductase subunit B	−3.2
dmsA	Putative anaerobic dimethyl sulfoxide reductase, subunit A pseudogene	−3.9
dmsC	Putative dimethyl sulfoxide reductase subunit C	−1.5
STM1498	Putative dimethyl sulfoxide reductase	−2.1
HYDROGEN SULFIDE PRODUCTION		
phsC	Hydrogen sulfide production: membrane anchoring protein	−2.7
phsB	Hydrogen sulfide production: iron- sulfur subunit; electron transfer	−2.6
phsA	Hydrogen sulfide production: membrane anchoring protein	−3.0

acid transport, and carbon compound degradation (**Table 1**, Supplementary Table S11) were found to be down-regulated by TC and EG. There were indications of down-regulation of mixed metabolism genes, including genes related to the glucose metabolism and genes associated with C4- (e.g., fumarate), non-glucose C6- (e.g., gluconate, fucose), and C9- (sialic acid) carbohydrate metabolisms. In addition, *melA*, *melB*, and *melR*, genes regulated by the melibiose operon, and mannose-specific PTS (phosphotransferase) family genes *manXYZ* were down-regulated by both plant-derived compounds (**Table 1**). A complete list of these genes is found in Supplementary Tables S8–S18.

A major set of genes involved in tetrathionate reduction (*ttr*), propanediol utilization (*pdu*), ethanolamine utilization (*eut*), and dimethyl sulfide reduction (*dms*) were down regulated by TC and EG (**Tables 1**–**3**). Moreover, the *cbi* locus genes associated with vitamin B12 synthesis (**Table 1**) were down-regulated by TC and EG. Furthermore, genes responsible for H_2S production, *phsABC,* were significantly down-regulated by both compounds (**Tables 2**, **3**).

Up-Regulated Genes

Besides the down-regulation of several genes indicated above, a few critical genes were up-regulated in response to TC and EG exposure (**Table 1**, Supplementary Table S19). The greatest increase in transcription was observed with heat shock proteins. The genes encoding two small heat shock proteins, *ibpA* and *ibpB,* were up-regulated by TC by 4.1- and 5.8-fold, respectively,

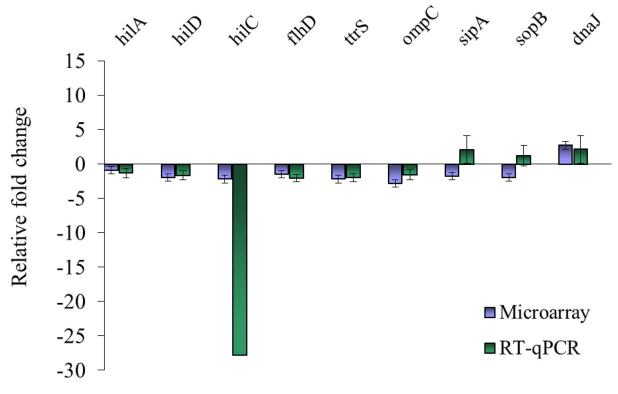


FIGURE 1 | Comparison of microarray and RT-qPCR data on select genes after exposure of *S*. Enteritidis PT8 to SICs of TC. Data are from three replicates.

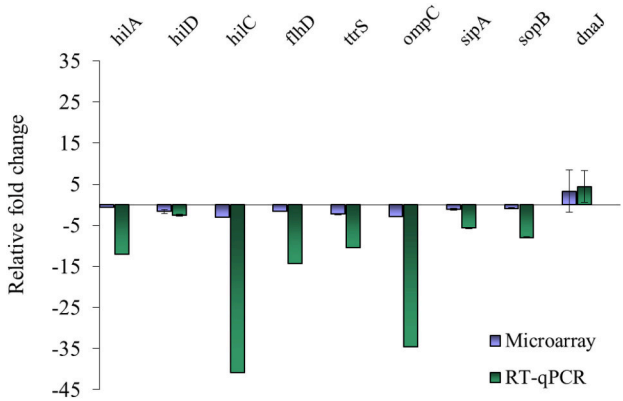


FIGURE 2 | Comparison of microarray and RT-qPCR data on select genes after exposure of *S*. Enteritidis PT8 to SICs of EG. Data are from three replicates.

and 4.3- and 3.5-fold, respectively, by EG. In addition, *dnaJ*, and the Hsp70 chaperone gene *dnaK* were up-regulated on exposure to both compounds. The microarray analysis revealed increased transcription of *acrA* and *acrB*, the acridine efflux pump genes, by EG (**Table 1**). Similarly, multiple antibiotic resistance protein (*mar*) genes were also up-regulated by TC and EG. However, *marR*, encoding the transcriptional repressor of the *marRAB* operon, was also found to be up-regulated by both plant - derived compounds ($P < 0.005$), probably as a compensatory mechanism.

Confirmation with RT-qPCR

Nine genes with significantly different transcription levels following TC or EG exposure as identified by microarray analysis were further analyzed by RT-qPCR. Regarding exposure to TC, microarray and RT-qPCR results were in agreement for all tested genes but *sipA* and *sopB* (**Figure 1**). Microarray data revealed that *sipA* and *sopB* were down-regulated by 1.8- and 2.0-fold, respectively, but in RT-qPCR analysis, a slight up-regulation of both genes on exposure to TC was observed. Regarding exposure to EG, *hilA*, *hilD*, *flhD*, *ttrS*, *ompC*, *sipA*, and *sopB* were found significantly down-regulated in microarray analysis and in RT-qPCR results (**Figure 2**).

DISCUSSION

Salmonella Enteritidis is a leading cause of foodborne salmonellosis worldwide. The increase in the number of outbreaks over the last few decades, where poultry and poultry products were implicated, has led to several pre- and post-harvest strategies to control the pathogen. We previously reported that supplementation of TC and EG in broilers (Kollanoor Johny et al., 2012a,b) and TC in layers (Upadhyaya et al., 2015) significantly reduced *S.* Enteritidis colonization in birds. We also observed that these compounds decreased *Salmonella* motility and invasion of avian epithelial cells *in vitro* (Kollanoor Johny et al., 2012b). Since analysis of transcriptional profiles of bacteria exposed to an antimicrobial molecule can yield new information regarding affected pathways and mechanisms of inhibition (Wilson et al., 1999; Gmuende et al., 2001), we conducted a microarray analysis of *S.* Enteritidis PT8 exposed to TC and EG. The goal was to delineate the genes affected by these plant-derived compounds, to gain insight into the molecular mechanism of how these compounds may cause reduced *S.* Enteritidis colonization in chickens.

A diverse repertoire of *Salmonella* virulence genes was down-regulated in response to treatment with TC and EG, including, critically, the genes involved in SPI-1 regulation. These genes are necessary for *Salmonella* uptake into non-phagocytic epithelial cells (McCormick, 2004). A direct control of SPI-1 genes is exerted by the activation by *hilA*-the major regulator of the island, resulting in the invasion of host tissue. Ellermeier and Slauch (2007) reported that other regulators, such as HilC and HilD work in conjunction with another regulator RtsA to express SPI-1. HilD, an AraC/XylS family member, binds directly to several sites within the *hilA* promoter and derepresses gene expression, which in turn results in the expression of other associated virulence genes. Both plant-derived compounds down-regulated the major regulators *hilA*, *hilD,* and *hilC*, although a greater effect was observed on *hilC* (**Table 1**, Supplementary Table S1). The T3SS encoded by SPI-1 is the pathogenic machinery to inject secretory proteins into host cells that help in the internalization of *Salmonella* (Kubori et al., 2000; Galan, 2001). The effector proteins are transported into the host cells with the help of three translocation proteins, *sipB*, *sipC* and *sipD* (Collazo and Galan, 1997; Galan, 2001). As revealed in the microarray data, TC down-regulated the effector protein genes (Supplementary Table S3).

Flagellar motility and chemotaxis are the two critical features by which bacteria survive under a wide variety of environments (Soutourina and Bertin, 2003), including the intestinal lumen and ceca. Flagellar motility is also required for colonization by *Salmonella* (Ciacci-Woolwine et al., 1998), and is considered a virulence factor in the bacterium (Josenhans and Suerbaum, 2002). In the regulation of flagella, the *flhDC* master operon

plays a key role (Kutsukake et al., 1990; Soutourina and Bertin, 2003). Among other genes, the master operon is necessary for transcriptional activation of the *fliA* gene, encoding σ^{28}, a sigma factor associated with the transcription of late flagellar genes, such as *fliC* (filament structural protein). It was interesting to note that that these associated genes were all down-regulated by EG (**Table 1**). Apart from the *flhCD*-regulated genes (early flagellar operon), the middle and late operon genes, such as *flgKLMN*, *fliZ*, *motAB*, and *cheAWYZ,* were also down-regulated by EG (**Table 3**, Supplementary Table S5). In contrast, TC did not exert strong effects on these genes.

Porins are outer membrane aqueous channels used in the diffusion of various compounds across the membrane that can also aid in adapting the bacteria to various conditions and confer antibacterial drug resistance (Gil et al., 2009). In the current study, TC and EG down-regulated porins, such as *ompW*, *ompC*, *ompS1*, and *nmpC* by 2-fold. Recently, Gil et al. (2009) reported that *ompW* expression in *Salmonella* was activated in response to oxidative stress. Both plant-derived compounds down-regulated *ompW* by 4.3- and 2.7-fold, respectively. Methner et al. (2004) reported that *ompC* deletion mutants of *S*. Enteritidis resulted in decreased virulence and colonization in chickens in conjunction with deletions of *rpoS* or *phoP*. Our results revealed that *ompC* was down-regulated by 2.8-fold by both compounds (**Table 1**). Porin *ompS1*, down-regulated by TC and EG in our study, is regulated by the EnvZ/OmpR two-component signal transduction system (Florez-Valdez et al., 2003). Rodriguez-Morales et al. (2006) reported that *ompS1* is required for virulence in *S*. Typhimurium in mice.

It was recently reported that during colonization in the gut *Salmonella* makes use of a wide range of carbon compounds, including D- glucose, melibiose, L-ascorbate, and other unusual compounds, such as sialic acid, ethanolamine, and propanediol resulting from the degradation of host mucosa (Dhawi et al., 2011). We observed that TC and EG down-regulated some of the glucose metabolism genes (*agp, pykA*), and TC reduced the transcription of *melABR* genes (Supplementary Table S8). Moreover, both plant-derived compounds were found to apparently reduce the bacterial capacity to utilize propanediol and ethanolamine as an energy source or tetrathionate as a terminal electron acceptor (**Table 1**), suggesting that TC and EG may interrupt the pathways critical for *Salmonella* survival and successful competition with the host microflora. During colonization of the host intestine, *Salmonella* can use energy and nutrient sources unavailable to commensal bacteria, including propanediol and ethanolamine. These compounds cannot be used for energy without a terminal electron acceptor, and the host flora mainly consists of mostly fermentative bacteria unable to perform this electron transport. *Salmonella,* however, is uniquely capable of using these compounds by employing tetrathionate as a terminal electron acceptor. When tetrathionate is used as an electron acceptor, it is reduced to thiosulfate, which is then regenerated via oxidation by the reactive oxygen compounds released by the host in response to invasion by *Salmonella*. *Salmonella* can therefore outcompete the host flora, resulting in inflammatory diarrhea, with *Salmonella* cells as major bacterial component of the excreta thus accomplishing transmission to the next host organism.

Harvey et al. (2011) reported that within the lumen of chicks, the degradation of 1, 2-propanediol occurs at a much higher rate, requiring cobalamin synthesis. The *pdu* operon required for propanediol utilization works in conjunction with *cob* and *cbi* operons involved in vitamin B12 synthesis. Our results revealed that TC and EG significantly down-regulated *pdu* and *cbi* operons (**Table 1**). It was previously observed that *Salmonella* could utilize ethanolamine, which is derived from host cells and membranes, as a sole source of carbon, nitrogen, and energy (Garsin, 2010). Catabolism of ethanolamine is under the control of the *eut* operon that primarily consists of 17 clustered genes (Kofoid et al., 1999) involved in the conversion of the compound to acetyl-CoA for energy (Harvey et al., 2011). The plant compound TC was found to down-regulate these genes (Supplementary Table S14). An anaerobic environment may exist in the chicken cecum (Harvey et al., 2011), and oxygen may not be present as a terminal electron acceptor. In that case, other electron acceptors are required, and ethanolamine and 1, 2-propanediol could be utilized in that function by *Salmonella* in the cecum (Price-Carter et al., 2001). Tetrathionate may be present in the cecum of chicks due to the presence of yolk sac, which is rich in sulfur. Tetrathionate is reduced to thiosulphide and H_2S, involving proteins encoded by *ttr* and *phs* (Harvey et al., 2011). Our results indicated that TC down-regulated the transcription of *ttr* genes (Supplementary Table S12), whereas both plant-derived compounds reduced *phsABC* transcription (Supplementary Table S18). Overall, TC and EG may disrupt *Salmonella*'s ability to use a unique energy source, possibly reducing its ability to compete successfully with the host's microflora.

Some heat shock genes and genes conferring antibiotic resistance were up-regulated by TC and EG. While the *mar* (multiple antibiotic resistance) locus genes were up-regulated by both TC and EG, the efflux pump genes *acrAB* were only up-regulated by EG. The MarA protein positively regulates transcription of *acrAB*, and it was therefore unexpected that the latter genes were not up-regulated after exposure to TC. However, the transcriptional repressor *marR* was also found to be up-regulated by both TC and EG, potentially indicating a compensatory mechanism on reducing *marAB*-related resistance. Heat shock proteins *dnaJ*, *dnaK*, *ibpA*, *ibpB*, and *htpG* were found to be up-regulated by both plant-derived compounds ($P < 0.005$). However, *clpPX*-mediated heat shock protection mechanisms were least affected (Supplementary Table S19).

Some limitations of this study include the use of nutrient broth for the gene expression studies as opposed to growth within a characterized chicken-derived intestinal cell line, although broth cultures have been previously used for similar experiments (Cao et al., 2011; Jessica et al., 2013; Upadhyay et al., 2013). To our knowledge, immortalized chicken intestinal cell lines are unavailable, and developing and maintaining a primary chicken intestinal cell line has its challenges, including the high variability in confluency across different propagations. In a separate study, we conducted invasion assays using the only available permanent

avian cancer cell line (Budgerigar Abdominal Tumor Cells; BATCs) and found that these two phytophenolics significantly reduced *Salmonella* invasion without affecting the viability of cells (Kollanoor Johny et al., 2012b). However, being a permanent cell line obtained from a different avian species (parakeets), a direct correlation of these results to a chicken-derived cell environment is not guaranteed. However, the selected phytophenolics reduced the cecal colonization of *S.* Enteritidis in broiler chickens in at least 5 independent *in vivo* experiments (Kollanoor Johny et al., 2012a,b), lending support to the notion that TC and EG directly affect *S.* Enteritidis virulence genes. However, this hypothesis needs to be further validated.

CONCLUSIONS

Genes required for *S.* Enteritidis PT8 virulence and colonization, including those conferring flagella-associated motility, those enabling invasion of epithelial cells, genes encoding T3SSs, genes regulating the synthesis of effector proteins delivered by T3SS, and genes encoding other surface virulence structures and OMPs were found to be down-regulated after exposure to subinhibitory concentrations of TC or EG. Moreover, the exposure to both phytochemicals brought about transcriptional changes of *S.* Enteritidis PT8 genes involved in the degradation of carbon compounds, those related to amino acid-, carbohydrate-, and lipid-transport, those associated with secondary metabolism, and genes encoding the biosynthesis of molybdopterin and vitamin B12. The genes regulating pathways associated with tetrathionate reduction, propanediol, and ethanolamine utilization, and H_2S production were also affected. Finally, a few genes regulating antibiotic resistance and heat shock were up-regulated by the plant-derived compounds, including transcriptional activators and repressors, with as yet undefined phenotypic net results.

METHODS

Bacterial Strain and Growth Conditions

S. Enteritidis PT8, obtained from the Connecticut Veterinary Diagnostic Medical Laboratory, was used for the experiments. This strain was used along with three other strains in our *in vitro* invasion and motility assays, and *in vivo* chicken experiments (Kollanoor Johny et al., 2008, 2010, 2012a,b,c). The strain was selected based on RT-qPCR analysis of virulence gene transcription (*hilA*, *hilD*, *motA*, *flhC*, and *invF*) of different phage types (PT-8, -13, and -13b) in response to subinhibitory concentrations of TC and EG (Kollanoor Johny et al., 2012b).

PT8 was grown overnight in Luria-Bertani (LB) broth (Difco) at 37°C with shaking at 150 rpm. For the transcription profiling experiments, 10 ml of an overnight culture was spun at 3,800 × *g* for 15 min, and the resulting pellet was resuspended in 10 ml phosphate buffered saline (PBS; pH 7.0). A 0.5 ml aliquot of the suspension was added to 500 ml of LB broth and grown with vigorous shaking at 37°C until it reached a late-log phase OD_{600} of 0.5. Assays were performed using 3 independent biological replicates for TC and EG, separately. The SIC of TC (0.01% vol/vol; 0.75 mM) or EG (0.04% vol/vol; 2.46 mM) (Kollanoor Johny et al., 2012b) was added to the culture and the mixture was vortexed for 1 min. The control flask was vortexed for 1 min as well. Exactly 100 ml were drawn from the broth before addition of TC or EG (time 0), and 30 min (time 30) after addition of the plant-derived compounds. The samples were drawn separately into 250 ml centrifuge tubes containing 16 ml of ice-cold ethanol/phenol stop solution (5% citrate-buffered phenol v/v), to halt the degradation of mRNA. The cells were spun at 8,000 × *g* for 2 min at 4°C, and the resulting pellet was stored at −80°C until use.

Total RNA Extraction

Total RNA from *S.* Enteritidis PT8 was extracted as previously described (Frye et al., 2006). The frozen pellet was lysed by resuspending in a final volume of a fresh solution of 10 ml 0.5 mg/ml lysozyme in TE (pH 8.0). A volume of 1 ml of 10% SDS was added, and the mixture was placed in a water bath at 64°C for 2 min. After incubation, 11 ml of 1 M sodium acetate solution (pH 5.2) was added to the tubes and mixed. An equal volume of phenol was added and mixed, followed by incubation at 64°C for 6 min. The tubes were then placed on ice to chill before they were spun at 10,000 × *g* for 10 min at 4°C. The aqueous layer was transferred to a fresh tube containing an equal volume of chloroform, mixed, and spun at 10,000 rpm for 5 min at 4°C. The aqueous layer from the samples was then transferred into fresh tubes, 1/10 volume of 3 M sodium acetate (pH 5.2) was added, and one volume of cold isopropanol. The tubes were incubated at −80°C for 20 min before they were spun at 14,000 × *g* for 25 min at 4°C. Isopropanol was carefully removed and the resulting pellet was washed with 40 ml 80% cold ethanol. The pellets were spun at 14,000 × *g* for 5 min at 4°C. Ethanol was carefully removed and the pellet was air dried. The pellet was resuspended in 2 ml of RNase-free DEPC-treated water. The sample was split into two and each was provided with 500 U of RNase inhibitor (Ribolock®, Fermentas), 250 U of DNase (Fermentas), 20 μl of 1 M Tris (pH 8.3) and 10 μl of 1 M MgCl, followed by mixing and incubation at 37°C for 30 min. The RNA sample was then extracted once each with phenol and phenol–chloroform and twice with chloroform. A 1/10th volume of 3 M sodium acetate at pH 5.2 was added, and the RNA was precipitated with isopropanol, washed with 80% ethanol and resuspended in nuclease-free water (Frye et al., 2006). The RNA concentration and purity were determined using a Nanodrop (Thermofisher Corp.).

Microarray, Target Preparation, and Hybridization

The *Salmonella* whole genome microarray used in this study has been previously described (Porwollik et al., 2003) and includes 5,776 PCR products representing more than 99% of the ORFs of *S.* Enteritidis PT4 as well as four other serotypes of *Salmonella*. The microarray hybridization and analysis were done by standard methods, as previously described for *Salmonella* (Porwollik et al., 2003; Frye et al., 2006). Fifty microgram of RNA was transcribed into cDNA and labeled with Cy3- or Cy5-conjugated dUTP using reverse transcriptase (Superscript II®; Invitrogen) and random hexamers as primers. Unincorporated nucleotides were removed using a PCR purification kit (Qiagen)

TABLE 4 | Primers used for RT-qPCR assays.

Gene target	Forward primer (5′–3′)	Reverse primer (5′–3′)
16S rRNA	CGTGTTGTGAAATGTTGGGTTAA	CCGCTGGCAACAAAGGATAA
hilA	TTGCTGACTCAATGCGTTAACA	CATTCTGCCAGCGCACAGTA
hilD	CAACGACTTGGCGCTCTCTAT	TCTCTGTGGGTACCGCCATT
hilC	CCAGTTTTCGCTTCAGACTTGA	CACCCGCAAATGGTCACA
flhD	CGTTTGATCGTCCAGGACAA	TGTTTGCCATCTCTTCGTTGAT
ttrS	CAAATATAATTGAGCGTGAGC AACA	TGGTTAATCCAGCACCAGCTAA
ompC	ACGCTGCTGCATAAAGTTGTCA	CCGATGTTCTGCCGGAGTT
sipA	TCTGCTTTTTTCCCACCATCA	AGATAAACTGCCTGACCCTAA AATTC
sopB	GTGCTGCAATAAGTTCGATAA GATTT	ACCGGCCAGCAACAAAAC
dnaJ	ATGGTCGTGTGATGCTGAAAGT	CCGCGCATACGGAACAG

according to the manufacturer's instructions. Equal volumes of labeled probes from 0 (Cy3-labeled control) and 30 min (Cy5-labeled treatment) were mixed with an equal volume of hybridization solution (50% formamide, 10 × SSC and 0.2% SDS). Slides were prehybridized in 25% formamide, 5 × SSC and 0.1% SDS at 42°C. Probes were hybridized simultaneously to a chip containing three replicate arrays spotted onto CMT-UltraGAPS® (Corning) slides. Slides were washed in stringency-controlled buffers and dried by centrifugation before scanning.

Scanning and Data Analysis

The slides were scanned using the ScanArray 5,000 laser scanner (GSI Lumonics). The signals were recorded with scanarray 2.1 software and then quantified using Quantarray 3.0 software (Packard BioScience) (Frye et al., 2006; Ledeboer et al., 2006). Arrays were scanned for high signal intensity and low background in either of the channels, to be included for analysis. The normalization of data considered the median intensity of the spot and the local background. The data were statistically analyzed using the Partek Discovery SuiteTM v 6.2 (Partek Inc., St. Louis, MO). Mixed effects ANOVA was performed, and a significant $P < 0.005$ for each comparison was determined using a false discovery rate of 0.05. A total of 11 (5 treated and 6 untreated) biological replicates (separate RNA preparations) were analyzed from experiments conducted on different days, and the data have been deposited in GEO (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE102477.

Real-Time Quantitative PCR (RT-qPCR)

An RT-qPCR analysis was conducted to confirm the differential expression of the selected microarray targets. The protocol of sample preparation and collection for microarray experiment was also used for preparation of RT-qPCR samples. Total RNA was extracted from three biological replicates of PT8 cultures exposed to TC or EG using the RNeasy minikit (Qiagen) according to manufacturer's instructions. The resulting RNA was treated with RNase-free DNase. The quantitation of RNA was done by measuring the absorbance at 260 and 280 nm (Nanodrop, Biorad). Complementary DNA (cDNA) was synthesized using the Superscript II Reverse transcriptase kit (Superscript™—Invitrogen, Carlsbad, CA). The cDNA was used as the template for the amplification of *Salmonella* genes selected based on the results from microarray, including *hilA*, *hilD*, *hilC, flhD, dnaJ, ompC, sipA, sopB,* and *ttrS*. The specific primers for the genes, and for 16S rRNA (endogenous control) (**Table 4**) were designed using Primer Express software (Applied Biosystems). The primers were synthesized by Integrated DNA Technologies (Foster City, CA). Real time-qPCR was performed with the ABI PRISM 7900 sequence detection system (Applied Biosystems), using the SYBR® Green assay (Applied Biosystems) under custom thermal cycling conditions (denaturation step at 95°C for 10 min, followed by 40 cycles of a denaturation step at 95°C for 15 s, and an annealing/elongation step at 60°C for 60 s). A default melting curve stage (95°C for 15 s, 0°C for 60 s, 95°C for 15 s) was also included. The biological replicates were analyzed in duplicate and normalized against 16S rRNA gene expression. The 16S rRNA gene was used to account for the variability between the samples since the transcript levels of the endogenous control were found to be not significantly different ($P > 0.05$) between the control- and the TC- or EG- treated cells. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to assess the relative changes in the mRNA expression levels between the control and TC-/EG-treated PT8 (Schmittgen and Livak, 2008).

AUTHORS CONTRIBUTIONS

AK carried out microarray experiments, RT-qPCR assays, involved in the study design and wrote the manuscript. JF trained AK to conduct microarray analysis, preliminary interpretation of statistical analysis and corrected the manuscript. AD and DD participated in the design of the study and corrected the manuscript. SP and MM designed and constructed the microarrays, interpreted the hybridization results and performed statistical analysis of the data. KV conceived and designed the study and corrected the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

This work was funded by the USDA-NIFA Agriculture and Food Research Initiative Grant no. 2009-03576 awarded to KV and DD. The authors would like to thank Lari Hiott at the Bacterial Epidemiology and Antimicrobial Resistance research unit at USDA, ARS, for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01828/full#supplementary-material

REFERENCES

Altekruse, S., Koehler, J., Hickman-Brenner, F., Tauxe, R. V., and Ferris, K. (1993). A comparison of *Salmonella* Enteritidis phage types from egg-associated outbreaks and implicated laying flocks. *Epidemiol. Infect.* 110, 17–22. doi: 10.1017/S0950268800050639

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in food – a review. *Int. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Campioni, F., Davis, M., Medeiros, M. I. C., Falcao, J. P., and Shah, D. H. (2013). MLVA typing reveals higher genetic homogeneity among *Salmonella* Enteritidis strains isolated from food, humans, and chickens in Brazil in comparison to the North American strains. *Int. J. Food Microbiol.* 162, 174–181. doi: 10.1016/j.ijfoodmicro.2013.01.008

Cao, B., Li, R., Xiong, S., Yao, F., Liu, X., Wang, M., et al. (2011). Use of a DNA microarray for detection and identification of bacterial pathogens associated with fishery products. *Appl. Environ. Microbiol.* 77, 8219–8225. doi: 10.1128/AEM.05914-11

Centers for Disease Control and Prevention (2010). *Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly through Food—10 States, 2009*. U.S. Department of Health and Human Services, Atlanta, GA: CDC.

Ciacci-Woolwine, F., Blomfield, I. C., Richardson, S. H., and Mizel, S. B. (1998). *Salmonella* flagellin induces tumor necrosis factor alfa in a human promocytic cell line. *Infect. Immun.* 6, 1127–1134.

Collazo, C. M., and Galan, J. E. (1997). The invasion-associated type III system of *S.* Typhimurium directs the translocation of Sip proteins into the host cell. *Mol. Microbiol.* 24, 747–756. doi: 10.1046/j.1365-2958.1997.3781740.x

Dhawi, A. A., Elazomi, A. M., Jones, A. M., Lovell, A., Li, H., Emes, R. D. et al. (2011). Adaptation to the chicken intestine in *Salmonella* Enteritidis PT4 studied by transcriptional analysis. *Vet. Microbiol.* 153, 198–204. doi: 10.1016/j.vetmic.2011.07.013

Ellermeier, J. R., and Slauch, J. M. (2007). Adaptation to the host environment: regulation of the SPI1 type III secretion system in *Salmonella enterica* serovar Typhimurium. *Curr. Opin. Microbiol.* 10, 24–29. doi: 10.1016/j.mib.2006.12.002

Florez-Valdez, M. A., Puente, J. L., and Calva, E. (2003). Negative osmoregulation of the *Salmonella* ompS1 porin gene independently of ompR in an hns background. *J. Bacteriol.* 185, 6497–6506. doi: 10.1128/JB.185.22.6497-6506.2003

Friedman, M. 1., Henika, P. R., and Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65, 1545–1560. doi: 10.4315/0362-028X-65.10.1545

Frye, J. G., Jessea, T., Longb, F., Rondeaub, G., Porwollikb, S., McClelland, M., et al. (2006). DNA microarray detection of antimicrobial resistance genes in diverse bacteria. *Intl. J. Antimicrob. Agents.* 27, 138–151. doi: 10.1016/j.ijantimicag.2005.09.021

Galan, J. E. (2001). *Salmonella* interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* 17, 53–86. doi: 10.1146/annurev.cellbio.17.1.53

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R. Humphrey, T. J. et al. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiol, Rev.* 33, 718–738. doi: 10.1111/j.1574-6976.2008.00161.x

Garsin, D. A. (2010). Ethanolamine utilization in bacterial pathogens: roles and regulation. *Nat. Rev. Microbiol.* 8, 290–295. doi: 10.1038/nrmicro2334

Gil, F., Hernandez-Lucas, I., Polanco, R., Pacheco, N., Collao, B., Villarreal, J. M., et al. (2009). SoxS regulates the expression of the *S.* Typhimurium ompW gene. *Microbiology* 155, 2490–2497. doi: 10.1099/mic.0.027433-0

Gmuende, H., Kuratli, K., Di Padova, K., Gray, C. P., Keck, W., and Evers, S. (2001). Gene expression changes triggeredf by exposure of *Haemophilus influenzae* to novobiocin or ciprofloxacin: combined transcription and translation analysis. *Genome Res.* 11, 28–42. doi: 10.1101/gr.157701

Gould, H. L., Walsh, K. A., Vieira, A. R., Herman, K., Williams, I. T., Hall, A. J., et al. (2013). Surveillance for foodborne disease outbreaks–United States, 1998–2008. *Morbid. Mortal. Weekly. Rep.* 62, 1–34. Available online at: https://www.cdc.gov/mmwr/preview/mmwrhtml/ss6202a1.htm

Guard-Petter, J. (2001). The chicken, the egg and *Salmonella* Enteritidis. *Environ. Microbiol.* 3, 421–430. doi: 10.1046/j.1462-2920.2001.00213.x

Harvey, P. C., Watson, M., Hulme, S., Jones, M. A., Lovell, M. A., Berchieri, A. Jr. et al. (2011). *Salmonella enterica* serovar Typhimurium colonizing the lumen of the chicken intestine are growing slowly and up-regulate a unique set of virulence and metabolism genes. *Infect. Immun.* 79, 4105–4121. doi: 10.1128/IAI.01390-10

Jessica, M. A. B., Richmond, G. E., Bailey, A. M., Ivens, A., and Piddock, L. J. V. (2013). Choice of bacterial growth medium alters the transcriptome and phenotype of *Salmonella enterica* serovar Typhimurium. *PLoS ONE* 8:e63912. doi: 10.1371/journal.pone.0063912

Josenhans, C., and Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *Intl. J. Med. Microbiol.* 291, 605–614. doi: 10.1078/1438-4221-00173

Keller, L. H., Benson, C. E., Krotec, K., and Eckroade, R. J. (1995). *Salmonella* Enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infect. Immun.* 63, 2443–2449.

Kofoid, E., Rappleye, C., Stojiljkovic, I., and Roth, J. (1999). The 17-gene ethanolamine (eut) operon of *Salmonella* Typhimurium encodes five homologues of carboxysome cell proteins. *J. Bacteriol.* 181, 5317–5329.

Kollanoor Johny, A., Darre, M. J., Donoghue, A. M., Donoghue, D. J., and Venkitanarayanan, K. (2010). Antibacterial effect of *trans*-cinnamaldehyde, eugenol, thymol and carvacrol against *Salmonella* Enteritidis and *Campylobacter jejuni in vitro*. *J. Appl. Poult. Res.* 19, 237–244. doi: 10.3382/japr.2010-00181

Kollanoor Johny, A., Darre, M. J., Hoagland, T. A., Schreiber, D. T., Donoghue, A. M., Donoghue, D. J., et al. (2008). Antibacterial Effect of *Trans*-cinnamaldehyde on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken drinking water. *J. Appl. Poult. Res.* 17, 490–497. doi: 10.3382/japr.2008-00051

Kollanoor Johny, A., Upadhyay, A., Upadhyaya, I., Baskaran, S. A., Moyoottu, S., Michael, D., et al. (2012a). Effect of therapeutic cinnamaldehyde and eugenol on *Salmonella* Enteritidis in market age broiler chicken. *J. Appl. Poult. Res.* 21, 816–822. doi: 10.3382/japr.2012-00540

Kollanoor Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A. R., Babapoor, S., March, B., et al. (2012b). Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds *trans*-cinnamaldehyde and eugenol. *Appl. Environ. Microbiol.* 78, 2981–2987. doi: 10.1128/AEM.07643-11

Kollanoor Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A., Hoagland, T. A., Darre, M. J., et al. (2012c). Caprylic acid reduces *Salmonella* Enteritidis populations in various segments of digestive tract and internal organs of 3- and 6-week-old broiler chickens, therapeutically. *Poult. Sci.* 91, 1686–1694. doi: 10.3382/ps.2011-01716

Kubori, T., Sukhan, A., Aizawa, S. I., and Galan, J. E. (2000). Molecular characterization and assembly of the needle complex of the *Salmonella* Typhimurium type III protein secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10225–10230. doi: 10.1073/pnas.170128997

Kutsukake, K., Ohya, Y., and Iino, T. (1990). Trancriptional analysis of the flagellar regulon of *S.* Typhimurium. *J. Bacteriol.* 172, 741–747. doi: 10.1128/jb.172.2.741-747.1990

Ledeboer, N. A., Frye, J. G., McClelland, M., and Jones, B. D. (2006). *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infect. Immun.* 74, 3156–3169. doi: 10.1128/IAI.01428-05

Marcus, R., Varma, J. K., Medus, C., Boothe, E. J., Anderson, B. J., Crume, T., et al. (2007). Re-assessment of risk factors for sporadic *Salmonella* serotype Enteritidis infections: a case-control study in five FoodNet Sites, 2002-2003. *Epidemiol. Infect.* 135, 84–92. doi: 10.1017/S0950268806006558

McCormick, B. A. (2004). "Invasion mechanisms of *Salmonella*," in *Bacterial Invasion of Host Cells*, 1st Edn., ed R. J. Lamont (New York, NY: Cambridge University Press), 328.

Methner, U., Barrow, P. A., Gregorova, D., and Rychlik, I. (2004). Intestinal colonization inhibition and virulence of *Salmonella* phoP, rpoS and ompC deletion mutants in chickens. *Vet. Microbiol.* 98, 37–43. doi: 10.1016/j.vetmic.2003.10.019

Porwollik, S., Frye, J., Florea, L. D., Blackmer, F., and McClelland, M. (2003). A non-redundant microarray of genes for two related bacteria. *Nucleic Acids Res.* 31, 1869–1876. doi: 10.1093/nar/gkg298

Price-Carter, M., Thingey, J., Bobik, T. A., and Roth, J. R. (2001). The alternative electron acceptor tetrathionate supports B12 dependent

anaerobic growth of *Salmonella enterica* serovar Typhimurium on ethanolamine or 1, 2–propanediol. *J. Bacteriol.* 183, 2463–2475. doi: 10.1128/JB.183.8.2463-2475.2001

Rodriguez-Morales, O., Fernandez-Mora, M., Hernandez-Lucas, I., Vazquez, A., Puente, J. L., and Calva, E. (2006). S. Typhimurium ompS1 and ompS2 mutants are attenuated for virulence in mice. *Infect. Immun.* 74, 1398–1402. doi: 10.1128/IAI.74.2.1398-1402.2006

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by comparative CT method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Soutourina, O. A., and Bertin, P. N. (2003). Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS Microbiol. Rev.* 27, 505–523. doi: 10.1016/S0168-6445(03)00064-0

Suhr, K., and Nielsen, P. V. (2003). Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol.* 94, 665–674. doi: 10.1046/j.1365-2672.2003.01896.x

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., and Venkitanarayanan, K. (2013). Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. *Food Microbiol.* 36, 79–89. doi: 10.1016/j.fm.2013.04.010

Upadhyaya, I., Upadhyay, A., Kollanoor-Johny, A., Mooyottu, S., Baskaran, S. A., Yin, H.-B., et al. (2015). In-Feed Supplementation of *trans*-cinnamaldehyde reduces layer-chicken egg-borne transmission of *Salmonella enterica* serovar enteritidis. *Appl. Environ. Microbiol.* 81, 2985–2994. doi: 10.1128/AEM.03809-14

Wilson, M., DeRisi, J., Kristensen, H. H., Imboden, P., Rane, S., Brown, P. O., et al. (1999). Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12833–12838. doi: 10.1073/pnas.96.22.12833

Disclaimer: Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that are suitable.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Kollanoor Johny, Frye, Donoghue, Donoghue, Porwollik, McClelland and Venkitanarayanan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 27 September 2017
doi: 10.3389/fmicb.2017.01893

Caco-2 Invasion by *Cronobacter sakazakii* and *Salmonella enterica* Exposed to Drying and Heat Treatments in Dried State in Milk Powder

*Emilie Lang[1,2], Stéphane Guyot[1], Pablo Alvarez-Martin[2], Jean-Marie Perrier-Cornet[1] and Patrick Gervais[1]**

[1] UMR PAM A 02.102 Procédés Alimentaires et Microbiologiques, Université de Bourgogne Franche-Comté/AgroSup Dijon, Dijon, France, [2] Novolyze, Dijon, France

Due to the ability of foodborne pathogens to survive in low moisture food, the decontamination of milk powder is an important issue in food protection. The safety of food products is, however, not always insured and the different steps in the processing of food involve physiological and metabolic changes in bacteria. Among these changes, virulence properties may also be affected. In this study, the effect of drying and successive thermal treatments on the invasion capacity of *Salmonella* Typhimurium, *Salmonella* Senftenberg, and *Cronobacter sakazakii* was assessed. Bacteria were dried on milk powder at three different water activity levels (0.25, 0.58, and 0.80) and heated at two different temperatures (90°C and 100°C) for 30 and 120 s. After recovery, stressed bacterial populations were placed in contact with Caco-2 cells to estimate their invasion capacity. Our results show that drying increases the invasion capacity of foodborne pathogens, but that heat treatment in the dried state did not exert a selective pressure on bacterial cells depending on their invasion capacity after drying. Taken together, our findings add to the sum of knowledge on food safety in dried food products and provide insight into the effects of food processing.

Keywords: ***Salmonella enterica***, ***Cronobacter sakazakii***, **Caco-2, invasion, stress**

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Séamus Fanning,
University College Dublin, Ireland
Stephen Forsythe,
Nottingham Trent University,
United Kingdom

****Correspondence:***
Patrick Gervais
patrick.gervais@u-bourgogne.fr

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 June 2017*
Accepted: *15 September 2017*
Published: *27 September 2017*

Citation:
Lang E, Guyot S, Alvarez-Martin P, Perrier-Cornet J-M and Gervais P (2017) Caco-2 Invasion by Cronobacter sakazakii and Salmonella enterica Exposed to Drying and Heat Treatments in Dried State in Milk Powder. Front. Microbiol. 8:1893. doi: 10.3389/fmicb.2017.01893

INTRODUCTION

Salmonella enterica is a Gram-negative, facultative anaerobic, motile and non-spore forming bacteria which causes human salmonellosis. It is a major pathogen in the food industry and is highly represented in outbreaks across the world, with nearly 100,000 cases every year in the European Union alone (Beuchat et al., 2013). Its target population is principally composed of infants and young children (0 – 4 years old). Salmonellosis generally causes nausea, vomiting, abdominal cramps, diarrhea (sometimes necrotizing), fever and headache (Hohmann, 2001). Due to the low infective dose (1–10 cells) required to cause illness in infants and immunocompromised populations, *Salmonella* is an important issue for food safety (Akhtar et al., 2014). In addition, *Cronobacter* (formerly *Enterobacter sakazakii*), another Gram-negative, facultative anaerobic, motile and non-spore forming bacteria, is considered an opportunistic pathogen which can

cause severe infections entailing a death rate of up to 80%, including meningitis, bacteremia or necrotizing enterocolitis in infants (Holý and Forsythe, 2014). The infective dose is unfortunately not well defined, and the incidence of this bacterium is largely underestimated. *Cronobacter* is also a major issue for producers of infant formula. These two bacteria are potential causes of severe infection following consumption of food products, especially powdered infant formula.

This is the reason why the *Codex Alimentarius*, which regulates food standards, imposes the absence of *Cronobacter* and *Salmonella* contamination in powdered infant formula, formula for special medical purposes and human milk fortifiers (Codex Alimentarius, 2008). Nevertheless, such contamination may elude food safety analysis (Cahill et al., 2008; Forsythe, 2014); in recent years, a certain number of cases of contamination by these two pathogens have been identified in infant formula and milk powder ($a_w \approx 0.25$–0.45). Contaminations in milk may occur during the transfer to spray-drying, during the spray-drying and during dried milk handling. This is reflected in outbreaks involving *Cronobacter*, such as the outbreaks in 1986 in Iceland (3 cases), in 1988 in the United States (4 cases), in 1998 in Belgium (12 cases), in 2001 in the United States (11 cases), in 2004 in France (3 cases) or in 2008 in the United States (2 cases). Outbreaks of *Salmonella enterica* have also been reported, such as the 1976 outbreak in Trinidad (3,000 cases), that in 1986 in the United Kingdom (76 cases), in 2005 in France (141 cases) or in 2008 in Spain (42 cases), all due to PIF or milk powder that from part of low water activity food products (Podolak et al., 2010; Beuchat et al., 2013; Forsythe, 2014). *S. enterica* is also clearly linked to outbreaks involving other low moisture food products (Beuchat et al., 2013; Burgess et al., 2016).

Foodborne bacteria encounter many stresses in food processing environments and in food products (Humphrey, 2004). Drying is one such stress and takes place during low moisture food production and during environmental contamination. Drying consists in a diminution of environmental water activity (a_w) which represents the water available for chemical and biochemical reactions. In a dried state, bacteria are more resistant to widely used decontamination processes, such as heat treatments (Rychlik and Barrow, 2005; Shaker et al., 2008; Guo and Gross, 2014). This resistance is partly due to the induction of a stress response by activation of the metabolic pathways which modify membrane composition and/or protein productions (Shen and Fang, 2012).

Stress perception also plays a role in other metabolic pathways, such as the activation of certain virulence genes governed by several two-component systems which sense environmental perturbations (Spector and Kenyon, 2012). For example, PhoQ-PhoP senses acid stress which is known to increase virulence properties in *Salmonella enterica*. In addition, EnvZ-OmpR, implied in osmotic change sensing, may control RNAm and protein regulating HilA, a central transcriptional activator in *S. enterica* virulence (Rychlik and Barrow, 2005). Ye et al. (2015) likewise suggest that osmotic changes are also related to the virulence of *C. sakazakii* (Ye et al., 2015). These authors directly observed that a virulence strain of this bacteria presented a higher expression and presence of EnvZ-OmpR than an attenuated strain (Giri et al., 2012).

In short, food processes can be stressful for foodborne pathogens and may impact bacterial virulence (Buchanan et al., 2000). Moreover, once in the dried state, a decontamination treatment is often applied to a dried food product to ensure food safety. As drying increases resistance to further decontamination treatment, it is possible to consider that the increase in virulence may impact pathogen survival of the heat treatment.

In this study, we describe the impact of drying and successive heat treatments on one virulence property of *C. sakazakii*, *S. enterica* subsp. *enterica* serovar Typhimurium and serovar Senftenberg. All experiments were performed in a food product dried at three different a_W levels (0.25, 0.58, and 0.80) and heated in the dried state at two different temperatures (90°C and 100°C). Invasion capacity in Caco-2 cells was subsequently performed using survival cells.

MATERIALS AND METHODS

Strain Cultivations

Salmonella enterica subspecies *enterica* serovar Typhimurium DT104 DSM 10506, *Salmonella enterica* subspecies *enterica* serovar Senftenberg 775W DSM 10062 and *Cronobacter sakazakii* CIP 103183T strains were used in the present study. Two serovars of *S. enterica* were tested regarding their respective behavior toward drying and heat treatment showed in literature (Beuchat et al., 2013). *S.* Typhimurium was chosen for its high thermal resistance in dried state and its relevance in outbreaks linked to low-moisture foods. *S.* Senftenberg was chosen for its high thermal resistance (Ng et al., 1969). Finally, *C. sakazakii* was chosen for its resistance to stress and its relevance in outbreaks (Dancer et al., 2009). All cultures were stored in Tryptic Soy Broth (TSB, Sigma–Aldrich) with 20% glycerol (Sigma–Aldrich) at −80°C. For recovery, bacteria were inoculated on Tryptic Soya Agar (TSA, Sigma–Aldrich) at 37°C for 24 h; five colonies of each bacterium were subsequently picked up in 50 mL of Tryptic Soya Broth (TSB, Sigma–Aldrich) and incubated for 8 h at 37°C. Suspensions were then diluted in 50 mL of new TSB in order to reach an Optical Density (OD) of 0.01 at 600 nm. Cultures in the stationary phase were obtained after 20 h at 37°C.

Inoculation of Powder

Milk powder was used in this study as a simplified model of dried food product. To obtain an inoculated milk powder (26% fat, Regilait, Saint-Martin-Belle-Roche, France), 50 mL cultures were centrifuged (3,400 *g*, 10 min at 25°C) and washed twice with 25 mL of PBS. In a final step, the supernatant was removed and cell pellets were weighed. Milk powder was added to the pellets at a 1:20 ratio (w_{pellet}:w_{powder}) and homogenized by means of a mortar for 30 min. Directly after inoculation, milk powder a_W was checked using an a_W meter (Aqualab, Decagon Devices, Inc, Dardilly, France) and found to be approximately 0.80. The cultivability of the bacteria was estimated using the spread plating method after incubation in TSA media for 24 h at 37°C and recorded as CFU/mL.

Drying Process

To dry inoculated milk powder, hermetic boxes with saturated salt solutions at the bottom which regulated the a_W and consequently the atmosphere RH were used. Potassium acetate and sodium bromide (both from Sigma–Aldrich) were used in this study to reach an a_w of 0.25 and 0.58. Atmospheres were maintained under convection using a ventilator (Sunon, Radiospare, France) as described in a previous study (Lemetais et al., 2012). For each strain, approximately 2 g of inoculated milk powder were spread on four small Petri dishes, which were then placed without the lids inside hermetic boxes for 16 h in order to reach the final a_W level. All drying processes were performed at room temperature.

Thermal Treatment

0.1 g of dried inoculated milk powder (Régilait, France) were put into a 0.2 mL tube and treated at two different holding temperatures (90°C and 100°C) for 0, 30, and 120 s in a thermocycler (Bioer, France). Samples were then cooled to 4°C to stop the impact of the thermal treatment. Milk powder was rehydrated by adding 1 mL of PBS and agitating for 30 s, as recommended by the supplier.

Virulence Assays

Caco-2 Cell Maintenance and Preparation

Caco-2 ACC 169 (DSMZ, Germany) was used in this study. Cells were maintained in flasks of 175 cm^2 in a complete medium, comprised of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1% Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100X, Invitrogen, France) and 2 mM L-glutamine (Invitrogen, France). Flasks were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. At confluence, complete medium was removed and cells were washed three times in PBS (pH 7.2, Invitrogen, France). 5 mL of 0.25% Trypsin-phenol red (Invitrogen, France) were added to cover the cell layers and flasks were incubated 15 min in a humidified atmosphere containing 5% CO_2 at 37°C, permitting to retrieve Caco-2 cells. The action of trypsin was stopped by adding 20 mL of complete medium. Viable cell concentration was estimated by means of trypan blue (Invitrogen, France). Caco-2 cells were seeded at a density of approximately 300,000 cells per well in 6-well tissue culture plates (Nunc, France), containing 3 mL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37°C for 15–17 days to obtain fully differentiated cell layers. During this period, the medium was changed every 2 days. Before use for virulence assays, cell layers were washed three times in PBS (pH 7.2) and 3 mL of complete medium without gentamicin were added.

Bacterial Sample Preparation

Control, dried and heated bacterial samples were washed three time in PBS by centrifugation (3,200 *g*, 10 min, 25°C). If necessary, several samples (taken from the same inoculated milk powder) treated in the same conditions were pooled together to reach the fixed bacterial concentration for the experiment. Counting by CFU was performed after incubation in TSA medium for 24 h at 37°C and results were expressed as $\log_{10}(N_1/N_0)$, where N_1 represented the CFU after drying and N_0 represented the initial contamination of inoculated milk powder before drying. After thermal treatment, results were expressed as $\log_{10}(N_2/N_1)$, where N_2 represented the contamination of milk powder after heat treatment. For each virulence assay, the bacterial concentration of each sample was adjusted to 10^7 CFU/mL. If necessary, several samples were pooled to reach this concentration.

Invasion in Caco-2 Cells

Ten microliter of prepared bacterial sample were added per well. Following incubation in humidified atmosphere containing 5% CO_2 at 37°C for 1 h 30, 30 µL of 10 mg/L gentamicin (concentration per well of 100 µg/mL) were added before incubation in humidified atmosphere containing 5% CO_2 at 37°C for 30 min. Medium was then removed and each well was washed three times with PBS (pH 7.2). Caco-2 cells were lysed with 1% Triton X-100 for 5 min. The cell lysates were then diluted and plated in TSA Petri dishes and incubated at 37°C for 24 h, permitting an estimation of the CFU.

Statistical Analyses

All experiments were performed independently five times. The effects of the factors on the thermal treatment were evaluated for each bacterium by analysis of variance (ANOVA) using R v3.4.0 software (R Development Core Team, 2008). Significance was evaluated when the *p*-value was equal to or less than 0.05; in this case a Tukey's HSD (Honest Significant Difference) test was performed to observe significant differences among conditions.

RESULTS

The effects of drying and heating in the dried state on the invasion of *C. sakazakii*, *S.* Typhimurium, and *S.* Senftenberg in Caco-2 cells are presented below.

Impact of Drying on Cultivability and Bacterial Virulence

The effect of drying on the invasion capacity of the studied foodborne pathogens is presented in **Figure 1**. ANOVA tests revealed a significant effect of the water activity level of the decimal logarithm of the bacterial population found after the invasion test. In the case of *C. sakazakii* (**Figure 1A**), the HSD test revealed two significantly different groups. The first group is represented by the pure culture, with a count of 3.9 log bacteria ("Control" in **Figure 1A**) after the invasion test, and the second is composed of the three different a_W levels (i.e., 0.80, 0.58, and 0.25) with a mean count of approximately 4.4 log bacteria. The difference between the means of the two HSD groups represented a count difference of 0.52 log. In the case of *S.* Typhimurium (**Figure 1B**), the HSD test revealed two significantly different groups as well. The first group is the pure culture, with 3.8 log bacteria ("Control" in **Figure 1B**) after the invasion test. The

second group is composed of the three different a_W levels with a count mean of approximately 4.3 log bacteria. In this case, the difference between the means of the two HSD groups represented a count difference of approximately 0.54 log. For *S.* Senftenberg (**Figure 1C**), the HSD test revealed two significantly different groups. The first one is composed of the pure culture and drying at 0.80 a_W, with counts of 3.9 and 4.0 log bacteria respectively after the invasion test ("Control" in **Figure 1C**). The second group is composed of the three different drying levels with a mean count of approximately 4.2 log bacteria. The difference between the means of the two HSD groups represented approximately 0.20 log. No differences were observed among the different drying levels, and the drying level at $a_W = 0.80$ belonged to both groups. This signifies that drying significantly increased invasion capacity at the beginning of the drying process in the three tested bacteria. This increase in invasion capacity was induced from the beginning of the drying as the effect was already significant for drying to an a_W of 0.80 in *C. sakazakii* and *S.* Typhimurium and between 0.80 and 0.58 in *S.* Senftenberg. Under this a_W level (0.25 and 0.58), no extended effect of drying was observed.

The loss of cultivability after drying at 0.25, 0.58 and 0.80 a_W is presented in **Figure 2**. In *C. sakazakii*, the loss of cultivability represented −0.57 log, −1.06, and −0.34 log after drying at 0.25, 0.58, and 0.80 a_W, respectively, while the virulence increase was represented by an invasion increase of 0.52 log in both cases (**Figure 1A**). In the same way, in the case of *S.* Typhimurium, at water activity levels of 0.25, 0.58, and 0.80, the uncultivable bacteria represented −0.83 log, −1.02 and −0.61 log of the initial bacteria respectively, while the virulence increase represented 0.54 log of invasive bacteria (**Figure 1B**). In *S.* Senftenberg, the invasion increase compensated for the loss of cultivability, which measured approximately −0.70 log, −1.38 and −0.55 log after drying at 0.25 and 0.58 a_W, respectively (**Figure 1C**).

Impact of Heating in the Dried State on the Cultivability and Virulence of Bacteria

The effect of heating in the dried state on the invasion capacity of studied foodborne pathogens is presented in **Figure 3**. ANOVA tests revealed no significant effect of heating conditions compared to previous drying (represented by "0 s" in **Figure 3**) on any of the bacteria after the invasion test. More exactly, heating did not increase the invasion capacity of dried bacteria.

The impact of heat treatment on bacterial cultivability is presented in **Figure 4**. The loss of cultivability was greatest in all bacteria at an a_W of 0.58. In the same way, this loss was greatest at high temperature (100°C) and for long treatment time (120 s). In all bacteria, the least loss of cultivability was observed in milk powder at an a_W of 0.25 treated at 90°C for 30 s. After 120 s at 100°C in milk powder at 0.58, cultivability of both *Salmonella* serovars was under the detection limit of the method. The loss of cultivability in all bacteria was greater than 1 log decrease and this loss of cultivability was in no case compensated for by an increase in virulence.

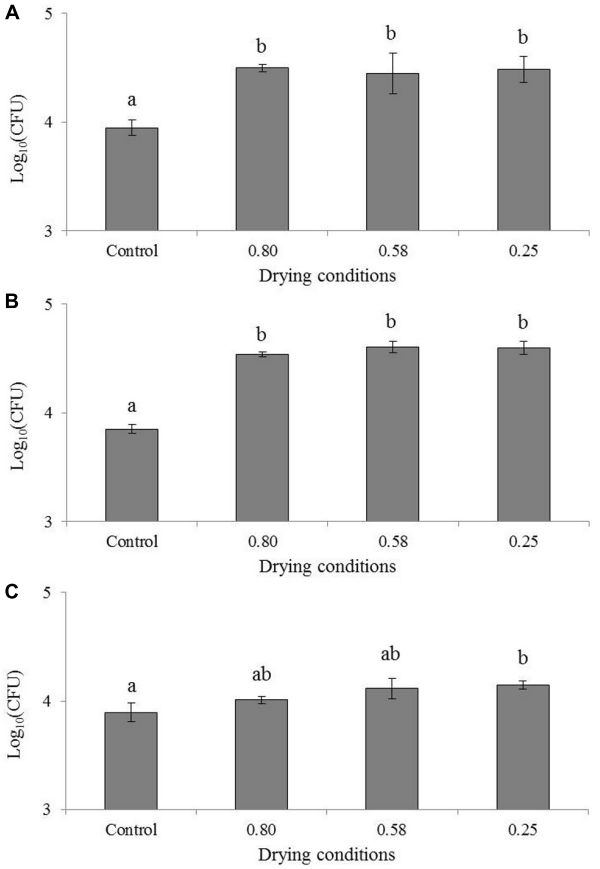


FIGURE 1 | Impact of drying on the invasion of *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg in Caco-2 cells. Results of invasion after 1 h 30 are presented in $\log_{10}$(CFU) for the same inoculum of **(A)** *Cronobacter sakazakii*, **(B)** *Salmonella* Typhimurium and **(C)** *Salmonella* Senftenberg. "Control" represents the invasion of pure culture, "0.80" is invasion directly after the inoculation of milk powder, "0.58" is invasion directly after drying to an a_W of 0.58 and "0.25" is invasion directly after drying to an a_W of 0.25. Error bars represent the standard deviations calculated on independent triplicates. Letters above bars represent the significant differences among conditions resulting from a Tukey's HSD test.

DISCUSSION

Drying and Invasion Capacity of Foodborne Pathogens

A threshold effect of a_W decrease was detected, higher than 0.80 for *S.* Typhimurium and *C. sakazakii*, and between 0.80 and 0.58 for *S.* Senftenberg and could correspond to the water activity limit above which metabolic pathways are still active, involving certain enzymes which require a lower water activity threshold to function (Lee and Kim, 1995).

Foodborne pathogens must adapt to several and successive environmental stresses in foods, gastrointestinal tract, and also host's phagosome to cause illness. All these stresses engage different and coordinate gene activation and expression pathways, as for acid, osmotic, antimicrobial peptide, or also nutrient starvation stresses. These perturbations activate

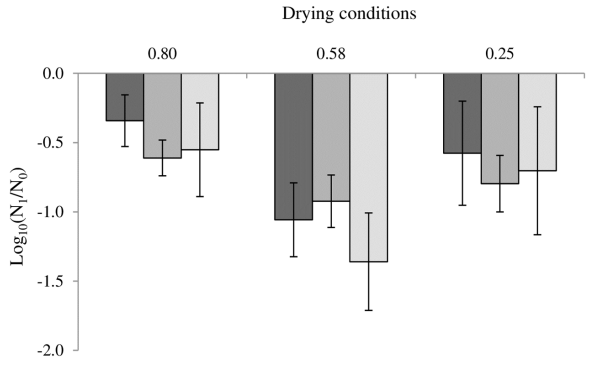


FIGURE 2 | Impact of drying on the cultivability of *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg. From darker to lighter, the results are presented for *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg. Error bars represent the standard deviations calculated on independent triplicates.

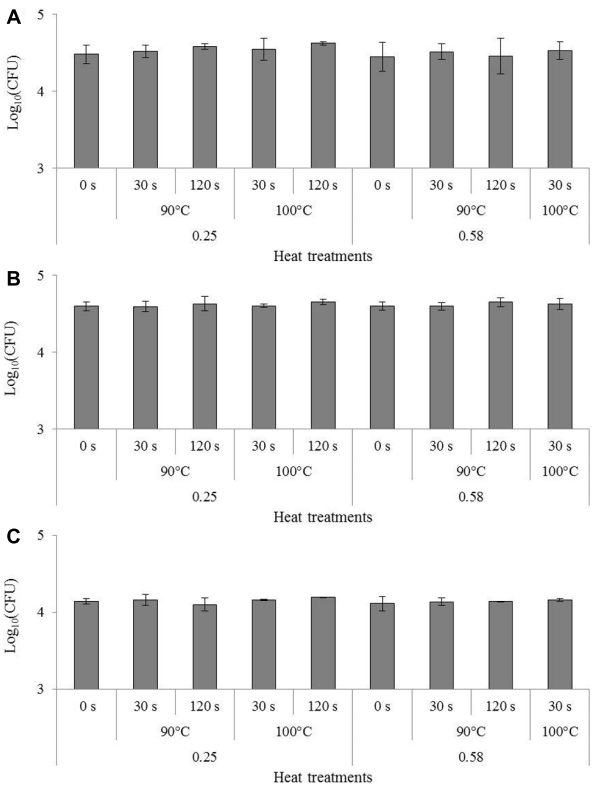


FIGURE 3 | Impact of heating in the dried state on the invasion of *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg in Caco-2 cells. Results of invasion after 1 h 30 are presented in $\log_{10}$(CFU) for the same inoculum of **(A)** *Cronobacter sakazakii*, **(B)** *Salmonella* Typhimurium and **(C)** *Salmonella* Senftenberg. Error bars represent the standard deviations calculated on independent triplicates. Letters above bars represent the significant differences among conditions resulting from a Tukey's HSD test.

generally several alternative metabolic pathways involved in stress adaptation, promoting activation or repression of multiple genes in response to a single environmental perturbation (Rychlik and Barrow, 2005; Shen and Fang, 2012; Guo and Gross, 2014; Feeney et al., 2015; Burgess et al., 2016). Regarding *S. enterica*, desiccation induces up-regulation of gene and transcriptional factors entailed in other stress responses, *rpoH* and *rpoE* genes encoding transcriptional factors involved in heat and oxidative stress response, respectively (Gruzdev et al., 2012). Also, Aviles et al. (2013) demonstrated that storage of *Salmonella* biofilm at $a_W = 0.3$ promoted an increased expression of stress response genes *rpoS* and *otsB* correlated with survival, indicating cross-protection to desiccation and acid stress. Also, in *Cronobacter*, a cross-protection is observed for several stresses. For example, Alvarez-Ordóñez et al. (2014) showed that *C. sakazakii* exposed to acid stress produces activation of several genes encoding chaperone proteins, as DnaJ also involved in heat stress response. Consequently, the way that bacteria undergo environmental perturbation as performed in this study could also promote their survival in gastrointestinal tract until epithelium cells.

Many stresses are known to impact the virulence capacity of foodborne pathogens, especially in the case *S. enterica* which is well studied (Rychlik and Barrow, 2005). The most completely studied stress response is acid stress response, occurring during the passage in the gastrointestinal track and which is under the control of the general stress response transcriptional factor RpoS (Fang et al., 1992; Gahan and Hill, 1999; Koutsoumanis and Sofos, 2004; Rychlik and Barrow, 2005). Moreover, RpoS is already shown to be linked with the invasion properties of bacterial cells and also known to play a role in osmotic stress, by activating the accumulation of ions and compatible solutes through *proU* activation (Csonka, 1989; Rychlik and Barrow, 2005; Du et al., 2011; Shen and Fang, 2012; Andino and Hanning, 2015). Consequently, osmotic stress could also activate such virulence properties. Although several studies have examined the impact of osmotic stress in liquid media (i.e., water/glycerol or salt solution), few are especially focused on desiccation. It seems that during desiccation, activation of the RpoE regulon is also recorded and this could impact the virulence property of *S. enterica* for which the RpoE transcriptional factor is essential for success inside the host (Li et al., 2015). Regarding *C. sakazakii*, the ability of this bacterium to survive osmotic or desiccation stress is often considered as remarkable compared to other Enterobacteriaceae (Burgess et al., 2016). *C. sakazakii* responds similarly to *Escherichia coli* or *Salmonella enterica*, i.e., first accumulating potassium and counter-ion, and secondly accumulating/synthesizing compatible solutes (Feeney et al., 2014). Nevertheless, the regulation pathways are not completely elucidated in *C. sakazakii*. It was already shown that *hfq* gene plays an important role in virulence and stress acclimation. Indeed, Kim et al. (2015) have showed that Δ*hfq* mutant presented a three-fold attenuation of invasion in animal cells and a lower resistance to oxidative stress (hydrogen peroxide, 100-fold). They also suggest that this gene plays an important role in regulation of multiple genes participating in virulence (Kim et al., 2015).

Thus, the way to undergo and survive drying could interfere in bacterial virulence. Indeed, Beaubrun et al. (2017) did not detect differences at the genomic level between two

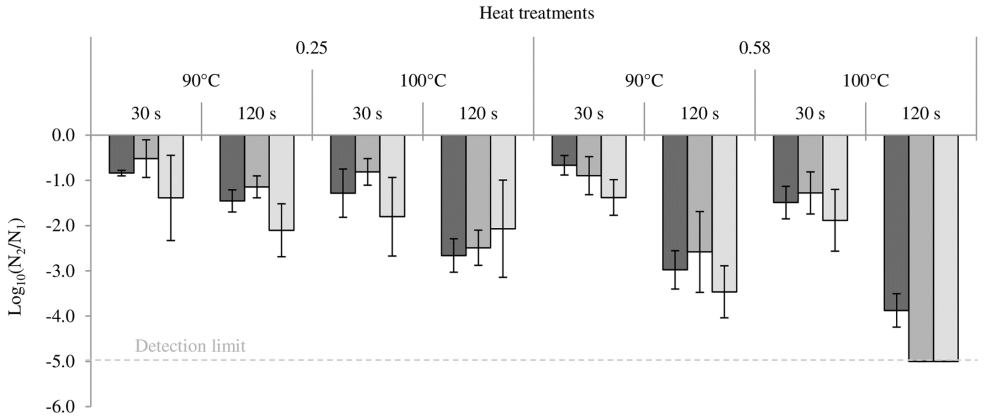


FIGURE 4 | Impact of heating on the cultivability of *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg. From darker to lighter, the results are presented for *Cronobacter sakazakii*, *Salmonella* Typhimurium and *Salmonella* Senftenberg. Error bars represent the standard deviations calculated on independent triplicates.

Salmonella Montevideo species (SAL242S and SAL242, atypical mucoid and non-mucoid strains, respectively). Nevertheless, they detected transcriptomic difference between these species, notably increased expression of EPS and SPI1 genes by SAL242S, responsive to mucoid and virulence protein production. They hypothesized that this is associated with post-transcriptional factors induced during environmental stress and that this increased expression of SPI1 genes may play a role in protecting *Salmonella* from environmental stressors (Beaubrun et al., 2017). Moreover, in studies concerning the transcriptome of desiccation stress, it has been clearly demonstrated that virulence is related to the stress response pathway (Li et al., 2012; Aviles et al., 2013). In *Cronobacter*, little knowledge was available. A recent study of Jing et al. (2016) showed that interaction between *C. sakazakii* and human intestinal epithelial cells induces modification of the bacterium transcriptome. Among upregulated genes, genes involved in the osmotic stress acclimation were identified. For instance, *proV* was upregulated 12-fold. This gene encodes for a glycine betaine/proline transport system ATP-binding protein, where glycine betaine and proline correspond to compatible solutes accumulated during osmotic stress (Feeney et al., 2014). In the same way, *kdpA*, *kdpB*, and *kdpC* were upregulated 27-, 15-, and 8-fold, respectively. These genes are associated with potassium transport system, implicated in first response to osmotic stress (Csonka and Hanson, 1991). Finally, Jing et al. (2016) also demonstrated that *betB* involved in the biosynthesis of osmoprotectant glycine betaine (Lamark et al., 1991) was upregulated 7-fold. Betaine aldehyde dehydrogenase (BetB) is an efficient osmotic regulator, which participates in catalyzing the oxidation of betaine aldehyde to glycine betaine (Kempf and Bremer, 1998). Taken together, all these informations suggest that genes associated with osmotic stress are important to infection of human intestinal epithelial cells by *C. sakazakii*. Consequently, resistance to drying could be linked to the disease-causing potential of the bacteria *C. sakazakii* and *S. enterica* and *vice versa*.

It is necessary to step back that drying has a potential negative impact on food safety and that drying conditions have to be managed to insure a minimal activation of several metabolic pathways which may be involved in increased invasion capacity. In addition, in our drying conditions the first step of a_W decrease was slow, i.e., 30 min to reach an a_W of 0.80. It is possible to assume that, as in the case of cultivability (Lang et al., 2017), a rapid drying of food products to reach a water activity under a physiologic threshold could limit the acclimation and the invasion capacity increase of foodborne pathogens.

Finally, rehydration of milk powder plays an important role for bacterial survival. Indeed, it was already shown that kinetics of rehydration can play on bacterial survival. The slower kinetics, the greater inactivation (Lang et al., 2016; Zoz et al., 2016). Previous works have also demonstrated that the temperature of rehydration allows an optimal bacterial survival in the approximate range of 35–45°C, depending on the bacterial strains.

Heat in Dried State and Invasion Capacity of Foodborne Pathogens

Even if drying leads to a microbial stability of food products over time, this process does not sterilize the product. Consequently, low moisture foods are microbiologically stable, but not microbiologically safe (Beuchat et al., 2013). For this reason, these food products, and particularly herbs and spices, are often exposed to a subsequent decontamination process (as heat treatment) (Grasso et al., 2014; Niemira, 2014). Indeed, dried food products are heated to insure food safety. Our results showed that heating did not increase the invasion capacity of dried bacteria, which can be explained by the low water activity aborting physiological metabolism; nor did heating decrease invasion capacity, which may be explained by a protection by drying of structures involved in the virulence pathway. Microorganisms are more resistant to the decontamination process when water activity is low. This is partially due to the low water activity which stabilizes physiological structures and also due to cross protection mechanisms. Notably, osmotic stress encourages the synthesis of HSP (Heat Shock Proteins),

a response which protects proteins and membrane from heat alteration (Richter et al., 2010; Shen and Fang, 2012). The fact that bacteria are able to engage certain mechanisms during drying also suggests that synthesized proteins may positively or negatively impact heat resistance. Indeed, virulence mechanisms involve the synthesis of several proteins and factors which could modify the membrane or cytosol composition and consequently influence stress resistance. For example, as some virulence proteins are membrane proteins, a modification of the membrane composition could impact the membrane phospholipid transition phase (Ibarra and Steele-Mortimer, 2009; Fàbrega and Vila, 2013) and, subsequently, impact bacterial resistance. In our case, no difference was detected between heated bacteria and dried bacteria, suggesting that the activation of virulence during drying does not interfere with heat sensitivity. Consequently, thermal treatment guarantees a great loss of cultivability and also does not increase the invasion properties of these two foodborne pathogens. If a maximal loss of cultivability can be guaranteed in dried food, thermal treatment could represent an effective tool in the quest for food safety.

CONCLUSION

This study has analyzed the impact of drying and successive heat treatment in three foodborne milk powder pathogens. While the impact of several stresses on virulence has already been demonstrated, this is the first record of the impact of aerial drying and of heating in the dried state on the invasion capacity of *Salmonella enterica* and *Cronobacter sakazakii*. Our results show that drying to the 0.80 water activity level significantly increases the invasion capacity of these bacteria (approximately four times as many bacteria entering Caco-2 cells), which almost compensates for the loss of cultivability observed after drying. Further deeper drying and subsequent heat treatments did not modify the invasion capacity of the three studied bacteria and did not offset the loss of cultivability noted after heat treatment. Taken together, these results provide a new perspective on food processing as well as insight into its impact on health in terms of foodborne pathogens in dried food products. Further experiment in other dried food products, such as PIF, spices, herbs, or flour, will permit to strengthen this study and our knowledge regarding bacterial virulence, food transformation, food conservation, and link with food safety.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work: EL and PG; the acquisition: EL; analysis and interpretation of data for the work: EL, SG, PA-M, J-MP-C, and PG. Drafting the work or revising it critically for important intellectual content: EL, SG, PA-M, J-MP-C, and PG. Final approval of the version to be published: EL, SG, PA-M, J-MP-C, and PG. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: EL, SG, PA-M, J-MP-C, and PG.

FUNDING

This work was supported by the Association Nationale de la Recherche et de la Technologie (grant number: 2012/1631, France) and by Novolyze (France), which are gratefully acknowledged.

ACKNOWLEDGMENT

We are thankful to Pr Frédéric DALLE for his help concerning Caco-2 cell culture, as well as to Amandine DUCREUX.

REFERENCES

Akhtar, S., Sarker, M. R., and Hossain, A. (2014). Microbiological food safety: a dilemma of developing societies. *Crit. Rev. Microbiol.* 40, 348–359. doi: 10.3109/1040841X.2012.742036

Alvarez-Ordóñez, A., Cummins, C., Deasy, T., Clifford, T., Begley, M., and Hill, C. (2014). Acid stress management by *Cronobacter sakazakii*. *Int. J. Food Microbiol.* 178, 21–28. doi: 10.1016/j.ijfoodmicro.2014.03.001

Andino, A., and Hanning, I. (2015). *Salmonella enterica*: survival, colonization, and virulence differences among serovars. *ScientificWorldJournal* 2015:520179. doi: 10.1155/2015/520179

Aviles, B., Klotz, C., Eifert, J., Williams, R., and Ponder, M. (2013). Biofilms promote survival and virulence of *Salmonella enterica* sv. Tennessee during prolonged dry storage and after passage through an *in vitro* digestion system. *Int. J. Food Microbiol.* 162, 252–259. doi: 10.1016/j.ijfoodmicro.2013.01.026

Beaubrun, J. J.-G., Tall, B. D., Flamer, M.-L., Patel, I., Gopinath, G., Auguste, W., et al. (2017). Increased secretion of exopolysaccharide and virulence potential of a mucoid variant of *Salmonella enterica* serovar Montevideo under environmental stress. *Microb. Pathog.* 103, 107–113. doi: 10.1016/j.micpath.2016.12.005

Beuchat, L. R., Komitopoulou, E., Beckers, H., Betts, R. P., Bourdichon, F., Fanning, S., et al. (2013). Low-water activity foods: increased concern as vehicles of foodborne pathogens. *J. Food Prot.* 76, 150–172. doi: 10.4315/0362-028X.JFP-12-211

Buchanan, R. L., Smith, J. L., and Long, W. (2000). Microbial risk assessment: dose-response relations and risk characterization. *Int. J. Food Microbiol.* 58, 159–172. doi: 10.1016/S0168-1605(00)00270-1

Burgess, C. M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., et al. (2016). The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. *Int. J. Food Microbiol.* 221, 37–53. doi: 10.1016/j.ijfoodmicro.2015.12.014

Cahill, S. M., Wachsmuth, I. K., Costarrica, M. D. L., and Ben Embarek, P. K. (2008). Powdered infant formula as a source of *Salmonella* infection in infants. *Clin. Infect. Dis.* 46, 268–273. doi: 10.1086/524737

Codex Alimentarius (2008). *Code of Hygienic Practice for Powdered Formulae for Infants and Young Children*. Rome: FAO.

Csonka, L., and Hanson, A. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* 45, 569–606. doi: 10.1146/annurev.mi.45.100191.003033

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* 53, 121–147.

Dancer, G. I., Mah, J. H., Rhee, M. S., Hwang, I. G., and Kang, D. H. (2009). Resistance of *Enterobacter* sakazakii (*Cronobacter* spp.) to environmental stresses. *J. Appl. Microbiol.* 107, 1606–1614. doi: 10.1111/j.1365-2672.2009.04347.x

Du, H., Wang, M., Luo, Z., Ni, B., Wang, F., Meng, Y., et al. (2011). Coregulation of gene expression by sigma factors RpoE and RpoS in *Salmonella enterica* serovar Typhi during hyperosmotic stress. *Curr. Microbiol* 62, 1483–1489. doi: 10.1007/s00284-011-9890-8

Fàbrega, A., and Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clin. Microbiol. Rev.* 26, 308–341. doi: 10.1128/CMR.00066-12

Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J., et al. (1992). The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. U.S.A.* 89, 11978–11982. doi: 10.1073/pnas.89.24.11978

Feeney, A., Johnston, C. D., Govender, R., O'Mahony, J., Coffey, A., and Sleator, R. D. (2014). Analysis of the role of the *Cronobacter sakazakii* ProP homologues in osmotolerance. *Gut Pathog* 6:15. doi: 10.1186/1757-4749-6-15

Feeney, A., Kropp, K. A., O'Connor, R., and Sleator, R. D. (2015). *Cronobacter sakazakii*: stress survival and virulence potential in an opportunistic foodborne pathogen. *Gut Microbes* 5, 711–718. doi: 10.4161/19490976.2014.983774

Forsythe, S. (2014). "Powdered infant formula," in *The Microbiological Safety of Low Water Activity Foods and Spices*, eds J. B. Gurtler, M. P. Doyle, and J. L. Kornacki (London: Springer), 177–211.

Gahan, C. G., and Hill, C. (1999). The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 50, 93–100. doi: 10.1016/S0168-1605(99)00079-3

Giri, C. P., Shima, K., Tall, B. D., Curtis, S., Sathyamoorthy, V., Hanisch, B., et al. (2012). *Cronobacter* spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells. *Microb. Pathog.* 52, 140–147. doi: 10.1016/j.micpath.2011.10.003

Grasso, E. M., Stam, C. N., Anderson, N. M., and Krishnamurthy, K. (2014). "Heat and steam treatments," in *The Microbiological Safety of Low Water Activity Foods and Spices*, eds J. B. Gurtler, M. P. Doyle, and J. L. Kornacki (Berlin: Springer), 403–424.

Gruzdev, N., McClelland, M., Porwollik, S., Ofaim, S., Pinto, R., and Saldinger-Sela, S. (2012). Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. *Appl. Environ. Microbiol.* 78, 7866–7875. doi: 10.1128/AEM.01822-12

Guo, M. S., and Gross, C. A. (2014). Stress-induced remodeling of the bacterial proteome. *Curr. Biol.* 24, R424–R434. doi: 10.1016/j.cub.2014.03.023

Hohmann, E. L. (2001). Nontyphoidal salmonellosis. *Clin. Infect. Dis.* 32, 263–269. doi: 10.1086/318457

Holý, O., and Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *J. Hosp. Infect.* 86, 169–177. doi: 10.1016/j.jhin.2013.09.011

Humphrey, T. (2004). *Salmonella*, stress responses and food safety. *Nat. Rev. Microbiol.* 2, 504–509. doi: 10.1038/nrmicro907

Ibarra, J. A., and Steele-Mortimer, O. (2009). *Salmonella* - the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cell. Microbiol.* 11, 1579–1586. doi: 10.1111/j.1462-5822.2009.01368.x

Jing, C. E., Du, X. J., Li, P., and Wang, S. (2016). Transcriptome analysis of *Cronobacter sakazakii* ATCC BAA-894 after interaction with human intestinal epithelial cell line HCT-8. *Appl. Microbiol. Biotechnol.* 100, 311–322. doi: 10.1007/s00253-015-7053-8

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* 170, 319–330. doi: 10.1007/s002030050649

Kim, S., Hwang, H., Kim, K., Yoon, H., Kang, D., and Ryu, S. (2015). hfq plays important roles in virulence and stress adaptation in *Cronobacter sakazakii* ATCC 29544. *Infect. Immun.* 83, 2089–2098. doi: 10.1128/IAI.03161-14

Koutsoumanis, K. P., and Sofos, J. N. (2004). Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habituation at different pH conditions. *Lett. Appl. Microbiol.* 38, 321–326. doi: 10.1111/j.1472-765X.2004.01491.x

Lamark, T., Kaasen, I., Eshoo, M. W., Falkenberg, P., Mcdougall, J., and Strom, A. R. (1991). DNA sequence and analysis of the bef genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *Mol. Microbiol.* 5, 1049–1064. doi: 10.1111/j.1365-2958.1991.tb01877.x

Lang, E., Iaconelli, C., Zoz, F., Guyot, S., Alvarez-Martin, P., Beney, L., et al. (2017). Drying parameters greatly affect the destruction of *Cronobacter sakazakii* and *Salmonella typhimurium* in standard buffer and milk. *Food Microbiol.* 62, 82–91. doi: 10.1016/j.fm.2016.10.005

Lang, E., Zoz, F., Iaconelli, C., Guyot, S., Alvarez-Martin, P., Beney, L., et al. (2016). Recovery estimation of dried foodborne pathogens is directly related to rehydration kinetics. *PLOS ONE* 11:e0160844. doi: 10.1371/journal.pone.0160844

Lee, S., and Kim, K. (1995). Effect of water activity on enzyme hydration and enzyme reaction rate in organic solvents. *J. Ferment. Bioeng.* 79, 473–478. doi: 10.1016/0922-338X(95)91264-6

Lemetais, G., Dupont, S., Beney, L., and Gervais, P. (2012). Air-drying kinetics affect yeast membrane organization and survival. *Appl. Microbiol. Biotechnol.* 96, 471–480. doi: 10.1007/s00253-012-4014-3

Li, H., Bhaskara, A., Megalis, C., and Tortorello, M. (2012). Transcriptomic analysis of *Salmonella* desiccation resistance. *Foodborne Pathog. Dis.* 9, 1143–1151. doi: 10.1089/fpd.2012.1254

Li, J., Nakayasu, E. S., Overall, C. C., Johnson, R. C., Kidwai, A. S., McDermott, J. E., et al. (2015). Global analysis of *Salmonella* alternative sigma factor E on protein translation. *J. Proteome Res.* 14, 1716–1726. doi: 10.1021/pr5010423

Ng, H., Bayne, H. G., and Garibaldi, J. A. (1969). Heat resistance of *Salmonella*: the uniqueness of *Salmonella senftenberg* 775W. *Appl. Microbiol.* 17, 78–82.

Niemira, B. A. (2014). "Irradiation, microwave, and alternative energy-based treatments for low-water activity foods," in *The Microbiological Safety of Low Water Activity Foods and Spices*, M. Doyle, J. Kornacki, and J. Gurtler (New York, NY: Springer), 389–401.

Podolak, R., Enache, E., Stone, W., Black, D. G., and Elliott, P. H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *J. Food Prot.* 73, 1919–1936. doi: 10.4315/0362-028X-73.10.1919

R Development Core Team (2008). *R: A Language and Computing, Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Richter, K., Haslbeck, M., and Buchner, J. (2010). The heat shock response: life on the verge of death. *Mol. Cell* 40, 253–266. doi: 10.1016/j.molcel.2010.10.006

Rychlik, I., and Barrow, P. A. (2005). *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. *FEMS Microbiol. Rev.* 29, 1021–1040. doi: 10.1016/j.femsre.2005.03.005

Shaker, R. R., Osaili, T. M., Abu Al-Hasan, A. S., Ayyash, M. M., and Forsythe, S. J. (2008). Effect of desiccation, starvation, heat, and cold stresses on the thermal resistance of *Enterobacter sakazakii* in rehydrated infant milk formula. *J. Food Sci.* 73, M354–M359. doi: 10.1111/j.1750-3841.2008.00880.x

Shen, S., and Fang, F. C. (2012). Integrated stress responses in *Salmonella*. *Int. J. Food Microbiol.* 152, 75–81. doi: 10.1016/j.ijfoodmicro.2011.04.017

Spector, M. P., and Kenyon, W. J. (2012). Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Res. Int.* 45, 455–481. doi: 10.1016/j.foodres.2011.06.056

Ye, Y., Gao, J., Jiao, R., Li, H., Wu, Q., Zhang, J., et al. (2015). The membrane proteins involved in virulence of *Cronobacter sakazakii* virulent G362 and attenuated L3101 isolates. *Front. Microbiol.* 6:1238. doi: 10.3389/fmicb.2015.01238

Zoz, F., Iaconelli, C., Lang, E., Iddir, H., Guyot, S., Grandvalet, C., et al. (2016). Control of relative air humidity as a potential means to improve hygiene on surfaces: a preliminary approach with *Listeria monocytogenes*. *PLOS ONE* 11:e0148418. doi: 10.1371/journal.pone.0148418

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Lang, Guyot, Alvarez-Martin, Perrier-Cornet and Gervais. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 September 2017
doi: 10.3389/fmicb.2017.01875

DNA-Sequence Based Typing of the *Cronobacter* Genus Using MLST, CRISPR-*cas* Array and Capsular Profiling

Pauline Ogrodzki[1] *and Stephen J. Forsythe*[2]*

[1] School of Science and Technology, Nottingham Trent University, Nottingham, United Kingdom, [2] foodmicrobe.com, Nottingham, United Kingdom

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Beatrix Stessl,
Veterinärmedizinische Universität Wien, Austria
Irshad Sulaiman,
United States Food and Drug Administration, United States
Songzhe Fu,
University of New South Wales, Australia

***Correspondence:**
Stephen J. Forsythe
steveforsythe@foodmicrobe.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 29 June 2017
Accepted: 13 September 2017
Published: 29 September 2017

Citation:
Ogrodzki P and Forsythe SJ (2017) DNA-Sequence Based Typing of the Cronobacter Genus Using MLST, CRISPR-cas Array and Capsular Profiling. Front. Microbiol. 8:1875. doi: 10.3389/fmicb.2017.01875

The *Cronobacter* genus is composed of seven species, within which a number of pathovars have been described. The most notable infections by *Cronobacter* spp. are of infants through the consumption of contaminated infant formula. The description of the genus has greatly improved in recent years through DNA sequencing techniques, and this has led to a robust means of identification. However some species are highly clonal and this limits the ability to discriminate between unrelated strains by some methods of genotyping. This article updates the application of three genotyping methods across the *Cronobacter* genus. The three genotyping methods were multilocus sequence typing (MLST), capsular profiling of the *K*-antigen and colanic acid (CA) biosynthesis regions, and CRISPR-*cas* array profiling. A total of 1654 MLST profiled and 286 whole genome sequenced strains, available by open access at the PubMLST *Cronobacter* database, were used this analysis. The predominance of *C. sakazakii* and *C. malonaticus* in clinical infections was confirmed. The majority of clinical strains being in the *C. sakazakii* clonal complexes (CC) 1 and 4, sequence types (ST) 8 and 12 and *C. malonaticus* ST7. The capsular profile K2:CA2, previously proposed as being strongly associated with *C. sakazakii* and *C. malonaticus* isolates from severe neonatal infections, was also found in *C. turicensis*, *C. dublinensis* and *C. universalis*. The majority of CRISPR-*cas* types across the genus was the I-E (Ecoli) type. Some strains of *C. dublinensis* and *C. muytjensii* encoded the I-F (Ypseudo) type, and others lacked the *cas* gene loci. The significance of the expanding profiling will be of benefit to researchers as well as governmental and industrial risk assessors.

Keywords: *Cronobacter*, genotyping, genomes, MLST, capsule, CRISPR-*cas* profiles

Abbreviations: BIGSdb, Bacterial Isolate Genome Sequence database; *Cas,* CRISPR-associated protein-coding genes; CC, clonal complex; Cg-MLST, core genome multilocus sequence typing; CRISPR, Clustered regularly interspaced short palindromic repeats; EGA, estimated gestation age; MLST, multilocus sequence typing; NGS, next generation sequencing; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; PIF, powdered infant formula; r-MLST, ribosomal multilocus sequence typing; ST, sequence type.

INTRODUCTION

Cronobacter spp. is well known with respect to outbreaks of severe infant infections (necrotizing enterocolitis and meningitis) in neonatal intensive care units. However, the majority of *Cronobacter* infections are in the adult population with various symptoms including urinary tract infections (Holy and Forsythe, 2014; Patrick et al., 2014; Alsonosi et al., 2015). The organism is also a commensal member of the human body flora. Bacterial analysis of throat swabs from over 45,000 outpatients during the period 2005–2011 recovered the organism from every age group, with the highest frequency (8.7/1000 patients sampled) from infants less than 1 year of age (Holý et al., 2014). *Cronobacter*, then known as *Enterobacter sakazakii*, was the first foodborne pathogen which the FAO-WHO aimed to control through reducing neonatal and infant exposure to contaminated reconstituted infant formula (Food and Agriculture Organization of the United Nations [FAO], 2004). This resulted in both improved microbiological criteria at point of production and revised hygienic practices of preparation (Food and Agriculture Organization of the United Nations [FAO], 2004, 2006; Food and Agriculture Organization of the United Nations [FAO] and World Health Organization [WHO], 2008). In addition, the first expert committee meeting made various recommendations, including the need for an internationally validated detection and molecular typing methods for *Cronobacter* spp. and other relevant microorganisms (Food and Agriculture Organization of the United Nations [FAO], 2004).

This paper considers how, since 2004, these recommendations have been met through molecular typing methods based on the application of NGS over conventional methods. Reviews of wider aspects of *Cronobacter*, such as environmental fitness and virulence traits, have been recently published and therefore will not be considered in detail here (Forsythe et al., 2014; Almajed and Forsythe, 2016).

Powdered infant formula (PIF) has been the main vector associated with *Cronobacter* spp. and therefore has been the focus for the reduction in neonatal infections. Consequently, the Food and Agriculture Organization of the United Nations [FAO] (2004) encouraged the establishment of detection and molecular typing schemes which could be used to monitor sources of *Cronobacter* in PIF. An initial challenge was the differentiation of *Cronobacter* spp. (then known as *Enterobacter sakazakii*) from closely related organisms which could be co-recovered from infant formula and its ingredients, i.e., *Franconibacter*, *Siccibacter* and *Enterobacter* spp. (Stephan et al., 2014). Therefore an accurate taxonomic description of *Cronobacter* spp. was necessary for both reliable detection method development and for appropriate regulatory control. It should also be noted that other sources have been reported including the water used for reconstitution of PIF reconstitution, and also enteral feeding tubes (Hurrell et al., 2009b; Broge and Lee, 2013; Hariri et al., 2013; Ravisankar et al., 2014).

Unfortunately, some *Cronobacter* detection methods have been based on poorly characterized, even misidentified, strains (Jackson and Forsythe, 2016). The various *Cronobacter* species were initially defined according to the 16 *Enterobacter sakazakii* biotypes, however some of the biotype index strains were assigned the wrong *Cronobacter* species and this has limited further development of accurate phenotypic methods for *Cronobacter* identification (Iversen et al., 2008; Baldwin et al., 2009; Joseph et al., 2013a; Jackson and Forsythe, 2016). Additionally, the earlier reliance on phenotyping methods led to a number of mistaken identifications in the literature (Caubilla-Barron et al., 2007; Townsend S. et al., 2008; Blažková et al., 2015; Jackson et al., 2015a; Ogrodzki and Forsythe, 2015).

Such misidentifications can cause further confusion for risk management and the control of infection, as well as misinformation on current issues such as carriage of antibiotic resistance. Therefore reliable and robust means of identifying and typing *Cronobacter* isolates are required and should be internationally accessible.

Although 16S rDNA sequence analysis is generally applicable for bacterial species identification, it is not a reliable method for members of the *Cronobacter* genus as it is unable to reliably differentiate between the two species *C. sakazakii* and *C. malonaticus* (Iversen et al., 2008; Baldwin et al., 2009). In order to overcome this limitation, Joseph et al. (2012b) used representative strains across the genus which had been selected using multilocus sequence analysis (MLSA) of 7 housekeeping genes; ATP synthase b chain (*atpD*), elongation factor G (*fusA*), glutaminyl tRNA synthetase (*glnS*), glutamate synthase large subunit (*gltB*), DNA gyrase subunit B (*gyrB*), translation initiation factor IF-2 (*infB*) and phosphoenolpyruvate synthase A (*ppsA*). This DNA-sequence based approach overcame the preconceived grouping of strains based on phenotyping, and supported the recognition of two further *Cronobacter* species; *C. universalis* and *C. condimenti* (Joseph et al., 2012a).

There are a number of centralized MLST databases which are internationally available covering bacteria and fungi with standardized allele profile determination programs. The three major bacterial MLST databases are those at the Institute of Pasteur[1], and the universities of Warwick[2], and Oxford[3]. Initially MLST used laboratory-based sequencing of individual genes, but nowadays it is more reliant on *in silico* analysis of whole genomes. The analysis of uploaded *Cronobacter* genome sequences is through the 'Bacterial Isolate Genomes Sequence Database' (BIGSdb) facility[4]. Furthermore the inclusion of whole genomes has enabled the *Cronobacter* MLST database to be expanded to ribosomal-MLST (r-MLST; 53 genes) and core genome MLST (cg-MLST; 1836 genes) analysis to provide greater resolution between strains and deeper bacterial population studies (Maiden et al., 2013; Forsythe et al., 2014; Jolley and Maiden, 2014). Phylogenetic comparison of 7-loci MLST, rMLST, cg-MLST and whole genome analysis has confirmed the reliability and robustness of the 7-loci MLST scheme for

[1]http://bigsdb.pasteur.fr/

[2]http://mlst.warwick.ac.uk/

[3]https://pubmlst.org

[4]https://pubmlst.org/cronobacter/

speciation and subtyping within the *Cronobacter* genus as well as differentiation from related genera. Additionally, *fusA* allele phylogeny corresponds to whole genome phylogeny and can be used for initial speciation of *Cronobacter* isolates (Joseph et al., 2012c; Forsythe et al., 2014).

The use of MLST, based on NGS, has therefore supported defining the *Cronobacter* genus. The genus is within the family *Enterobacteriaceae,* with the nearest relatives being the *Franconibacter* and *Siccibacter* genera, as well as the more familiar genera of *Enterobacter*, *Citrobacter* and *Pantoea* (Stephan et al., 2014; Jackson et al., 2015b). According to the MLST phylogenetic analysis, the genus split from its nearest ancestor in the *Enterobacteriaceae* family *ca.* 45–68 million years ago, at the same time as the *Salmonella* genus diverged into its species and subspecies (McQuiston et al., 2008; Joseph and Forsythe, 2012).

Using *in vitro* virulence studies, it has been shown that *Cronobacter* spp. can invade intestinal and brain cells, as well as persist and even replicate in macrophages (Townsend et al., 2007; Townsend S.M. et al., 2008; Almajed and Forsythe, 2016). The most reported *Cronobacter* species in clinical cases are *C. sakazakii* and *C. malonaticus* in infant and adults, respectively (Forsythe et al., 2014; Alsonosi et al., 2015). A number of virulence traits have been proposed which may account for these clinical differences. Nevertheless, there have been insufficient large scale comparative whole genome studies to establish their contribution to severe clinical presentations. Of particular interest has been the predominance of *C. sakazakii* in neonatal infections, especially CC4 and ST12. However, studies have not identified traits unique to specific *Cronobacter* pathovars (Kucerova et al., 2010, 2011; Joseph and Forsythe, 2011; Joseph et al., 2012b; Masood et al., 2015). This could be due to unrecognized synergy between different virulence traits and environmental fitness traits leading to increased neonatal exposure and severity of infection (Hariri et al., 2013).

Cronobacter sakazakii strains are able to use sialic acid as a carbon source for growth (Joseph et al., 2013b). This could be a highly significant host-adaptation trait since sialic acid is found in breast milk, mucin and gangliosides (Almagro-Moreno and Boyd, 2009). Laboratory studies have shown that *C. sakazakii* is able to grow on both sialic acid and ganglioside (GM1) as a sole carbon source (Joseph et al., 2013b) and could enable the degradation of intestinal mucin, facilitating access to intestinal cells. Due to its association with brain development, sialic acid is an ingredient in PIF and therefore an additional *C. sakazakii* growth substrate following rehydration. In contrast, unlike other *Cronobacter* species, *C. sakazakii* strains are unable to grow on malonic acid. This organic acid is found in plants and the ability to utilize it is regarded as reflecting the initial plant-association of the *Cronobacter* genus (Schmid et al., 2009). The adaptation of *C. sakazakii* to a new ecosystem, with the subsequent loss of malonic acid utilization and gain in sialic acid utilization might therefore contribute to its pathogenicity toward neonates (Joseph et al., 2013b). The mechanisms and routes of exposure by which *C. malonaticus* infect adults more than infants are as yet unknown. In the *Cronobacter* genera, a total of 10 fimbrial families have been identified. *C. sakazakii* lack curli fimbriae, but are unique in encoding for β–fimbriae (Joseph et al., 2012b). The clinical significance of this has not been investigated, but might contribute to the greater predominance of *C. sakazakii* in infant infections through attachment to host cells. A range of other virulence traits have been reported. These include *Cronobacter* plasminogen activator (cpa) (Franco et al., 2011), iron utilization (Grim et al., 2012), outer membrane vesicle release causing host cell cytopathogenicity (Alzahrani et al., 2015; Kothary et al., 2017), as well as heavy metal resistance traits (copper, silver, zinc, tellurite) (Joseph et al., 2012b; Chaturvedi et al., 2015).

Cronobacter spp. produce capsular material composed of the *O* and *K*-antigens, colanic acid, Enterobacteriacae common antigen, and cellulose (Ogrodzki and Forsythe, 2015). The capsular material is composed of water-saturated, high molecular weight polysaccharides. It plays a role in virulence by enabling organisms to evade host response mechanisms (serum resistance and phagocytosis), as well as facilitating biofilm formation and desiccation survival (Willis and Whitfield, 2013). *Cronobacter* biofilms have been found in hospitals on equipment used to prepare infant formula, feeding bottles, and neonatal enteral feeding tubes (Iversen and Forsythe, 2003; Iversen et al., 2004; Kim et al., 2006; Hurrell et al., 2009a,b; Holy and Forsythe, 2014; Jackson et al., 2015a). The biofilms on enteral feeding tubes could act as loci for neonatal infection (Hurrell et al., 2009a,b). Under some growth conditions, the organism produces excessive capsule material which may protect the organism from disinfectants in food production environments, and enable long-term persistence under desiccation conditions, such as in PIF for over 2 years (Caubilla-Barron and Forsythe, 2007). It is therefore plausible that the strong association between *C. sakazakii* CC4 and neonatal meningitis is due to its environmental fitness of desiccation survival, resulting in its persistence in PIF and the environment of PIF manufacturing plants (Muller et al., 2013; Power et al., 2013; Sonbol et al., 2013; Fei et al., 2015, 2017). Therefore the greater incidence of *C. sakazakii* CC4 in PIF could lead to an increased risk of exposure and incidence of infection rather than being due to the organism encoding for specific pathogenicity traits.

A capsular profiling scheme for *Cronobacter* based on the *K*-antigen and colanic acid (CA) biosynthesis encoding genes has been proposed (Ogrodzki and Forsythe, 2015). This scheme was based on the analysis of 104 *Cronobacter* genomes and revealed that strains of *C. sakazakii* and *C. malonaticus* isolated from cases with the most severe neonatal clinical presentations of invasive meningitis and necrotizing enterocolitis (NEC), had a definable capsular profile which differed from strains associated with less severe clinical cases. They reported that all ($n = 54$) strains of *C. sakazakii* CC4 and ST12 strains known for being associated with severe neonatal infections of meningitis and NEC, had the capsular profile K2:CA2 (Joseph and Forsythe, 2011, 2012; Joseph et al., 2012c; Hariri et al., 2013; Forsythe et al., 2014). Of particular interest was that this particular capsule profile was also found in the isolates of the few *C. sakazakii* non-CC4 cerebral spinal fluid (CSF) cases, including a single *C. malonaticus* fatal case (Ogrodzki and Forsythe, 2015). Also of interest was that two *C. turicensis* strains encoding for sialic acid utilization also had the capsular profile K2:CA2. Since the earlier study by

Ogrodzki and Forsythe (2015), a large number of *Cronobacter* genomes have become available and therefore a wider evaluation of capsular profiling across the genus is now feasible.

The establishment of internationally standardized molecular typing methods applicable across the *Cronobacter* genus is necessary given the severe outcomes of infections in neonates and the attributed link to contaminated PIF on the international market (Food and Agriculture Organization of the United Nations [FAO], 2004, 2006; Food and Agriculture Organization of the United Nations [FAO] and World Health Organization [WHO], 2008). Although it is generally possible to differentiate *Cronobacter* species by biochemical profiling, molecular methods are increasingly used as a more rapid and reliable tool to study bacterial genomic diversity and to track sources of infection. Since *Cronobacter* is ubiquitous, such typing schemes are applicable for both epidemiological and environmental investigations. For epidemiological analysis (i.e., tracing source and dissemination during an outbreak), PFGE with two restriction enzymes (*Xba*1 and *Spe*1) has in the past been the most common method (van Acker et al., 2001; Himelright et al., 2002). The method is limited, however, as not all *Cronobacter* strains can be typed due to intrinsic DNase activity, non-identical strains can give the same PFGE profile and the method does not give the relationship between strains (Centers for Disease Control and Prevention [CDC], 2010; Alsonosi et al., 2015). Due to these limitations of PFGE, the Centers for Disease Control and Prevention (CDC) is transitioning to using whole genome sequencing as the basis for PulseNet surveillance (Carleton and Gerner-Smidt, 2016; Nadon et al., 2017).

A number of typing methods for *Cronobacter* have been published, in particular PCR-probe *O*-antigen serotyping (Mullane et al., 2008; Jarvis et al., 2011; Sun et al., 2011). Initially 7 serotypes were defined for *C. sakazakii* and 2 in *C. malonaticus*. However, some strains of *C. malonaticus* were mis-identified as *C. sakazakii* by Sun et al. (2011) and were assigned *C. sakazakii* serotypes O:5 and O:6. Consequently, Blažková et al. (2015) proposed the O-antigen scheme should be revised with additional recognition of 7 new and 2 re-assigned serotypes. Chemical analysis of the *O*-polysaccharide units from many strains has confirmed the molecular *Cronobacter* serotypes. However three structures have been determined for *C. sakazakii* O:2 strains (Arbatsky et al., 2010; Czerwicka et al., 2010; Maclean et al., 2010). This is probably due to variants in the *O*-antigen genes which occur outside the target region of the PCR primers.

The PCR-probe O-serotyping has been superseded by allele profiling of *gnd* and *galF* (encoding 6-phosphogluconate dehydrogenase and UTP-glucose-1-phosphate uridylyltransferase subunits, respectively) (Ogrodzki and Forsythe, 2015). This DNA-sequence based approach is a more reliable and expansive method for *O*-antigen determination within *Cronobacter*. It has increased the definable number of serotypes in the *Cronobacter* genus from 18 to 34 (Ogrodzki and Forsythe, 2015).

MLST analysis has not only been able to support taxonomic revisions, but can differentiate strains to a greater degree than other methods; >500 defined STs compared with 34 for O-serotyping. It has also revealed a remarkably strong clonal nature in the *Cronobacter* genus, in particular within the clinically relevant *C. sakazakii* and *C. malonaticus* species (Baldwin et al., 2009; Joseph et al., 2012c). This has subsequently led to the recognition of particular pathovars (Joseph and Forsythe, 2011; Hariri et al., 2013; Sonbol et al., 2013; Forsythe et al., 2014). *C. sakazakii* CC4 is a DNA sequence defined evolutionary lineage which is especially associated with neonatal meningitis. *C. sakazakii* ST12 is associated with cases of NEC and not neonatal meningitis or septicaemia. *C. sakazakii* CC1 strains are primarily isolated from infant formula and clinical sources, whereas *C. sakazakii* ST8 are isolated from clinical and non-formula food sources. *C. malonaticus* adult infections are almost exclusively CC7.

While this clonal recognition is useful for the identification of *Cronobacter* pathovars, it is counter-productive for microbial source tracking as unrelated strains will occur in the same ST. This may explain the observation that the same PFGE pulsotype can be obtained for unrelated clinical *C. sakazakii* strains (Forsythe et al., 2014; Alsonosi et al., 2015). In order to address this issue, two further typing methods have been applied to discriminate strains within a given ST; capsule profiling and 'CRISPRs' and CRISPR-associated genes (*cas*) protein-coding genes (CRISPR-*cas*) array profiling. These were chosen as two independent typing tools as capsular genes do not follow phylogeny, and the CRISPR-*cas* array reflects the exposure of strains to phages and plasmids (Ogrodzki and Forsythe, 2015; Zeng et al., 2017).

There are a number of different CRISPR-*cas* systems, often named according to their first identification organism, i.e., *E. coli* (type I-E) and *Yersinia pseudotuberculosis* (type I-F) (Makarova et al., 2015). In general, CRISPR-*cas* systems may have up to three sections (a) *cas* genes (b) an AT-rich leader sequence upstream of the array and (c) a CRISPR array, composed of short (∼24–48 nucleotide) direct repeat sequences separated by similarly sized, unique spacers which are usually derived from mobile genetic elements such as bacteriophages and plasmids (Grissa et al., 2007b; Makarova et al., 2015). CRISPR-*cas* systems may have a number of roles such as adaptive immunity to phages and plasmids, as well as bacterial virulence and stress response (Barrangou et al., 2007; Shariat and Dudley, 2014).

Many applications have been identified for the CRISPR-*cas* system such as gene editing, evolutionary and phylogenetic studies, as well as genotyping for epidemiological investigations (Fricke et al., 2011; Makarova et al., 2015). CRISPR arrays may differ between closely related strains due to their different exposure histories to phages and plasmids, leading to differences in their spacer acquisitions. Therefore these loci can be used for molecular subtyping, offering greater discrimination between strains than MLST and PFGE, especially useful for highly clonal species, such as *C. sakazakii*.

This paper considers the diversity of *Cronobacter* across the genus according to 7-loci MLST, the association between capsule profile of the K-antigen and colanic acid biosynthesis genes with clinical presentation, and compares CRISPR-*cas* array profiling between the highly clonal *Cronobacter* species *C. sakazakii* with less clonal species *C. dublinensis*. Of particular relevance is the

availability of over twice the number of genomes (280 compared with 104) since previous publications on capsule and CRISPR-*cas* array profiling (Forsythe et al., 2014; Ogrodzki and Forsythe, 2015, 2016).

MATERIALS AND METHODS

Strains Used in This Study

A total of 1654 STd strains and 275 genomes were included in this study (**Table 1**). Strains from patients less than 1 year in age were defined as infant in origin, those from patients ages 1–15 were termed child, and those above 15 were regarded as adult isolates. Additional metadata can be obtained from the open access *Cronobacter* PubMLST database; http://pubmlst.org/cronobacter/.

Seven Loci MLST Analysis

As per Forsythe et al. (2014), DNA sequences collated at http://pubmlst.org/cronobacter/ were investigated. Concatenated sequences of seven loci from 521 STs were downloaded in FASTA format using the Export/Sequences option. These sequences were aligned in MEGA version 6.05 using the ClustalW algorithm (Tamura et al., 2013). The final alignment spanned 3036 bp and was analyzed using the default pipeline in SplitsTree4 (UncorrectedP to calculate distances and NeighborNet to build the network) (Kloepper and Huson, 2008).

Goeburst Analysis

Phyloviz version 2.0 tool was used with goeBURST Full MST algorithm. Level 3 (TLV) was chosen which represents the Locus Variant level and removes of all the links greater than the number selected (Nascimento et al., 2017).

Bacterial Genome Analysis

As per Ogrodzki and Forsythe (2015, 2016). A total of 275 genomes were analyzed for this study, which were the total number of genomes available (March 2017). Of particular interest were 41 whole genome sequenced isolates were chosen as representatives of *C. dublinensis* and *C. muytjensii*. They were geographically dispersed over 12 countries and temporally spread over 50 years (**Table 1**). Additional metadata can be obtain from the open access *Cronobacter* PubMLST database; http://pubmlst.org/cronobacter/.

Defining CRISPR-*cas* Arrays

CrisprFinder tool was used to identify number of Crispr arrays, including the number and length of DR and spacers sequences; http://crispr.i2bc.paris-saclay.fr/ (Grissa et al., 2007a)

Phylogenetic Analysis

As per Ogrodzki and Forsythe (2015, 2016). DNA sequences were carefully curated prior to and after alignment and phylogenetic analyses in order to maximize the quality of the results using the satisfactory default parameters for the latter analyses. DNA sequences were aligned in MEGA version 6.05 using the ClustalW algorithm (Tamura et al., 2013) set to default parameters settings. The phylogenetic trees were generated using the Maximum Likelihood (ML) method based on the Tamura-Nei model with the additional parameters set to default settings. All phylogenetic trees are drawn to scale with branch lengths measured in the number of substitutions per site.

DNA Sequences

As per Ogrodzki and Forsythe (2016). Whole genome DNA sequences collated at http://pubmlst.org/cronobacter/ were investigated. *In silico* analyses were carried out using search options, such as BLAST, on the *Cronobacter* PubMLST portal accessible at: http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cronobacter_isolates.

DNA Annotation and Visualization Tools

As per Ogrodzki and Forsythe (2015, 2016). Nucleotide sequences were extracted from the corresponding genome assemblies in the *Cronobacter* PubMLST database.

Bacterial DNA sequences were investigated using the genome browser and annotation tool Artemis (Carver et al., 2012).

TABLE 1 | Summary of *Cronobacter* isolates in the *Cronobacter* PubMLST database.

						Source				
Species	**Number of strains (%)**	**Number of STs[a]**	**Number of genomes**	**Earliest isolate**	**Countries**	**Clinical**	**Infant formula**	**Food and ingredients**	**Environmental**	**Other**
C. sakazakii	1126 (68.1)	236	155	1950	32	14.5[b]	21.5	43.4	17.6	3.0
C. malonaticus	222 (13.4)	94	55	1973	17	26.4	13.7	44.8	8.5	6.6
C. dublinensis	155 (9.4)	107	31	1956	12	3.0	5.3	63.9	25.6	2.3
C. turicensis	76 (4.6)	46	14	1970	13	8.0	4.0	52.0	31.4	6.7
C. muytjensii	57 (3.4)	28	10	1988	12	1.8	10.7	53.6	1.8	32.1
C. universalis	16 (1.0)	9	8	1956	6	5.6	0.0	55.6	22.2	16.7
C. condimenti	2 (0.1)	1	2	2010	1	0.0	0.0	100	0	0
Total	1654	521	275		36	14.2[c]	17.5	46.4	17.2	4.7

[a]*Sequence type;* [b]*Percentage of species total;* [c]*Percentage of genus total.*

RESULTS

Cronobacter Diversity and Source According to MLST

The MLST scheme for *Cronobacter* open access database[5] was used as the source of strain sequences and metadata. At the time of writing this database contained over 1654 strain profiles, including >270 whole genomes, along with associated metadata such as source, date of isolation, and clinical presentations (**Table 1**). The strains had been collected from various sources (clinical, food and environmental) and countries over a 60 year period. Since the database also contains the metadata for over 1600 isolates, an informed understanding of the diversity and sources of the *Cronobacter* genus can be obtained. The database can also be used for the retrospective analysis of strains from earlier studies.

Investigating 1654 isolate entries in the *Cronobacter* MLST database revealed the temporal, geographic and source diversity of the organism (**Table 1**). *Cronobacter* strains have been isolated from 36 countries, and are from clinical (14.2%), infant formula (17.5%), food and food ingredients (46.4%), and environmental (17.2%). The earliest isolate (*C. sakazakii* NCIMB 8282) was isolated from dried milk powder in 1950.

Given the primary interest in *Cronobacter* is due to clinical infections, Goeburst analysis was used to visualize the diversity of clinical isolates according to their ST, patient age and site of isolation; **Figure 1**. The majority of strains were from *C. sakazakii* (68.1%) and *C. malonaticus* (13.4%). Out of a total of 236 defined STs for *C. sakazakii*, the major STs recovered from clinical sources were STs 1, 4, 8, and 12. ST4 was the most numerous (208 strains out of 1126) ST in the database. The ST4 isolates were primarily from infants (53%), and secondly adults (37%); **Figure 1A**. Other *C. sakazakii* STs of note were STs 1, 8, and 12 which were primarily composed of infant isolates; 79% ($n = 14$), 14% ($n = 14$) and 67% ($n = 9$), respectively. As shown in **Figure 1B**, the site of isolation for *C. sakazakii* ST4 was most frequently sputum (31%), CSF (16%) and feces (17%). Isolates of *C. sakazakii* ST8 were also from a range of sources (throat, fecal, sputum, CSF), whereas ST1 were from fewer sites; primarily CSF (36%) for ST1, and feces and throat (33% each) for ST12. *C. malonaticus* ST7 was the major (29% n = 199) *C. malonaticus* ST. Sixteen *C. malonaticus* ST7 strains had detailed clinical information. These had been recovered from throat, feces, and sputum samples; **Figure 1B**. The remaining 5 *Cronobacter* species together composed 18.5% of the database, and contained only 13 clinical isolates in total (**Table 1**).

[5]www.pubMLST.org/cronobacter/

Capsule Profiling and Distribution of Sialic Acid Utilization Genes across the *Cronobacter* Genus

Table 2 summarizes the occurrence of K-antigen and colanic acid biosynthesis genes across 275 whole genome strains from the *Cronobacter* genus. The K2:CA2 profile, previously reported to be associated with neonatal meningitis cases, was primarily found in clinical isolates ($n = 59/275$). Most ($n = 84/103$) were *C. sakazakii* strains, in 16 STs. The K2:CA2 profile was found in 10 *C. malonaticus* strains of ST60 and ST307, of which 7 were clinical in origin. Previously only one *C. malonaticus* reported case of severe meningitis in an infant had been documented (Ogrodzki and Forsythe, 2015). This was a fatal meningitis case of an infant born with an EGA of 32 weeks in America (2011). The culture sequenced was a blood isolate, and was presumed to be the infectious organism of the brain. This single case was proposed by Ogrodzki and Forsythe (2015) as possible outlier evidence of K2:CA2 link to meningitis. In this paper a second *C. malonaticus* K2:CA2 isolate (ID 1494) is reported. This strain was isolated in China (2014) from the CSF of an infant born with an EGA of 36 weeks, who was fed fortified breast milk and developed

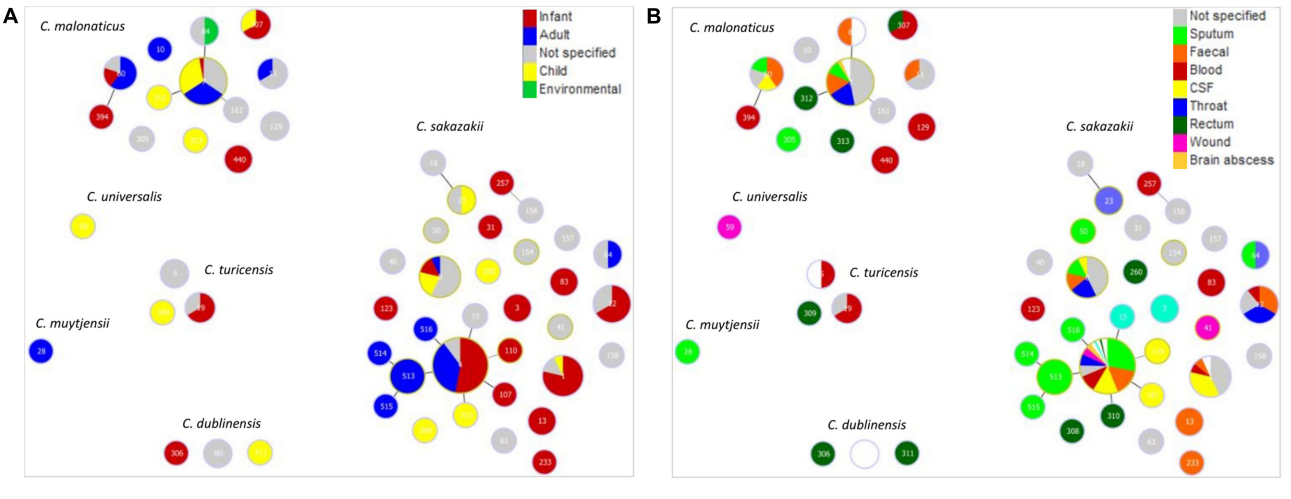


FIGURE 1 | Genetic relationship of clinical *Cronobacter* isolates according to **(A)** patient age along with clinically relevant environment and **(B)** isolation site. The minimum spanning tree was constructed using goeBurst analysis of 53 STs from 234 strains. The size of the circle is proportional to the number of strains.

TABLE 2 | Distribution of capsule profile (*K*-antigen and colanic acid genes) and presence of sialic acid utilization genes across the *Cronobacter* genus.

Capsule					Source		
Capsule profile	Species	Sequence type (ST)	Number of strains	Number of strains encoding for sialic acid utilization genes[a]	Clinical	Non-clinical	Unknown
K2:CA2	*C. sakazakii*	CC4[b], 12, 13, 31, 40, 184, 3, 233, 136, 83	84	84	52	32	0
	C. malonaticus	60, 307	10	0	7	2	1
	C. turicensis	35, 342	3	3	0	1	1
	C. turicensis	24, 252, 387	4	0	0	5	0
	C. dublinensis	301	1	0	0	0	1
	C. universalis	137	1	0	0	1	0
	Subtotal (24)[c]		103	87	59	41	3
K1:CA1	*C. sakazakii, C. malonaticus, C. muytjensii, C. dublinensis* (19)		89	44	31	56	2
K2:CA1	*C. sakazakii, C. universalis* (13)		15	14	3	10	2
K1:CA2	All *Cronobacter* species (46)		68	17	9	40	15
	Subtotal (60)		172	75[d]	43	106	23
	Total		275	162	102	147	26

[a]*Presence of yhch-nanKTAR;* [b]*Clonal complex (CC);* [c]*Total number of sequence types is given in parenthesis;* [d]*All were strains of C. sakazakii (n = 71) and C. turicensis (n = 4).*

clinical symptoms of meningitis on day 11. Of the remaining *C. malonaticus* strains with the K2:CA2 profile, three were from adults aged 26–82 years (no clinical symptoms available). There were no clinical details for the remaining 2 *C. malonaticus* strains. The K2:CA2 profile was also found in a total of 9 *C. turicensis*, *C. dublinensis* and *C. universalis* strains, none of which were clinical in origin. The second most frequent capsule profile was K1:CA1 ($n = 89/275$), of which 31 were clinical in origin but did not correspond to any specific clinical presentation (**Table 2**). This profile was not found in *C. condimenti*, *C. universalis,* or *C. turicensis*.

All *C. sakazakii* strains encoded for the sialic acid utilization genes (*yhch-nanKTAR*); **Table 2**. These genes were not found in any other species, except 7 (out of 14) *C. turicensis* strains, of which 3 had the K2:CA2 capsular profile and the remainder had the profile K1:CA2. The *C. turicensis* strains encoding for sialic acid utilization were in five different STs; 24, 35, 252, 342, and 387. The sialic acid utilization genes were not found in any other *Cronobacter* species.

CRISPR-*cas* Operon Architecture

Table 3 presents the detailed CRISPR-*cas* profiling of 100 isolates. The previously published data for the four clinically significant *C. sakazakii* pathovars (CC1, CC4, ST8 and ST12) are shown for comparative purposes with *C. dublinensis* and *C. muytjensii*. The latter two species were chosen for detailed analysis as they had not been the focus of earlier CRISPR-*cas* array studies (Ogrodzki and Forsythe, 2016; Zeng et al., 2017). *C. muytjensii* and *C. dublinensis* do not show such strong clonality compared with *C. sakazakii* and *C. malonaticus* resulting in the higher proportion of unique STs for the number of strains in the *Cronobacter* database (**Table 1**). It was therefore predicted that the diversity of the CRISPR-*cas* profiles might be greater in *C. dublinensis* and *C. muytjensii.* The genomes of *C. dublinensis* and *C. muytjensii* strains studied were widely temporally (58 years) and globally (11 countries) distributed in their origin and therefore representative of the diversity of the two species.

Twenty genomes of source distributed and ST diverse *C. dublinensis* strains were analyzed. Thirteen genomes revealed CRISPR-*cas* arrays with the same general I-E structure, also known as Ecoli or CASS2 type, due to the presence of *cas3, cse1, cse2, cas7, cas5, cas6e, cas1,* and *cas2.* There was a maximum of 4 CRISPR arrays per strain, with up to 66 spacers. Four genomes (ST 80, 95 and 409) contained I-F (Ypest or CASS3) type CRISPR-*cas* structure, encoding for *cas1, cas2-3, csy1, csy2, csy3,* and *cas6f.* The strains had up to 5 CRISPR arrays per strains, and up to 47 spacers. As shown in **Table 3**, the two strains from ST80 differed in their operon structure; one being I-E and the other I-F. Three genomes from STs 70, 74 and 389 did not encode for any *cas* genes, yet did encode for one CRISPR array composed of 14 spacers.

Ten *C. muytjensii* genomes which were diverse with respect to their temporal, source and ST were analyzed. Five ST81 strains contained the I-F (Ypest or CASS3) type CRISPR-*cas* structure, with a maximum of 5 CRISPR arrays, and 45 spacers per strain. Two genomes (ST 407 and ST411) revealed CRISPR-*cas* arrays with the general I-E (Ecoli or CASS2) type CRISPR-*cas* structure. There was a maximum of 2 CRISPR arrays per strain, with up to 20 spacers. Three genomes from STs 294, 347, and 403 did not encode for any *cas* genes, but did encode for one CRISPR array composed of 15 spacers.

For completeness of the relative comparative analysis, the CRISPR-*cas* profiles of the species types strains of the remaining three *Cronobacter* species, *C turicensis*, *C. universalis,* and *C. condimenti,* were determined and included in the analysis

TABLE 3 | CRISPR-*cas* operon structure and array profile variation in *Cronobacter* spp.

Cronobacter species	Sequence type or clonal complex	Number of strains	Operon structure type[a]	Maximum number of CRISPR arrays per strain	Maximum number of spacers per strain	Reference
C. dublinensis	76, 77, 79, 80, 106, 213, 301, 341, 346, 388, 389	13	I-E[b]	4	66	This study
C. dublinensis	80, 95, 409	4	I-F[c]	5	47	This study
C. dublinensis	70, 74, 389	3	No *cas* genes	1	14	This study
C. muytjensii	407, 411	2	I-E	2	20	This study
C. muytjensii	81	5	I-F	3	45	This study
C. muytjensii	294, 347, 403	3	No *cas* genes	1	15	This study
C. sakazakii	CC4	25	I-E	2	23	[1]
C. sakazakii	CC1	29	I-E	3	30	[1]
C. sakazakii	12	8	I-E	3	11	[1]
C. sakazakii	8	8	I-E	4	18	[1]

[a]*As defined by Makarova et al 2011;* [b]*I-E (Ecoli or CASS2) composed of cas3, cse1, cse2, cas7, cas5, cas6e, cas1, cas2 ;*[c]*I-F (Ypest or CASS3) composed of cas1, cas2-3, csy1, csy2, csy3, cas6f;*[d]*Species type strain analyzed.*

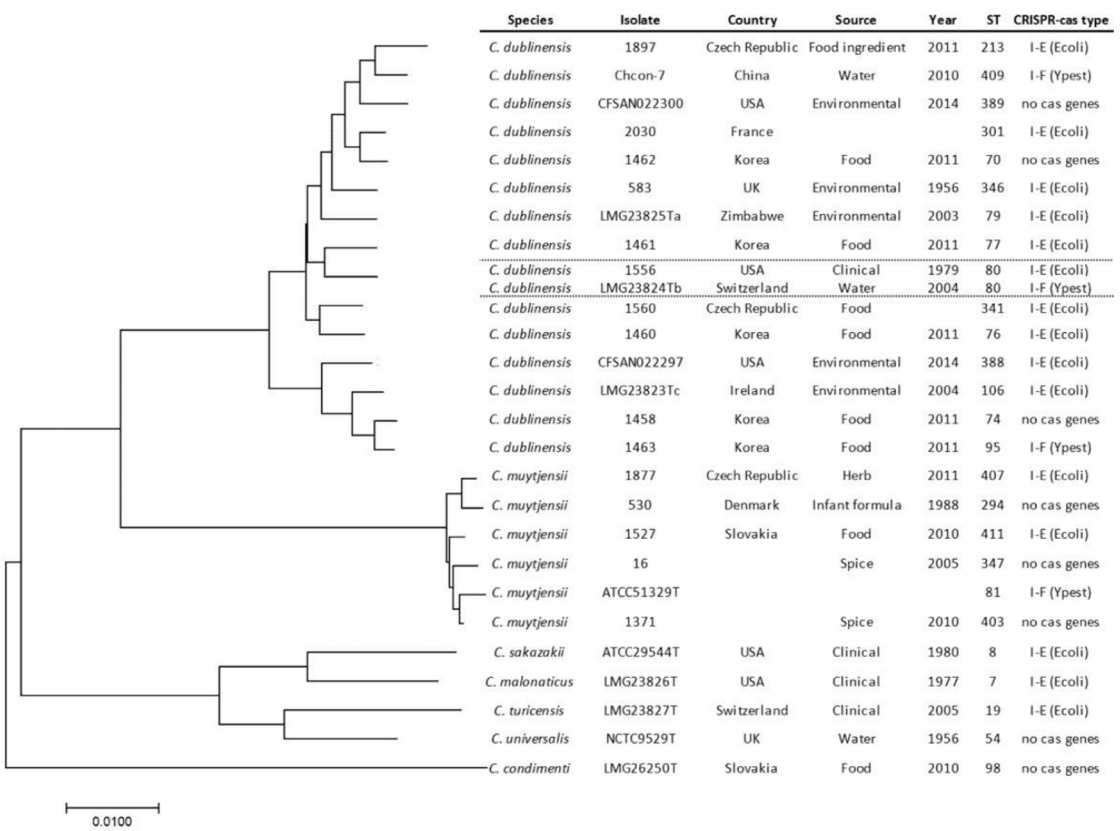


FIGURE 2 | MLST phylogenetic tree of *C. dublinensis* (16 STs) and *C. muytjensii* (6 STs) and species type strains of *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. universalis*, and *C. condimenti.* The phylogenetic tree is drawn to scale with branch lengths measured in the number of substitutions per site. Scale bar represents nucleotide substitutions.

(**Table 3**). The type strains for *C. universalis* and *C. condimenti* did not contain any *cas* genes.

In order to investigate any phylogenetic relationship with the CRISPR-*cas* type across the *C. dublinensis* and *C. muytjensii* species, phylogenetic analysis of the *Cronobacter* genus based on 7-loci MLST is shown in **Figure 2**. The *C. dublinensis* species cluster is very diverse, compared with *C. muytjensii*, yet can be divided into two subgroups. The smaller subgroup contains the *C. dublinensis* subsp. *dublinensis* type strain, whereas *C. dublinensis* subsp. *lactaridii* and *C. dublinensis* subsp. *lausanensis* are in the larger cluster. There was no phylogenetic association between the distribution of the I-E and

I-F type CRISPR-*cas* operon structures across *C. dublinensis* and *C. muytjensis*, or across the whole genus. In addition, the two *C. dublinensis* ST80 strains (isolated in 1979 and 2004 in United States and Switzerland respectively) differed in their CRISPR-*cas* type (**Table 3**).

DISCUSSION

Diversity of *Cronobacter* Species According to 7-Loci MLST

Due to initial concerns of the association between *Cronobacter* in PIF and infant infection, the organism became the focused attention for new identification and typing schemes. The primary goals being to reduce the risk of neonate exposure to the organism, and facilitate monitoring for the organism in PIF ingredients and associated manufacturing environments. The differentiation of the organism from closely related genera, such as *Franconibacter* and *Siccibacter*, has been achieved through a series of taxonomic revisions describing the *Cronobacter* genus (Stephan et al., 2014; Jackson et al., 2015b). While the considerable diversity within the *Cronobacter* genus has been recognized through DNA-sequence based typing schemes. These achievements have been through the application of NGS, and the centralization of the sequences in an open access database repository along with associated metadata. Therefore meeting the recommendations of the FAO-WHO expert committee as detailed in the Introduction (Food and Agriculture Organization of the United Nations [FAO], 2004).

The curated open access *Cronobacter* PubMLST database has been established at the University of Oxford for the genus along with associated metadata for each deposited strain; http://pubmlst.org/cronobacter/ (Forsythe et al., 2014). This database has enabled the recognition of identifiable *Cronobacter* clonal lineages within the genus as pathogenic variants, whereas others are primarily commensal organisms of the environment. The original 7-loci MLST scheme is congruent with both 53-loci rMLST and 1865-loci cg-MLST as well as whole genome phylogeny [Forsythe et al., 2014].

Although the *Cronobacter* PubMLST database cannot be used for direct enumeration purposes, the submission of new strains and sequences to the database will reflect the diversity of the genus with respect to global distribution and source variation. Additionally, the choice of strains genotyped and genome sequenced by different research groups will have been for different rationales, i.e., clinical significance, representative of species, or diversity study. Consequently, outbreak investigations may result in repeated isolation of indistinguishable strains from the same location being deposited in the database. Nevertheless this study of 1654 strains and 275 genomes is the most comprehensive to date and will serve as a reference for further specific studies and can be compared with the earlier analysis of 1000 strains (Forsythe et al., 2014).

All *Cronobacter* species have been isolated from food and food ingredients, and this source comprises 46.4% of the total profiled strains in the database (**Table 1**). These are primarily from starch-based ingredients, cake mixes, packet soup, salads, flavored teas as well as herbs and spices. Another source for *Cronobacter* spp. given in the database is the environment (17.2% entries) which included the manufacturing environment as well as the natural environment with isolates from water and soil. Despite reconstitution water being reported as the source of one reported serious *C. sakazakii* infection, water as a source of the bacterium has not received much attention (Hariri et al., 2013; Liu et al., 2013). The remaining strains in the database were clinical (14.2%) and PIF (17.5%) in origin. The major species were *C. sakazakii* and *C. malonaticus* (68.1 and 13.4%, respectively), which have been isolated from 32 countries, the earliest isolate being from dried milk powder in 1950 and the genome of which has been published (Masood et al., 2013).

The designation 'clinical' needs to be used with caution as it can mean a plethora of sources; symptomatic and asymptomatic patients, from normally sterile sites and non-sterile sites. For example, some strains of *C. sakazakii* isolated from neonatal feeding tubes are designated as 'clinical' as this was deemed more appropriate than 'environmental,' even though there were no associated infections reported. Consequently we have focused on isolates of clinical significance, such as those from CSF and blood with respect to proposing specific pathovars in the genus.

The four major pathovars in the *Cronobacter* genus are (i) *C. sakazakii* CC4, which is more predominantly associated with neonatal meningitis, (ii) *C. sakazakii* ST12 with neonatal necrotizing enterocolitis, (iii) *C. sakazakii* CC1 strains are primarily isolates from infant formula and clinical sources, and (iv) *C. sakazakii* ST8 were isolated from clinical and non-formula food sources (Forsythe et al., 2014). The reason for the predominance of these pathovars could be due to their persistence during the manufacture and storage of infant formula which would result in increased neonatal exposure, as well as encoding for virulence genes (Joseph and Forsythe, 2011).

The pathovar *C. sakazakii* CC4 comprises single (SLV), double (DLV) and triple (TLV) loci variants of *C. sakazakii* ST4. This clonal complex was the major one recovered from clinical sources (208/1126). They were primarily from infants (53%) as well as adults (37%) (**Figure 1A**). The reason for *C. sakazakii* CC4 association with neonatal meningitis is uncertain since no unique virulence traits had been determined in this ST (Masood et al., 2015). However, it could be linked to environmental fitness, in particular desiccation resistance accounting for the reported recovery of *C. sakazakii* CC4 strains from PIF and infant formula manufacturing plants in China, Ireland, Switzerland, Australia, and Germany (Craven et al., 2010; Reich et al., 2010; Jacobs et al., 2011; Sonbol et al., 2013; Yan et al., 2013). Fei et al. (2015) reported that, according to PFGE, environmental isolates of *C. sakazakii* CC4 from a PIF manufacturing plant in Switzerland were indistinguishable from those in the finished product. Therefore *C. sakazakii* CC4 may be an environmentally persistent clonal complex which due to increased neonatal exposure results in infant infections. Other *C. sakazakii* STs of isolated from infants include CC1 (79%), ST8 (14%), and ST12 (67%). The strains had been recovered from a number of sites; throat, sputum, feces, blood, and CSF.

Twenty-nine percent of *C. malonaticus* strains ($n = 199$) recorded in the database are in clonal complex 7 (**Table 1**). Strains

in this complex have been isolated over the past 30 years. Strains of clinical origin had been recovered from throat, feces, and sputum samples; **Figure 1B**. It is of note that the only reported fatal neonatal meningitis cases which has been attributed to *C. malonaticus* were not from this ST. These will be discussed further below with respect to capsule profiling. The database only contained three *C. malonaticus* CC7 isolates from PIF, and there were no isolates from infant formula or milk powder manufacturing plants. This probably reflects a low incidence of this clonal complex in PIF and the manufacturing environment.

It is pertinent to emphasize that some of the predominant clinical STs were also found in the environment and this includes flies (*Musca domestica* and *Sarcophaga haemorrhoidalis*) which carry the pathovars *C. sakazakii* CC4 and ST8, as well as *C. malonaticus* ST7. Pava-Ripoll et al. (2012) reported that *Cronobacter* spp. were isolated from 14% of the flies they analyzed.

As given in **Table 1**, the remaining five species represented 18.5% of strains in the database. These 306 strains were composed of 191 STs. These species had primarily been isolated from environmental sites and are of less clinical relevance. The issue of whether only the clinically significance *Cronobacter* species need to be controlled in PIF has not been evaluated by regulatory authorities. However, the lack of epidemiological evidence of infection from all species may not be substantiated due to the frequent misidentification of *Cronobacte*r strains following the routine use of phenotyping for identification (Jackson and Forsythe, 2016).

Diversity of *Cronobacter* Species According to Capsular Profiling

This new study enabled capsular profiling to be undertaken using genomes from across the whole *Cronobacter* genus, instead of being focussed on *C. sakazakii* and *C. malonaticus* as per the previous studies (Ogrodzki and Forsythe, 2015, 2016). Previously, the variation in the *K*-antigen and colanic acid biosynthesis encoding regions were profiled as a novel means of differentiating strains of *C. sakazakii* and *C. malonaticus* (Ogrodzki and Forsythe, 2015). The K-antigen region found was homologous to the well described K-antigen gene cluster from *E. coli* and was composed of three regions. However while *E. coli* has 60 K-antigen variants, the study of *Cronobacer* spp. by Ogrodzki and Forsythe (2015) found only two variants of the loci (designated K1 and K2). The K-antigen Region 1 (*kpsEDCS*) and Region 3 (*kpsTM*) were conserved across the two species, however there were only two variants of Region 2, designated as K1 and K2, which extended into the *kpsS* gene. The Region 2 variants differed in their length (2.28 and 3.78 kb) and CG% content (34.7 and 42.8%). They both encoded for glycosyltransferases genes for which there were no specific nearest matches (<50% similarity) in any BLAST search. Presumably the 2 variants reflect differences in the synthesized K-antigen which is exported to the cell surface, and also corresponds with the differences in the *kpsS* sequence.

This new study, using 275 genomes, shows the *K*-antigen region was present in all *Cronobacter* strains and species across the whole genus, with the exception of *C. dublinensis* ST389. Nevertheless there were no further variants have been found in this wider study compared to that of Ogrodzki and Forsythe (2015). The composition of the *K*-antigen specific capsular polysaccharide remains unknown, though it could be an important virulence or environmental fitness trait.

Previous analysis of the colanic acid biosynthesis region of *C. sakazakii* and *C. malonaticus* revealed there were two variants of the colanic acid biosynthesis gene cluster. CA2 differed from CA1 in the absence of the *galE* (encoding for UDP-N-acetyl glucosamine 4-epimerase). It is predictable that the chemical composition of the colanic acid produced by the two variants will differ, though the affect on virulence or environmental fitness trait is uncertain. The colanic acid biosynthesis region was present in all *Cronobacter* strains and species across the whole genus based on the analysis of 275 genomes. Nevertheless, as for the *K*-antigen region, there were no further variants found in this wider study. The variation is the K-antigen and colanic acid biosynthesis genes therefore do not follow the phylogeny of the genus.

Cronobacter sakazakii CC4 strains are associated with neonatal meningitis, and have the K2:CA2 capsule profile (Ogrodzki and Forsythe, 2015). Although this could be attributed to clonal inheritance, a limited number of non-*C. sakazakii* CC4 strains from meningitis cases have been collated from the PubMLST database. It is therefore notable that the K2:CA2 profile was also found in three other species *C. malonaticus* ($n = 10$), *C. turicensis* ($n = 7$), and *C. universalis* ($n = 1$) as well as the more distantly related *C. dublinensis* ($n = 1$); (**Table 2**). Therefore this capsule profile is neither phylogenetically nor clonaly related. In this new study, more strains of *C. malonaticus* ST60 and ST307 are shown to encode for the K2:CA2 profile. Previously only one *C. malonaticus* reported meningitis case had been documented (Ogrodzki and Forsythe, 2015). This strain (1569, ST307) was a blood isolate from a meningitis case in the United States, and was purported as being the infectious organism. This single case was taken as a possible outlier evidence for K2:CA2 profile link to meningitis. In this paper a second *C. malonaticus* K2:CA2 isolate of significance is reported. The strain Chcon-9 (ID 1494) was a CSF isolate, kindly deposited by Dr Jing-hua Cui (CDC Beijing). This second example was isolated in China (2014) from the CSF of an infant born with an EGA of 36 weeks, who had been fed fortified breast milk before developing clinical symptoms of meningitis on day 11. Strain Chcon-9 is ST60 not ST307 as per the previous *C. malonaticus* example. This latter observation further confirms the lack of congruence between the capsule profile and phylogeny. However, the remaining *C. malonaticus* ST60 strains of clinical origin also had the K2:CA2 profile and were from adults aged 26–82 years (no clinical symptoms available). The apparent correlation between capsule profile and severe infant meningitis gives a clear direction for further meningitis research with the bacterium.

None of the 55 *C. malonaticus* strains encoded for the sialic acid utilization genes (*yhch-nanKTAR*); (**Table 2**). This trait had previously been proposed as an important virulence trait in *C. sakazakii* given the occurrence of sialic acid in breast milk, mucin and gangliosides. Strains of *C. sakazakii* are able

to grow on sialic acid and the ganglioside GM1 as a sole carbon source (Joseph et al., 2013b). This ability may explain the more frequent occurrence of *C. sakazakii* in severe meningitic infections compared with *C. malonaticus*.

Of the *C. turicensis* strains encoding for K2:CA2, none were clinical in origin (**Table 2**). The strains were from 5 STs which were not phylogenetically closely related (Joseph et al., 2012b). Three of these also encoded for the sialic acid utilization genes (*yhch-nanKTAR*), and a further 4 strains also encoded for these genes but had the capsule profile K1:CA2. These were the only strains outside the *C. sakazakii* species which encoded for the sialic acid utilization genes. Similar to the strains encoding for K2:CA2, the *yhch-nanKTAR* encoding strains which were identified in six different STs, clustered together according to phylogenetic analysis (Joseph et al., 2012c). The *C. turicensis* type strain lacked the sialic acid utilizing genes despite being closely related to these 6 STs. This would account for the earlier reported absence of sialic acid utilization in *C. turicensis* in laboratory studies by (Joseph et al., 2013b). Whether the acquisition of the sialic acid utilization trait by a phylogenetic cluster within *C. turicensis* is of ecological significance is uncertain given none of the strains were clinical in origin.

The K2:CA2 profile was also detected in one strain each of *C. dublinensis* (2030) and *C. universalis* (1883); (**Table 2**). Unfortunately the specific site of isolation and clinical presentation of *C. dublinensis* strain 2030 (synonym CDC 28–83) are unknown and was previously misidentified as *C. sakazakii* (Miled-Bennour et al., 2010). Currently this is the only ST301 strain in the *Cronobacter* PubMLST database, and hence the only one available for genome sequencing. The nearest related ST is ST70 for which there are 2 strains (1130 and 1462) in the database (**Figure 2**). Both strains were from Korea, one of which was from follow-up formula and the other was a food isolate (Chap et al., 2009). Neither of these strains have been sequenced to date. Whether other traits of significance occur in ST301 can be investigated in the future using comparative genomic analysis via the PubMLST *Cronobacter* database. This was considered as being outside the scope of this study. The single *C. universalis* strain (1883) encoding for K2:CA2 was isolated during a food survey in the Czech Republic (synonym DEM 3321) (Killer et al., 2015).

The occurrence of the K2:CA2 profile therefore does not follow phylogeny within the *Cronobacter* genus. Instead, there is only an association within *C. sakazakii* for severe cases of infection (NEC and meningitis) and *C. malonaticus* with severe cases of meningitis. Additionally, none of the *C. turicensis* strains encoding for K2:CA2 had been isolated from CSF. The co-occurrence of the sialic acid utilization genes did not occur in the two isolates of *C. malonaticus* isolated from meningitis cases. This observation does not discount the potential contribution of the sialic acid utilization genes in *C. sakazakii* meningitis, but does infer that it is not essential.

The *Cronobacter* genus is predicted to have evolved in the Palaeogene period of the Cenozoic era when early flowering plants evolved and coincides with the suspected natural plant habitat of the organism. The earliest branches of the genus lead to *C. dublinensis* and *C. muytjensii,* whereas *C. sakazakii is* predicted to have evolved more recently (Joseph and Forsythe, 2012; Grim et al., 2013). Possibly linked to this, **Table 1** shows the considerable genetic diversity of *C. dublinensis* and *C. muytjensii,* as reflected by the relatively large number of STs for total number of strains (107/155 and 28/57) compared with the relative smaller number of *C. sakazakii* STs (236/1126) reflecting the considerable clonality within the latter species. Such clonality results in a low amount of genomic difference between unrelated strains and therefore limits the use of conventional genotyping methods such as PFGE.

Diversity of *Cronobacter* Species According to CRISPR-*cas* Array Profiling

CRISPR-*cas* array content has previously been strongly associated with sequence based phylogeny and hence could be used as a rapid lineage based detection method. This analysis has been applied to various *Enterobacteriaceae* (*Yersinia, Salmonella* and *E. coli*) for phylogenetic, evolutionary and virulence related analysis (Makarova et al., 2015). It has also been considered as a discriminatory tool for epidemiological purposes since bacterial strains from the same geographical and temporal region should acquire the same spacers due to localized exposure to phages and plasmids, differentiating them from unrelated strains. In *Cronobacter* it was proposed that the strong clonal lineages of *C. sakazakii* resulted in a constraint in the CRISPR spacer array diversity (Ogrodzki and Forsythe, 2016). To consider if the CRISPR-*cas* array diversity varied across the genus, the arrays were determined for the less clonal species, *C. dublinensis* and *C. muytjensii* and compared with *C. sakazakii*.

Thirty *C. muytjensii* ($n = 10$) and *C. dublinensis* ($n = 20$) isolates were subject to detailed CRISPR-*cas* analysis. These two species were chosen since MLSA analysis had shown their greater diversity and more frequent novel sequence typing than the more clonal species *C. sakazakii* which has previously been analyzed. It was predicted that there would also be greater CRISPR-*cas* diversity in these two species given the probable reduction in clonal constraint of genomic recombination. The selected strains had been isolated from 11 countries over a 58 year period.

As shown in **Table 3**, both *C. dublinensis* and *C. muytjensii* included two CRISPR-*cas* operon architectures of I-E (Ecoli) and I-F (Ypseudo). The distribution of the operon types were not phylogenetically related as shown in **Figure 2**. There were a considerably high number of direct repeats and spacers in the two species, generating up to 5 CRISPR arrays and up to 66 spacers per strain compared to a maximum of 4 CRISPR arrays and 31 spacers per strain across 4 clonal *C. sakazakii* pathovars (Ogrodzki and Forsythe, 2016). Some strains in *C. dublinensis* and *C. muytjensii* lacked *cas* genes. The lack of *cas* genes was also found in the type strains of *C. universalis* and *C. condimenti* species.

Horizontal gene transfer of CRISPR and *cas* genes can occur between strains of the same species and even distant species and genera (Makarova et al., 2015). Subsequently not all strains within a species will necessarily possess the same sets of CRISPR-*cas* genes. As given in **Table 3**, within the *Cronobacter* genera there is a considerable variation in CRISPR-*cas* arrays, and

even operon type and presence. Similarly, CRISPR-*cas* genes are present in enterohemorrhagic (EHEC) and Shiga toxin producing *E. coli* serotypes, but not the extra intestinal (ExPEC) *E. coli* phylogenetic group B2. Consequently, it has been proposed that the type I-E CRISPR-*cas* system could have alternative functions, such as gene expression and virulence (Touchon et al., 2011; Yin et al., 2013; García-Gutiérrez et al., 2015). Whether this relates to the variation in CRISPR-*cas* arrays in *Cronobacter* is currently unclear.

This is the first study to identify the wide-spread phylogenetic distribution of CRISPR-cas arrays across the *Cronobacter* genus, as opposed to just *C. sakazakii* (Ogrodzki and Forsythe, 2016; Zeng et al., 2017). This analysis has confirmed that differentiation within clonal lineages can be achieved using genotyping based on CRISPR-*cas* array variability.

Genotyping across the *Cronobacter* Genus

Due to the increasing number of genome and allele sequences (MLST alleles and whole genomes) deposited in the PubMLST *Cronobacter* database, this article not only updates researchers regarding the diversity of strains within the *Cronobacter* genus as revealed by 7-loci MLST, it has also investigated the occurrence of the capsule profiles and CRISPR-*cas* arrays beyond the initial studies of *C. sakazakii* and *C. malonaticus*.

Genotyping of *Cronobacter* spp. using MLST has considerably improved our understanding of the bacterial population diversity across the genus, and led to the recognition of clonal lineages and pathovars. However, like PFGE, MLST is unable to discriminate between unrelated strains within a clonal lineage (Alsonosi et al., 2015). Therefore more discriminatory DNA-sequence based methods need to be developed. Given the increasing availability and lowering costs of NGS tools, there is an increasing trend for genome-based genotyping methods, such as CRISPR-*cas* array profiling. Such analysis may also provide additional understanding of the diversity of the species and potential virulence mechanisms.

Given the expanding nature of the *Cronobacter* PubMLST database, the figures used were as of April 2017 and may differ in precise values when accessed later. Nevertheless the general consensus of *Cronobacter* diversity will be the same. The online *Cronobacter* PubMLST database has enabled open access to considerable information on *Cronobacter* isolates which can be interrogated by researchers, industry and regulatory authorities for taxonomic, and genotyping purposes. The curation of metadata of the isolates is standardized and therefore facilitates an international contribution to collating information.

ETHICS STATEMENT

All clinical data are taken from previous publications associated with the sequenced bacterial strains.

AUTHOR CONTRIBUTIONS

PO: performed the genomic analysis and collated the data. SF: Initiated the study, wrote the manuscript, and managed the project. Both authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors gratefully thank Nottingham Trent University for their financial support (PO). This publication made use of the *Cronobacter* Multi Locus Sequence Typing website http://pubmlst.org/cronobacter/) developed by Keith Jolley and sited at the University of Oxford (Maiden et al., 2013). The development of this site has been funded by the Wellcome Trust. The authors thank the numerous collaborators who have submitted sequences to the open access *Cronobacter* PubMLST database.

REFERENCES

Almagro-Moreno, S., and Boyd, E. F. (2009). Insights into the evolution of sialic acid catabolism among bacteria. *BMC Evol. Biol.* 9:118. doi: 10.1186/1471-2148-9-118

Almajed, F. S., and Forsythe, S. J. (2016). *Cronobacter sakazakii* clinical isolates overcome host barriers and evade the immune response. *Microb. Pathog.* 90, 55–63. doi: 10.1016/j.micpath.2015.11.014

Alsonosi, A., Hariri, S., Kajsik, M., Orieskova, M., Hanulik, V., Roderova, M., et al. (2015). The speciation and genotyping of *Cronobacter* isolates from hospitalised patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 1979–1988. doi: 10.1007/s10096-015-2440-8

Alzahrani, H., Winter, J., Boocock, D., De Girolamo, L., and Forsythe, S. J. (2015). Characterization of outer membrane vesicles from a neonatal meningitic strain of *Cronobacter sakazakii*. *FEMS Microbiol. Lett.* 362:fnv085. doi: 10.1093/femsle/fnv085

Arbatsky, N. P., Wang, M., Shashkov, A. S., Chizhov, A. O., Feng, L., Knirel, Y. A., et al. (2010). Structure of the O-polysaccharide of *Cronobacter sakazakii* O2 with a randomly O-acetylated l-rhamnose residue. *Carbohydr. Res.* 345, 2090–2094. doi: 10.1016/j.carres.2010.07.014

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., et al. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiol.* 9:223. doi: 10.1186/1471-2180-9-223

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Blažková, M., Javùrková, B., Vlach, J. J., Göselová, S., Karamonová, L., Ogrodzki, P., et al. (2015). Diversity of O antigens within the genus *Cronobacter*: from disorder to order. *Appl. Environ. Microbiol.* 81, 5574–5582. doi: 10.1128/AEM.00277-15

Broge, T., and Lee, A. (2013). A case of *Cronobacter* (*Enterobacter sakazakii*) bacteremia in a breastfed infant. *J. Pediatr. Infect. Dis. Soc.* 2, e1–e2. doi: 10.1093/jpids/pit021

Carleton, H. A., and Gerner-Smidt, P. (2016). Whole-genome sequencing is taking over foodborne disease surveillance. *Microbe Mag.* 11, 311–317. doi: 10.1128/microbe.11.311.1

Carver, T., Harris, S. R., Berriman, M., Parkhill, J., and McQuillan, J. A. (2012). Artemis: an integrated platform for visualization and analysis of

high-throughput sequence-based experimental data. *Bioinformatics* 28, 464–469. doi: 10.1093/bioinformatics/btr703

Caubilla-Barron, J., and Forsythe, S. J. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other *Enterobacteriaceae* in dehydrated powdered infant formula. *J. Food Prot.* 70, 2111–2117. doi: 10.4315/0362-028X-70.9.2111

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., et al. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *J. Clin. Microbiol.* 45, 3979–3985. doi: 10.1128/JCM.01075-07

Centers for Disease Control and Prevention [CDC] (2010). *Multistate Outbreak of Human Salmonella Enteritidis Infections Associated with Shell Eggs*. Available at: https://www.cdc.gov/salmonella/2010/shell-eggs-12-2-10.html [accessed May 2, 2017].

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., et al. (2009). International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. *Int. J. Food Microbiol.* 136, 185–188. doi: 10.1016/j.ijfoodmicro.2009.08.005

Chaturvedi, N., Kajsik, M., Forsythe, S., and Pandey, P. N. (2015). Protein sequences insight into heavy metal tolerance in *Cronobacter sakazakii* BAA-894 encoded by plasmid pESA3. *Arch. Microbiol.* 197, 1141–1149. doi: 10.1007/s00203-015-1147-7

Craven, H. M., McAuley, C. M., Duffy, L. L., and Fegan, N. (2010). Distribution, prevalence and persistence of *Cronobacter* (*Enterobacter sakazakii*) in the nonprocessing and processing environments of five milk powder factories. *J. Appl. Microbiol.* 109, 1044–1052. doi: 10.1111/j.1365-2672.2010.04733.x

Czerwicka, M. M., Forsythe, S. J., Bychowska, A., Dziadziuszko, H., Kunikowska, D., Stepnowski, P., et al. (2010). Structure of the O-polysaccharide isolated from *Cronobacter sakazakii* 767. *Carbohydr. Res.* 345, 908–913. doi: 10.1016/j.carres.2010.01.020

Fei, P., Jiang, Y., Feng, J., Forsythe, S. J., Li, R., Zhou, Y., et al. (2017). Antibiotic and desiccation resistance of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and processing environments. *Front. Microbiol.* 8:316. doi: 10.3389/fmicb.2017.00316

Fei, P., Man, C., Lou, B., Forsythe, S. J., Chai, Y., Li, R., et al. (2015). Genotyping and source tracking of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and an infant formula production factory in China. *Appl. Environ. Microbiol.* 81, 5430–5439. doi: 10.1128/AEM.01390-15

Food and Agriculture Organization of the United Nations [FAO] (2004). *Enterobacter sakazakii and other Microorganisms in Powdered Infant Formula, Microbiological Risk Assessment Series n. 6*. Rome: Food and Agriculture Organization of the United Nations. doi: 10.1111/j.1740-8709.2004.00008.x

Food and Agriculture Organization of the United Nations [FAO] (2006). *Expert Meeting on Enterobacter sakazakii and Salmonella in Powdered Infant Formula, Microbiological Risk Assessment Series n. 10*. Geneva: World Health Organization.

Food and Agriculture Organization of the United Nations [FAO] and World Health Organization [WHO] (2008). *Enterobacter sakazakii (Cronobacter spp.) in Powdered Follow-up Formulae, Microbiological Risk Assessment Series n. 15*. Rome: FAO.

Forsythe, S. J., Dickins, B., and Jolley, K. A. (2014). *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. *BMC Genomics* 15:1121. doi: 10.1186/1471-2164-15-1121

Franco, A. A., Kothary, M. H., Gopinath, G., Jarvis, K. G., Grim, C. J., Hu, L., et al. (2011). Cpa, the outer membrane protease of *Cronobacter sakazakii*, activates plasminogen and mediates resistance to serum bactericidal activity. *Infect. Immun.* 79, 1578–1587. doi: 10.1128/IAI.01165-10

Fricke, W. F., Mammel, M. K., McDermott, P. F., Tartera, C., White, D. G., Leclerc, J. E., et al. (2011). Comparative genomics of 28 *Salmonella enterica* isolates: evidence for CRISPR-mediated adaptive sublineage evolution. *J. Bacteriol.* 193, 3556–3568. doi: 10.1128/JB.00297-11

García-Gutiérrez, E., Almendros, C., Mojica, F. J. M., Guzmán, N. M., and García-Martínez, J. (2015). CRISPR content correlates with the pathogenic potential of *Escherichia coli*. *PLOS ONE* 10:e0131935. doi: 10.1371/journal.pone.0131935

Grim, C., Kothary, M., Gopinath, G., Jarvis, K., Beaubrun, J. J., McClelland, M., et al. (2012). Identification and characterization of *Cronobacter* iron acquisition systems. *Appl. Environ. Microbiol.* 78, 6035–6050. doi: 10.1128/AEM.01457-12

Grim, C. J., Kotewicz, M. L., Power, K. A., Gopinath, G., Franco, A. A., Jarvis, K. G., et al. (2013). Pan-genome analysis of the emerging foodborne pathogen *Cronobacter* spp. suggests a species-level bidirectional divergence driven by niche adaptation. *BMC Genomics* 14:366. doi: 10.1186/1471-2164-14-366

Grissa, I., Vergnaud, G., and Pourcel, C. (2007a). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res.* 35, W52–W57. doi: 10.1093/nar/gkm360

Grissa, I., Vergnaud, G., and Pourcel, C. (2007b). The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics* 8:172. doi: 10.1186/1471-2105-8-172

Hariri, S., Joseph, S., and Forsythe, S. J. (2013). *Cronobacter sakazakii* ST4 strains and neonatal meningitis, United States. *Emerg. Infect. Dis.* 19, 175–177. doi: 10.3201/eid1901.120649

Himelright, I., Harris, E., and Lorch, V. A. M. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula–Tennessee, 2001. *MMWR Morb. Mortal. Wkly. Rep.* 51, 297–300.

Holy, O., and Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *J. Hosp. Infect.* 86, 169–177. doi: 10.1016/j.jhin.2013.09.011

Holý, O., Petrželová, J., Hanulík, V., Chromá, M., Matoušková, I., and Forsythe, S. J. (2014). Epidemiology of *Cronobacter* spp. isolates from patients admitted to the Olomouc University Hospital (Czech Republic). *Epidemiol. Mikrobiol. Imunol.* 63, 69–72.

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., and Forsythe, S. J. (2009a). Biofilm formation on enteral feeding tubes by *Cronobacter sakazakii*, *Salmonella* serovars and other *Enterobacteriaceae*. *Int. J. Food Microbiol.* 136, 227–231. doi: 10.1016/j.ijfoodmicro.2009.08.007

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., Hilton, A., Armstrong, R., et al. (2009b). Neonatal enteral feeding tubes as loci for colonisation by members of the *Enterobacteriaceae*. *BMC Infect. Dis.* 9:146. doi: 10.1186/1471-2334-9-146

Iversen, C., and Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends Food Sci. Technol.* 14, 443–454. doi: 10.1016/S0924-2244(03)00155-9

Iversen, C., Lane, M., and Forsythe, S. J. (2004). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Lett. Appl. Microbiol.* 38, 378–382. doi: 10.1111/j.1472-765X.2004.01507.x

Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., et al. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* 58(Pt 6), 1442–1447. doi: 10.1099/ijs.0.65577-0

Jackson, E. E., Flores, J. P., Fernandez-Escartin, E., and Forsythe, S. J. (2015a). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *J. Food Prot.* 78, 1191–1196. doi: 10.4315/0362-028X.JFP-14-563

Jackson, E. E., Masood, N., Ibrahim, K., Urvoy, N., Hariri, S., and Forsythe, S. J. (2015b). Description of *Siccibacter colletis* sp. nov., a novel species isolated from plant material, and emended description of *Siccibacter turicensis*. *Int. J. Syst. Evol. Microbiol.* 65(Pt 4), 1335–1341. doi: 10.1099/ijs.0.000108

Jackson, E. E., and Forsythe, S. J. (2016). Comparative study of *Cronobacter* identification according to phenotyping methods. *BMC Microbiol.* 16:146. doi: 10.1186/s12866-016-0768-6

Jacobs, C., Braun, P., and Hammer, P. (2011). Reservoir and routes of transmission of *Enterobacter sakazakii* (*Cronobacter* spp.) in a milk powder-producing plant. *J. Dairy Sci.* 94, 3801–3810. doi: 10.3168/jds.2011-4318

Jarvis, K. G., Grim, C. J., Franco, A. A., Gopinath, G., Sathyamoorthy, V., Hu, L., et al. (2011). Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. *Appl. Environ. Microbiol.* 77, 4017–4026. doi: 10.1128/AEM.00162-11

Jolley, K. A., and Maiden, M. C. J. (2014). Using multilocus sequence typing to study bacterial variation: prospects in the genomic era. *Future Microbiol.* 9, 623–630. doi: 10.2217/fmb.14.24

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., and Forsythe, S. J. (2012a). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *Int. J. Syst. Evol. Microbiol.* 62, 1277–1283. doi: 10.1099/ijs.0.032292-0

Joseph, S., Desai, P., Ji, Y., Cummings, C. A., Shih, R., Degoricija, L., et al. (2012b). Comparative analysis of genome sequences covering the seven *Cronobacter* species. *PLOS ONE* 7:e49455. doi: 10.1371/journal.pone.0049455

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., and Forsythe, S. J. (2012c). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. *J. Clin. Microbiol.* 50, 3031–3039. doi: 10.1128/JCM.00905-12

Joseph, S., and Forsythe, S. J. (2011). Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerg. Infect. Dis.* 17, 1713–1715. doi: 10.3201/eid1709.110260

Joseph, S., and Forsythe, S. J. (2012). Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by multilocus sequence typing and analysis. *Front. Microbiol.* 3:397. doi: 10.3389/fmicb.2012.00397

Joseph, S., Hariri, S., and Forsythe, S. J. (2013a). Lack of continuity between *Cronobacter* biotypes and species as determined using multilocus sequence typing. *Mol. Cell. Probes* 27, 137–139. doi: 10.1016/j.mcp.2013.02.002

Joseph, S., Hariri, S., Masood, N., and Forsythe, S. (2013b). Sialic acid utilization by *Cronobacter sakazakii*. *Microb. Inform. Exp.* 3:3. doi: 10.1186/2042-5783-3-3

Killer, J., Skrivanova, E., Hochel, I., and Marounek, M. (2015). Multilocus sequence typing of *Cronobacter* strains isolated from retail foods and environmental samples. *Foodborne Pathog. Dis.* 12, 514–521. doi: 10.1089/fpd.2014.1884

Kim, H., Ryu, J.-H., and Beuchat, L. R. (2006). Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. *Appl. Environ. Microbiol.* 72, 5846–5856. doi: 10.1128/AEM.00654-06

Kloepper, T. H., and Huson, D. H. (2008). Drawing explicit phylogenetic networks and their integration into SplitsTree. *BMC Evol. Biol.* 8:22. doi: 10.1186/1471-2148-8-22

Kothary, M. H., Gopinath, G. R., Gangiredla, J., Rallabhandi, P. V., Harrison, L. M., Yan, Q. Q., et al. (2017). Analysis and characterization of proteins associated with outer membrane vesicles secreted by *Cronobacter* spp. *Front. Microbiol.* 8:134. doi: 10.3389/fmicb.2017.00134

Kucerova, E., Clifton, S. W., Xia, X.-Q., Long, F., Porwollik, S., Fulton, L., et al. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLOS ONE* 5:e9556. doi: 10.1371/journal.pone.0009556

Kucerova, E., Joseph, S., and Forsythe, S. (2011). The *Cronobacter* genus: ubiquity and diversity. *Qual. Assur. Saf. Crops Foods* 3, 104–122. doi: 10.1111/j.1757-837X.2011.00104.x

Liu, H., Yang, Y., Cui, J., Liu, L., Liu, H., Hu, G., et al. (2013). Evaluation and implementation of a membrane filter method for *Cronobacter* detection in drinking water. *FEMS Microbiol. Lett.* 344, 60–68. doi: 10.1111/1574-6968.12155

Maclean, L. L., Vinogradov, E., Pagotto, F., Farber, J. M., and Perry, M. B. (2010). The structure of the O-antigen of *Cronobacter sakazakii* HPB 2855 isolate involved in a neonatal infection. *Carbohydr. Res.* 345, 1932–1937. doi: 10.1016/j.carres.2010.06.020

Maiden, M. C. J., Jansen van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., et al. (2013). MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat. Rev. Microbiol.* 11, 728–736. doi: 10.1038/nrmicro3093

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* 13, 722–736. doi: 10.1038/nrmicro3569

Masood, N., Moore, K., Farbos, A., Hariri, S., Paszkiewicz, K., Dickins, B., et al. (2013). Draft genome sequence of the earliest *Cronobacter sakazakii* sequence Type 4 strain, NCIMB 8272. *Genome Announc.* 1:e00782-13. doi: 10.1128/genomeA.00782-13

Masood, N., Moore, K., Farbos, A., Paszkiewicz, K., Dickins, B., McNally, A., et al. (2015). Genomic dissection of the 1994 *Cronobacter sakazakii* outbreak in a French neonatal intensive care unit. *BMC Genomics* 16:750. doi: 10.1186/s12864-015-1961-y

McQuiston, J. R., Herrera-Leon, S., Wertheim, B. C., Doyle, J., Fields, P. I., Tauxe, R. V., et al. (2008). Molecular phylogeny of the Salmonellae: relationships among *Salmonella* species and subspecies determined from four housekeeping genes and evidence of lateral gene transfer events. *J. Bacteriol.* 190, 7060–7067. doi: 10.1128/JB.01552-07

Miled-Bennour, R., Ells, T. C., Pagotto, F. J., Farber, J. M., Kérouanton, A., Meheut, T., et al. (2010). Genotypic and phenotypic characterisation of a collection of *Cronobacter* (*Enterobacter sakazakii*) isolates. *Int. J. Food Microbiol.* 139, 116–125. doi: 10.1016/j.ijfoodmicro.2010.01.045

Mullane, N., O'Gaora, P., Nally, J. E., Iversen, C., Whyte, P., Wall, P. G., et al. (2008). Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. *Appl. Environ. Microbiol.* 74, 3783–3794. doi: 10.1128/AEM.02302-07

Muller, A., Stephan, R., Fricker-Feer, C., and Lehner, A. (2013). Genetic diversity of *Cronobacter sakazakii* isolates collected from a Swiss infant formula production facility. *J. Food Prot.* 76, 883–887. doi: 10.4315/0362-028X.JFP-12-521

Nadon, C., Van Walle, I., Gerner-smidt, P., Campos, J., Chinen, I., and Concepcion-acevedo, J. (2017). PulseNet International: vision for the implementation of whole genome sequencing (WGS) for global food- borne disease surveillance. *Euro Surveill.* 22:30544. doi: 10.2807/1560-7917.ES.2017.22.23.30544

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A. P., Carriço, J. A., and Vaz, C. (2017). PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* 33, 128–129. doi: 10.1093/bioinformatics/btw582

Ogrodzki, P., and Forsythe, S. (2015). Capsular profiling of the *Cronobacter* genus and the association of specific *Cronobacter sakazakii* and *C. malonaticus* capsule types with neonatal meningitis and necrotizing enterocolitis. *BMC Genomics* 16:758. doi: 10.1186/s12864-015-1960-z

Ogrodzki, P., and Forsythe, S. J. (2016). CRISPR – cas loci profiling of *Cronobacter sakazakii* pathovars. *Future Microbiol.* 11, 1507–1519. doi: 10.2217/fmb-2016-0070

Patrick, M. E., Mahon, B. E., Greene, S. A., Rounds, J., Cronquist, A., Wymore, K., et al. (2014). Incidence of *Cronobacter* spp. infections, United States, 2003-2009. *Emerg. Infect. Dis.* 20, 1520–1523. doi: 10.3201/eid2009.140545

Pava-Ripoll, M., Pearson, R. E. G., Miller, A. K., and Ziobro, G. C. (2012). Prevalence and relative risk of *Cronobacter* spp., *Salmonella* spp., and *Listeria monocytogenes* associated with the body surfaces and guts of individual filth flies. *Appl. Environ. Microbiol.* 78, 7891–7902. doi: 10.1128/AEM.02195-12

Power, K. A., Yan, Q., Fox, E. M., Cooney, S., and Fanning, S. (2013). Genome sequence of *Cronobacter sakazakii* SP291, a persistent thermotolerant isolate derived from a factory producing powdered infant formula. *Genome Announc.* 1:e0008213. doi: 10.1128/genomeA.00082-13

Ravisankar, S., Syed, S. S., Garg, P., and Higginson, J. (2014). Is *Cronobacter sakazakii* infection possible in an exclusively breastfed premature neonate in the neonatal intensive care unit? *J. Perinatol.* 34, 408–409. doi: 10.1038/jp.2014.14

Reich, F., Konig, R., von Wiese, W., and Klein, G. (2010). Prevalence of *Cronobacter* spp. in a powdered infant formula processing environment. *Int. J. Food Microbiol.* 140, 214–217. doi: 10.1016/j.ijfoodmicro.2010.03.031

Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A., et al. (2009). Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Res. Microbiol.* 160, 608–614. doi: 10.1016/j.resmic.2009.08.013

Shariat, N., and Dudley, E. G. (2014). CRISPRs: molecular signatures used for pathogen subtyping. *Appl. Environ. Microbiol.* 80, 430–439. doi: 10.1128/AEM.02790-13

Sonbol, H., Joseph, S., McAuley, C. M., Craven, H. M., and Forsythe, S. J. (2013). Multilocus sequence typing of *Cronobacter* spp. from powdered infant formula and milk powder production factories. *Int. Dairy J.* 30, 1–7. doi: 10.1016/j.idairyj.2012.11.004

Stephan, R., Grim, C. J., Gopinath, G. R., Mammel, M. K., Sathyamoorthy, V., Trach, L. H., et al. (2014). Re-examination of the taxonomic status of *Enterobacter helveticus*, *Enterobacter pulveris* and *Enterobacter turicensis* as members of the genus *Cronobacter* and their reclassification in the genera *Franconibacter* gen. nov. and *Siccibacter* gen. nov. as Francon. *Int. J. Syst. Evol. Microbiol.* 64(Pt 10), 3402–3410. doi: 10.1099/ijs.0.059832-0

Sun, Y., Wang, M., Liu, H., Wang, J., He, X., Zeng, J., et al. (2011). Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* 77, 2209–2214. doi: 10.1128/AEM.02229-10

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Touchon, M., Charpentier, S., Clermont, O., Rocha, E. P. C., Denamur, E., and Branger, C. (2011). CRISPR distribution within the *Escherichia coli* species is not suggestive of immunity-associated diversifying selection. *J. Bacteriol.* 193, 2460–2467. doi: 10.1128/JB.01307-10

Townsend, S., Hurrell, E., and Forsythe, S. (2008). Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. *BMC Microbiol.* 8:64. doi: 10.1186/1471-2180-8-64

Townsend, S. M., Hurrell, E., Caubilla-Barron, J., Loc-Carrillo, C., and Forsythe, S. J. (2008). Characterization of an extended-spectrum beta-lactamase *Enterobacter hormaechei* nosocomial outbreak, and other *Enterobacter hormaechei* misidentified as *Cronobacter* (*Enterobacter*) *sakazakii*. *Microbiology* 154(Pt 12), 3659–3667. doi: 10.1099/mic.0.2008/021980-0

Townsend, S. M., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J. G., Forsythe, S., et al. (2007). *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. *Microbiology* 153(Pt 10), 3538–3547. doi: 10.1099/mic.0.2007/009316-0

van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A., and Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J. Clin. Microbiol.* 39, 293–297. doi: 10.1128/JCM.39.1.293-297.2001

Willis, L. M., and Whitfield, C. (2013). Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. *Carbohydr. Res.* 378, 35–44. doi: 10.1016/j.carres.2013.05.007

Yan, Q., Power, K. A., Cooney, S., Fox, E., Gopinath, G. R., Grim, C. J., et al. (2013). Complete genome sequence and phenotype microarray analysis of *Cronobacter sakazakii* SP291: a persistent isolate cultured from a powdered infant formula production facility. *Front. Microbiol.* 4:256. doi; 10.3389/fmicb.2013.00256

Yin, S., Jensen, M. A., Bai, J., Debroy, C., Barrangou, R., and Dudley, E. G. (2013). The evolutionary divergence of Shiga toxin-producing *Escherichia coli* is reflected in clustered regularly interspaced short palindromic repeat (CRISPR) spacer composition. *Appl. Environ. Microbiol.* 79, 5710–5720. doi: 10.1128/AEM.00950-13

Zeng, H., Zhang, J., Li, C., Xie, T., Ling, N., Wu, Q., et al. (2017). The driving force of prophages and CRISPR-Cas system in the evolution of *Cronobacter sakazakii*. *Sci. Rep.* 7:40206. doi: 10.1038/srep40206

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Ogrodzki and Forsythe. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 October 2017
doi: 10.3389/fmicb.2017.02106

Prevalence and Antibiotic Resistance of Non-typhoidal *Salmonella* Isolated from Raw Chicken Carcasses of Commercial Broilers and Spent Hens in Tai'an, China

*Song Li[1], Yufa Zhou[2] and Zengmin Miao[3]**

[1] College of Basic Medicine, Taishan Medical University, Tai'an, China, [2] Center for Disease Control, Veterinary Bureau of Daiyue, Tai'an, China, [3] College of Life Sciences, Taishan Medical University, Tai'an, China

The present study was aimed to determine the prevalence and characteristics of *Salmonella* isolated from meat samples of commercial broilers (CB) and spent hens (SH). Between March and June 2016, 200 retail raw chicken carcasses (100 from CB and 100 from SH) were obtained from local supermarkets in Tai'an city of China, and *Salmonella* isolates were then analyzed for antibiotic resistance, serotype, β-lactamase genes, and the presence of class 1 integron. Forty *Salmonella* strains were obtained in this study (CB: 21/100, 21%; SH: 19/100, 19%). Three serotypes were identified in 40 *Salmonella*, and *S.* Enteritidis (CB: 15/21, 71.4%; SH: 10/19, 52.6%) was the dominant serotype, followed by *S.* Typhimurium (CB: 4/21, 19%; SH: 6/19, 31.6%) and *S.* Derby (CB: 2/21, 9.5%; SH: 3/19, 15.8%). Among 21 *Salmonella* isolated from CB, high antibiotic resistance rates were found for ampicillin (20/21, 95.2%), nalidixic acid (18/21, 85.7%), cefotaxime (17/21, 81%), and tetracycline (13/21, 61.9%); class 1 integron was observed in seven isolates (7/21, 33.3%), and gene cassettes included an empty integron (0.15 kb, $n = 1$), *aadA2* (1.2 kb, $n = 3$), *drfA1-aadA1* (1.4 kb, $n = 1$), and *drfA17-aadA5* (1.7 kb, $n = 2$); bla_{TEM-1} was the dominant β-lactamase gene (21/21, 100%), followed by $bla_{CTX-M-55}$ (7/21, 33.3%). Among 19 *Salmonella* isolated from SH, high antibiotic resistance rates were found for nalidixic acid (19/19, 100%), tetracycline (19/19, 100%), ampicillin (18/19, 94.7%), and ciprofloxacin (13/19, 68.4%); class 1 integron was observed in two isolates (2/19, 10.5%), and gene cassettes included *drfA17-aadA5* (1.7 kb, $n = 1$) and *drfA1-aadA1* (1.4 kb, $n = 1$); bla_{TEM-1} was the dominant β-lactamase gene (19/19, 100%), followed by $bla_{CTX-M-55}$ (2/19, 10.5%) and bla_{CMY-2} (1/19, 5.3%). Collectively, antibiotic-resistant *Salmonella* can be widely detected in retail raw chicken carcasses of CB and SH, and therefore can pose a serious risk to public health.

Keywords: antibiotic resistance, β-lactamase gene, class 1 integron, *Salmonella,* serotype

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Zhao Chen, University of California, Davis, United States
Ben Davies Tall, United States Food and Drug Administration, United States

****Correspondence:***
Zengmin Miao
zengminmiao@126.com;
zengminmiao@sina.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 July 2017*
Accepted: *16 October 2017*
Published: *31 October 2017*

Citation:
Li S, Zhou Y and Miao Z (2017) Prevalence and Antibiotic Resistance of Non-typhoidal Salmonella Isolated from Raw Chicken Carcasses of Commercial Broilers and Spent Hens in Tai'an, China. Front. Microbiol. 8:2106. doi: 10.3389/fmicb.2017.02106

INTRODUCTION

Salmonella is a notorious human pathogen and can lead to acute intestinal disease outbreaks in humans through consumption of contaminated foods (Pegues et al., 2006). It has been widely recognized that poultry products, such as eggs and meats, are a crucial transmission vehicle for *Salmonella* (Kusunoki et al., 2000; Betancor et al., 2010; Painter et al., 2013; Antunes et al., 2016).

At present and even for a long time in the future, antibiotic-based treatment for human salmonellosis infection is the most effective method in clinical practice (Ribeiro et al., 2011; Crump et al., 2015). It is therefore pivotal to use antibiotics to prevent and control *Salmonella* infections.

However, the widespread use and even abuse of antibiotics in animal husbandry have facilitated the emergence and dissemination of antibiotic resistance in *Salmonella*, which has posed a serious challenge for the health of animals and humans (Marshall and Levy, 2011; Mukerji et al., 2017). Noticeably, numerous studies in recent years have indicated that extended-spectrum β-lactamase (ESBL)-producing *Salmonella* has been frequently isolated from food-producing animals and animal-derived foods in many countries of the world, including China (Wu et al., 2013; Chon et al., 2015; Franco et al., 2015; Hu et al., 2015; Noda et al., 2015; Ziech et al., 2016; Zhao et al., 2017). ESBL-producing *Salmonella* is able to inactivate and hydrolyze the β-lactam ring in β-lactam antibiotics and third- and fourth-generation cephalosporins, leading to the increase of treatment cost and even to therapy failure, which has triggered a serious public concern (Bonnet, 2004; Pitout and Laupland, 2008). In addition, the class 1 integrons are frequently observed among antibiotic-resistant *Salmonella*, which contributes to the spread of antibiotic resistance genes among bacteria (Wannaprasat et al., 2011).

Therefore, understanding the prevalence and characteristics of *Salmonella* isolated from meat samples of food animal origins is of importance for developing effective treatment strategies to control and prevent *Salmonella* infections in humans and animals. However, information about the occurrence and characteristics of *Salmonella* in chicken meats in China is poorly documented. In China, two main chicken breeds, including introduced commercial broilers (CB) and spent hens (SH), are widely reared and are important sources of chicken meat (Chen et al., 2016). This study was therefore undertaken to determine the prevalence and characteristics of *Salmonella* recovered from retail chicken carcasses of CB and SH in Tai'an region, China.

MATERIALS AND METHODS

Sample Collection

Between March and June 2016, 200 retail raw chicken carcasses without giblets (100 from CB and 100 from SH) were obtained from local supermarkets in Tai'an city, China. These supermarkets had areas of 5,000–10,000 m^2, offering various foods and household products, in which raw chicken carcasses were sold refrigerated in a meat department. After purchase, the samples were stored in an icebox and immediately transported into our microbiology laboratory for further processing within 24 h.

Salmonella Isolation and Serotype Identification

Salmonella isolation was conducted as previously described (Choi et al., 2015). Briefly, each chicken carcass was mixed with 400 ml of buffered peptone water (BPW; Hopebiol,

TABLE 1 | Antibiotic resistance phenotype, presence of class 1 integron, and β-lactamase genes in *Salmonella* isolated from CB.

Isolates	Serovar	Antibiotic resistance phenotype[a]	Integron/β-lactamase genes
CB-1	*S.* Derby	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}
CB-12	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	(*aadA2*)/bla_{TEM-1}
CB-14	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-18	*S.* Derby	AMP, CTX, NAL	(*drfA1-aadA1*)/bla_{TEM-1}
CB-33	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-42	*S.* Typhimurium	AMP, GEN, SPT	(*aadA2*)/bla_{TEM-1}
CB-48	*S.* Typhimurium	AMP, SPT	bla_{TEM-1}
CB-49	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-51	*S.* Enteritidis	AMP, CTX, NAL	bla_{TEM-1}
CB-58	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}
CB-59	*S.* Enteritidis	AMP, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-63	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	(*drfA17-aadA5*)/bla_{TEM-1}, $bla_{CTX-M-55}$
CB-65	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}
CB-71	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	(*drfA17-aadA5*)/bla_{TEM-1}
CB-79	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-81	*S.* Typhimurium	AMP, SPT	(*aadA2*)/bla_{TEM-1}
CB-88	*S.* Enteritidis	NAL	bla_{TEM-1}
CB-89	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
CB-91	*S.* Enteritidis	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}
CB-96	*S.* Enteritidis	AMP, CTX, NAL	empty integron/bla_{TEM-1}
CB-98	*S.* Typhimurium	AMP, CTX, NAL	bla_{TEM-1}

[a]*AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CTX, cefotaxime; CIP, ciprofloxacin; FFC, florfenicol; GEN, gentamicin; NAL, nalidixic acid; SPT, spectinomycin; TET, tetracycline; SXT, sulfamethoxazole/trimethoprim.*

TABLE 2 | Antibiotic resistance phenotype, presence of class 1 integron, and β-lactamase genes in *Salmonella* isolated from SH.

Isolates	Serovar	Antibiotic resistance phenotype[b]	Integron/ β-lactamase genes
SH-3	*S.* Enteritidis	NAL	bla_{TEM-1}
SH-5	*S.* Enteritidis	AMP, NAL, TET	bla_{TEM-1}
SH-8	*S.* Enteritidis	AMP, CTX, CIP, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
SH-13	*S.* Derby	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-21	*S.* Enteritidis	AMP, NAL, TET	bla_{TEM-1}
SH-26	*S.* Enteritidis	AMP, NAL, TET	bla_{TEM-1}
SH-33	*S.* Enteritidis	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-35	*S.* Enteritidis	AMP, CIP, CTX, NAL, TET	(*drfA17-aadA5*)/bla_{TEM-1}
SH-48	*S.* Enteritidis	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-50	*S.* Enteritidis	AMP, CIP, NAL, TET	bla_{TEM-1}
SH53	*S.* Derby	AMP, NAL, TET	bla_{TEM-1}
SH-61	*S.* Typhimurium	AMP, CTX, CIP, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
SH-66	*S.* Typhimurium	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-73	*S.* Typhimurium	AMP, NAL, TET	bla_{TEM-1}
SH-75	*S.* Typhimurium	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-88	*S.* Typhimurium	AMP, CIP, CTX, GEN, NAL, TET	(*drfA1-aadA1*)/bla_{TEM-1}, bla_{CMY-2}
SH-90	*S.* Typhimurium	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-93	*S.* Enteritidis	AMP, CIP, NAL, TET	bla_{TEM-1}
SH-96	*S.* Derby	AMP, CIP, NAL, TET	bla_{TEM-1}

[b]*AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CTX, cefotaxime; CIP, ciprofloxacin; FFC, florfenicol; GEN, gentamicin; NAL, nalidixic acid; SPT, spectinomycin; TET, tetracycline; and SXT, sulfamethoxazole/trimethoprim.*

Qingdao, China) contained in a sterile plastic bag to rinse for 1 min by gentle shaking. Twenty-five milliliter of the rinsate was mixed with 25 mL of 2 × BPW and the mixture was incubated overnight at 37°C. A 100 μL aliquot was removed form the BPW enrichment broth and inoculated into Rappaport-Vassiliadis soy peptone broth (10 mL) (RVS; Hopebiol, Qingdao, China), which was incubated for 24 h at 42°C. One loopful of the RVS culture was streaked onto a xylose lysine desoxycholate agar plate (XLD; Hopebiol, Qingdao, China), which was incubated overnight at 37°C. The suspected *Salmonella* colony (red colonies with black centers) on the XLD plates from each chicken meat sample was identified by biochemical confirmation using biochemical tubes (Hopebiol, Qingdao, China), and the results were interpreted according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004).

According to the Kauffmann-White scheme, slide agglutination tests were used to serotype *Salmonella* isolates in this study (S&A Reagents Lab, Bangkok, Thailand).

Antimicrobial Susceptibility Testing

Based on the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2013), the disk diffusion method was employed to determine antibiotic susceptibilities of *Salmonella* strains. Antibiotics used in this study were amoxicillin/clavulanic acid (20/10 μg), ampicillin (10μg), cefotaxime (30 μg), ciprofloxacin (5 μg), florfenicol (30 μg), gentamicin (10 μg), nalidixic acid (10 μg), spectinomycin (10 μg), tetracycline (30 μg) and sulfamethoxazole/trimethoprim (1.25/23.75 μg) (Hopebiol, Qingdao, China). *Salmonella* strains resistant to no less than three classes of antibiotics were defined as multidrug-resistant (MDR) isolates. *Escherichia coli* ATCC 25922 was used in this study as quality control strain.

Detection of β-Lactamase Gene

According to the method previously described (Batchelor et al., 2005; Rayamajhi et al., 2008; Li et al., 2013), polymerase chain reaction (PCR) was used to determine the presence of β-lactamase genes (bla_{TEM}, bla_{PSE-1}, bla_{CMY-2}, bla_{SHV}, bla_{DHA-1}, bla_{OXA}, and bla_{CTX-M}). For isolates carrying bla_{CTX-M} genes, bla_{CTX-M} gene group was further identified by using PCR (Kim et al., 2015). The PCR products were sequenced (Sunny, Shanghai, China), and the sequences were analyzed and aligned using the NCBI BLAST program[1].

Detection of Class I Integrons

Based on the primers previously described, PCR was used to analyze the presence of class 1 integron (Guerra et al., 2001; Kerrn et al., 2002). Additionally, PCR was employed to amplify gene cassettes within the variable region of class 1 integron according to the methods described previously (Sandvang et al., 1998). The amplification fragments were cloned into the pMD18-T vector (Takara, Dalian, China), which were sequenced (Sunny, Shanghai, China).

Statistical Analyses

Fisher's exact test was used to compare the prevalence of *Salmonella* and proportions of class 1 integron in *Salmonella* in CB and SH using SPSS 16.0 software (IBM, United States). *P*-values of less than 0.05 were defined as difference significance.

RESULTS

Salmonella Prevalence

A total of 40 *Salmonella* strains (40/200, 20%) were isolated from the foods, and the prevalence in CB was 21% (21/100) and 19% (19/100) in SH. No significant difference was found in *Salmonella* prevalence between CB and SH samples ($P > 0.05$).

Serotyping and Antimicrobial Susceptibility Testing

Three serotypes were identified in 40 *Salmonella* strains. *S.* Enteritidis (CB: 15/21, 71.4%; SH: 10/19, 52.6%) was the dominant serotype, followed by *S.* Typhimurium (CB: 4/21, 19%; SH: 6/19, 31.6%) and *S.* Derby (CB: 2/21, 9.5%; SH: 3/19, 15.8%) (**Tables 1**, **2**).

[1]http://www.ncbi.nlm.nih.gov/BLAST

Prevalence of Class 1 Integron and β-Lactamase Genes

Among 21 *Salmonella* isolated from CB, class 1 integron was observed in seven isolates (7/21, 33.3%), and gene cassettes included an empty integron (0.15 kb, $n = 1$), *aadA2* (1.2 kb, $n = 3$), *drfA1-aadA1* (1.4 kb, $n = 1$), and *drfA17-aadA5* (1.7 kb, $n = 2$); $bla_{\mathrm{TEM-1}}$ was the dominant β-lactamase gene (21/21, 100%), followed by $bla_{\mathrm{CTX-M-55}}$ (7/21, 33.3%) (**Table 1**). Among 19 *Salmonella* isolated from SH, class 1 integron was observed in two isolates (2/19, 10.5%), and gene cassettes included *drfA17-aadA5* (1.7 kb, $n = 1$) and *drfA1-aadA1* (1.4 kb, $n = 1$); $bla_{\mathrm{TEM-1}}$ was the dominant β-lactamase gene (19/19, 100%), followed by $bla_{\mathrm{CTX-M-55}}$ (2/19, 10.5%) and $bla_{\mathrm{CMY-2}}$ (1/19, 5.3%) (**Table 2**). Of note, the proportion of class 1 integron targets detected in *Salmonella* strains from CB samples was higher than that found in SH samples (33.3% vs. 10.5%, $P < 0.05$).

DISCUSSION

In the present study, 20% of 200 retail chicken carcasses were *Salmonella* positive. The prevalence of *Salmonella* in poultry meat products in other parts of China has been reported by others to be approximately 36.1% (Cui H.X. et al., 2009) and 28.3% (Li et al., 2013). The prevalence in other regions of the world was 15.6% in chicken carcasses in EU (European Food Safety Authority [EFSA], 2010) and 45.8% in retail chicken meat in Korea (Park et al., 2017). These investigations indicated that *Salmonella* contamination is widely distributed in poultry meats. Of note, it is difficult to compare the prevalence of *Salmonella* among different studies, because the difference may be associated with geographical differences, sampling seasons, sample types, methodology of isolation and culture, and environments of slaughterhouses and marketing areas (Yan et al., 2010; Yang et al., 2010).

Salmonella Enteritidis was the most commonly isolated serotype in this study, and has been widely isolated in chickens, eggs, and chicken meats in China (Yang et al., 2010; Lu et al., 2011; Long et al., 2016). In addition, *S.* Enteritidis is the leading cause of *Salmonella* related food-borne outbreaks in humans worldwide (Galanis et al., 2006). Of note, *S.* Typhimurium is the main serotype isolated from humans in China (Deng et al., 2012) and *S.* Derby is the most common serotype isolated from infants and toddlers in China (Cui S. et al., 2009), which suggested that an association may exist between *Salmonella*-contaminated food and salmonellosis in these age groups.

Similar antibiotic resistance patterns were observed in *Salmonella* isolated from CB and SH. Of 21 *Salmonella* isolated from CB, high antibiotic resistance rates were found for ampicillin (20/21, 95.2%), nalidixic acid (18/21, 85.7%), cefotaxime (17/21, 81%), and tetracycline (13/21, 61.9%); and 18 out of 21 *Salmonella* were MDR isolates (85.7%). Among 19 *Salmonella* isolated from SH, high antibiotic resistance rates were found for nalidixic acid (19/19, 100%), tetracycline (19/19, 100%), ampicillin (18/19, 94.7%), and ciprofloxacin (13/19, 68.4%); and 17 of 19 *Salmonella* were MDR strains (89.5%). No significant difference ($P > 0.05$) in the prevalence of MDR *Salmonella* between CB and SH. Of note, co-resistance to ciprofloxacin and cefotaxime in these *Salmonella* strains would limit therapeutic options in clinical practice (Whichard et al., 2007).

All class 1 integron-positive isolates in this study exhibited resistance to at least two classes of antibiotics, which supports the hypothesis that there is a strong association between the presence of class I integron and the emerging of MDR in *Salmonella* (Wannaprasat et al., 2011; Firoozeh et al., 2012).

All *Salmonella* isolates in this study carried $bla_{\mathrm{TEM-1}}$ genes, 38 of which showed were resistant to ampicillin. Noticeably, one $bla_{\mathrm{CMY-2}}$- producing *Salmonella* isolate was detected, which has been observed in chicken meat in 2010-2011 in Sichuan province of China (Li et al., 2013). Because $bla_{\mathrm{CMY-2}}$ can encode antibiotic resistance to third-generation cephalosporins, which is frequently used to treat cases of salmonellosis (Gonzalez-Sanz et al., 2009), the dissemination of $bla_{\mathrm{CMY-2}}$- positive *Salmonella* via poultry meat products has pivotal public health implications. Therefore, the poultry industry should follow prudent management by establishing more effective disinfection guidelines to reduce the population of antibiotic-resistant pathogens. Moreover, a moderate use of antibiotics may help prevent the occurrence of antibiotic resistance in pathogens (Chen and Jiang, 2014).

CONCLUSION

To our best knowledge, this is the first study in China comparing the prevalence and characteristics of *Salmonella* isolated from chicken meat samples of CB and SH. Regardless of chicken meat type, 25% (10/40) of the *Salmonella* isolates in this study carried ESBL-producing genes; 22.5% (9/40) of the *Salmonella* isolates contained class 1 integrons. Therefore, the reasonable use of antibiotics in animal husbandry should be taken, and continued long-term surveillance of *Salmonella* in animal-derived foods is warranted.

AUTHOR CONTRIBUTIONS

ZM designed the study; SL and YZ collected samples and conducted the experiments; ZM, SL, and YZ analyzed data and wrote the manuscript.

REFERENCES

Antunes, P., Mourão, J., Campos, J., and Peixe, L. (2016). Salmonellosis: the role of poultry meat. *Clin. Microbiol. Infect.* 2, 110–121. doi: 10.1016/j.cmi.2015.12.004

Batchelor, M., Hopkins, K., Threlfall, E. J., Clifton-Hadley, F. A., Stallwood, A. D., Davies, R. H., et al. (2005). $bla_{(\mathrm{CTX-M})}$ genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. *Antimicrob. Agents Chemother.* 49, 1319–1322. doi: 10.1128/AAC.49.4.1319-1322.2005

Betancor, L., Pereira, M., Martinez, A., Giossa, G., Fookes, M., Flores, K., et al. (2010). Prevalence of *Salmonella enterica* in poultry and eggs in Uruguay during an epidemic due to *Salmonella enterica* serovar Enteritidis. *J. Clin. Microbiol.* 48, 2413–2423. doi: 10.1128/JCM.02137-09

Bonnet, R. (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* 48, 1–14. doi: 10.1128/AAC.48.1.1-14.2004

Chen, Y., Qiao, Y., Xiao, Y., Chen, H., Zhao, L., Huang, M., et al. (2016). Differences in physicochemical and nutritional properties of breast and thigh meat from crossbred chickens, commercial broilers, and spent hens. *Asian Australas. J. Anim. Sci.* 29, 855–864. doi: 10.5713/ajas.15.0840

Chen, Z., and Jiang, X. (2014). Microbiological safety of chicken litter or chicken litter-based organic fertilizers: a review. *Agriculture* 4, 1–29. doi: 10.3390/agriculture4010001

Choi, D., Chon, J. W., Kim, H. S., Kim, D. H., Lim, J. S., Yim, J. H., et al. (2015). Incidence, antimicrobial resistance, and molecular characteristics of nontyphoidal *Salmonella* including extended-spectrum β-lactamase producers in retail chicken meat. *J. Food Prot.* 78, 1932–1937. doi: 10.4315/0362-028X.JFP-15-145

Chon, J. W., Jung, H. I., Kuk, M., Kim, Y. J., Seo, K. H., and Kim, S. K. (2015). High occurrence of extended-spectrum β-lactamase-producing *Salmonella* in broiler carcasses from poultry slaughterhouses in South Korea. *Foodborne Pathog. Dis.* 12, 190–196. doi: 10.1089/fpd.2014.1847

Clinical and Laboratory Standards Institute [CLSI] (2013). *Performance Standards for Antimicrobial Susceptibility Testing, 23rd Informational Supplement M100-S23*. Wayne, PA: Clinical and Laboratory Standards Institute.

Crump, J. A., Sjölund-Karlsson, M., Gordon, M. A., and Parry, C. M. (2015). Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive *Salmonella* infections. *Clin. Microbiol. Rev.* 28, 901–937. doi: 10.1128/CMR.00002-15

Cui, H. X., Zhang, X. L., Liao, X. G., Zhang, D., Hu, D., Zhang, M., et al. (2009). Monitoring and analysis for food-borne pathogens in Henan in 2007. *Chin. J. Health Lab Technol.* 19, 173–175.

Cui, S., Li, J., Sun, Z., Hu, C., Jin, S., Li, F., et al. (2009). Characterization of *Salmonella enterica* isolates from infants and toddlers in Wuhan, China. *J. Antimicrob. Chemother.* 63, 87–94. doi: 10.1093/jac/dkn452

Deng, X., Ran, L., Wu, S., Ke, B., He, D., Yang, X., et al. (2012). Laboratory-based surveillance of non-typhoidal *Salmonella* infections in Guangdong Province, China. *Foodborne Pathog. Dis.* 9, 305–312. doi: 10.1089/fpd.2011.1008

European Food Safety Authority [EFSA] (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008, part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA J.* 8, 1503.

Firoozeh, F., Zahraei-Salehi, T., Shahcheraghi, F., Karimi, V., and Aslani, M. M. (2012). Characterization of class I integrons among *Salmonella enterica* serovar Enteritidis isolated from humans and poultry. *FEMS Immunol. Med. Microbiol.* 64, 237–243. doi: 10.1111/j.1574-695X.2011.00883.x

Franco, A., Leekitcharoenphon, P., Feltrin, F., Alba, P., Cordaro, G., Iurescia, M., et al. (2015). Emergence of a clonal lineage of multidrug-resistant ESBL-producing *Salmonella* Infantis transmitted from broilers and broiler meat to humans in Italy between 2011 and 2014. *PLOS ONE* 10:e0144802. doi: 10.1371/journal.pone.0144802

Galanis, E., Lo Fo Wong, D. M., Patrick, M. E., Binsztein, N., Cieslik, A., Chalermchikit, T., et al. (2006). Web-based surveillance and global *Salmonella* distribution, 2000-2002. *Emerg. Infect. Dis.* 12, 381–388. doi: 10.3201/eid1205.050854

Garrity, G. M., Bell, J. A., and Lilburn, T. G. (2004). *Bergey's Manual of Systematic Bacteriology*, 2nd Edn. New York, NY: Springer.

Gonzalez-Sanz, R., Herrera-Leon, S., de la Fuente, M., Arroyo, M., and Echeita, M. A. (2009). Emergence of extended-spectrum beta-lactamases and AmpC-type beta-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. *J. Antimicrob. Chemother.* 64, 1181–1186. doi: 10.1093/jac/dkp361

Guerra, B., Soto, S. M., Arguelles, J. M., and Mendoza, M. C. (2001). Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4,5,12:i:-]. *Antimicrob. Agents Chemother.* 45, 1305–1308. doi: 10.1128/AAC.45.4.1305-1308.2001

Hu, Y. J., Wang, Y. R., and Li, F. Q. (2015). Study on simultaneous contamination of *Salmonella* and *Campylobacter* in retail chicken carcasses in Beijing. *J. Hyg. Res.* 44, 68–72.

Kerrn, M. B., Klemmensen, T., Frimodt-Moller, N., and Espersen, F. (2002). Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of sul genes conferring sulphonamide resistance. *J. Antimicrob. Chemother.* 50, 513–516. doi: 10.1093/jac/dkf164

Kim, H. S., Chon, J. W., Kim, Y. J., Kim, D. H., Kim, M. S., and Seo, K. H. (2015). Prevalence and characterization of extended-spectrum-β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in ready-to-eat vegetables. *Int. J. Food Microbiol.* 207, 83–86. doi: 10.1016/j.ijfoodmicro.2015.04.049

Kusunoki, K., Jin, M., Iwaya, M., Ishikami, T., Kurimoto, K., Saito, K., et al. (2000). *Salmonella* contamination in domestic raw chickens in Tama, Tokyo, and serovar or drug-resistance of isolates (1992-1999). *Jpn. J. Food Microbiol.* 17, 207–212. doi: 10.5803/jsfm.17.207

Li, R., Lai, J., Wang, Y., Liu, S., Li, Y., Liu, K., et al. (2013). Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. *Int. J. Food Microbiol.* 163, 14–18. doi: 10.1016/j.ijfoodmicro.2013.01.020

Long, M., Lai, H., Deng, W., Zhou, K., Li, B., Liu, S., et al. (2016). Disinfectant susceptibility of different *Salmonella* serotypes isolated from chicken and egg production chains. *J. Appl. Microbiol.* 121, 672–681. doi: 10.1111/jam.13184

Lu, Y., Wu, C. M., Wu, G. J., Zhao, H. Y., He, T., Cao, X. Y., et al. (2011). Prevalence of antimicrobial resistance among *Salmonella* isolates from chicken in china. *Foodborne Pathog. Dis.* 8, 45–53. doi: 10.1089/fpd.2010.0605

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/CMR.00002-11

Mukerji, S., O'Dea, M., Barton, M., Kirkwood, R., Lee, T., and Abraham, S. (2017). Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact. *Essays Biochem.* 61, 23–35. doi: 10.1042/EBC20160055

Noda, T., Murakami, K., Etoh, Y., Okamoto, F., Yatsuyanagi, J., Sera, N., et al. (2015). Increase in resistance to extended-spectrum cephalosporins in *Salmonella* isolated from retail chicken products in Japan. *PLOS ONE* 10:e0116927. doi: 10.1371/journal.pone.0116927

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., et al. (2013). Attribution of food-borne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerg. Infect. Dis.* 19, 407. doi: 10.3201/eid1903.111866

Park, J. H., Kim, H. S., Yim, J. H., Kim, Y. J., Kim, D. H., Chon, J. W., et al. (2017). Comparison of the isolation rates and characteristics of *Salmonella* isolated from antibiotic-free and conventional chicken meat samples. *Poult. Sci.* doi: 10.3382/ps/pex055 [Epub ahead of print].

Pegues, D. A., Ohl, M. E., and Miller, S. I. (2006). Nontyphoidal salmonellosis. *Trop. Infect. Dis.* 1, 241–254.

Pitout, J. D., and Laupland, K. B. (2008). Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: an emerging public health concern. *Lancet Infect. Dis.* 8, 159–166. doi: 10.1016/S1473-3099(08)70041-0

Rayamajhi, N., Kang, S. G., Lee, D. Y., Kang, M. L., Lee, S. I., Park, K. Y., et al. (2008). Characterization of TEM-, SHV- and AmpC-type beta-lactamases from cephalosporin-resistant *Enterobacteriaceae* isolated from swine. *Int. J. Food Microbiol.* 124, 183–187. doi: 10.1016/j.ijfoodmicro.2008.03.009

Ribeiro, V. B., Lincopan, N., Landgraf, M., Franco, B. D., and Destro, M. T. (2011). Characterization of class I integrons and antibiotic resistance genes in multidrug-resistant *Salmonella enterica* isolates from foodstuff and related sources. *Braz. J. Microbiol.* 42, 685–692. doi: 10.1590/S1517-838220110002000033

Sandvang, D., Aarestrup, F. M., and Jensen, L. B. (1998). Characterization of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol. Lett.* 160, 37–41. doi: 10.1111/j.1574-6968.1998.tb12887.x

Wannaprasat, W., Padungtod, P., and Chuanchuen, R. (2011). Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. *Int. J. Antimicrob. Agents* 37, 457–461. doi: 10.1016/j.ijantimicag.2010.12.001

Whichard, J. M., Gay, K., Stevenson, J. E., Joyce, K. J., Cooper, K. L., Omondi, M., et al. (2007). Human *Salmonella* and concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins. *Emerg. Infect. Dis.* 13, 1681–1688. doi: 10.3201/eid1311.061438

Wu, H., Xia, X., Cui, Y., Hu, Y., Xi, M., Wang, X., et al. (2013). Prevalence of extended-spectrum β-lactamase-producing *Salmonella* on retail chicken in six provinces and two national cities in the People's Republic of China. *J. Food Prot.* 76, 2040–2044. doi: 10.4315/0362-028X.JFP-13-224

Yan, H., Li, L., Alam, M. J., Shinoda, S., Miyoshi, S., and Shi, L. (2010). Prevalence and antimicrobial resistance of *Salmonella* in retail foods in northern China. *Int. J. Food Microbiol.* 143, 230–234. doi: 10.1016/j.ijfoodmicro.2010.07.034

Yang, B., Qu, D., Zhang, X., Shen, J., Cui, S., Shi, Y., et al. (2010). Prevalence and characterization of *Salmonella* serovars in retail meats of marketplace in Shaanxi, China. *Int. J. Food Microbiol.* 141, 63–72. doi: 10.1016/j.ijfoodmicro.2010.04.015

Zhao, X., Ye, C., Chang, W., and Sun, S. (2017). Serotype distribution, antimicrobial resistance, and class 1 integrons profiles of *Salmonella* from animals in slaughterhouses in Shandong province, China. *Front. Microbiol.* 8:1049. doi: 10.3389/fmicb.2017.01049

Ziech, R. E., Lampugnani, C., Perin, A. P., Sereno, M. J., Sfaciotte, R. A., Viana, C., et al. (2016). Multidrug resistance and ESBL-producing *Salmonella* spp. isolated from broiler processing plants. *Braz. J. Microbiol.* 47, 191–195. doi: 10.1016/j.bjm.2015.11.021

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Li, Zhou and Miao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 November 2017
doi: 10.3389/fmicb.2017.02159

Combination of Microfluidic Loop-Mediated Isothermal Amplification with Gold Nanoparticles for Rapid Detection of *Salmonella* spp. in Food Samples

Alejandro Garrido-Maestu, Sarah Azinheiro, Joana Carvalho, Sara Abalde-Cela, Enrique Carbó-Argibay, Lorena Diéguez, Marek Piotrowski, Yury V. Kolen'ko and Marta Prado*

International Iberian Nanotechnology Laboratory, Braga, Portugal

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Soohyoun Ahn, University of Florida, United States
Lucia Galli, National University of La Plata, Argentina

***Correspondence:**
Alejandro Garrido-Maestu
alejandro.garrido@inl.int

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *09 August 2017*
Accepted: *20 October 2017*
Published: *06 November 2017*

Citation:
Garrido-Maestu A, Azinheiro S, Carvalho J, Abalde-Cela S, Carbó-Argibay E, Diéguez L, Piotrowski M, Kolen'ko YV and Prado M (2017) Combination of Microfluidic Loop-Mediated Isothermal Amplification with Gold Nanoparticles for Rapid Detection of Salmonella spp. in Food Samples. Front. Microbiol. 8:2159. doi: 10.3389/fmicb.2017.02159

Foodborne diseases are an important cause of morbidity and mortality. According to the World Health Organization, there are 31 main global hazards, which caused in 2010 600 million foodborne illnesses and 420000 deaths. Among them, *Salmonella* spp. is one of the most important human pathogens, accounting for more than 90000 cases in Europe and even more in the United States per year. In the current study we report the development, and thorough evaluation in food samples, of a microfluidic system combining loop-mediated isothermal amplification with gold nanoparticles (AuNPs). This system is intended for low-cost, *in situ*, detection of different pathogens, as the proposed methodology can be extrapolated to different microorganisms. A very low limit of detection (10 cfu/25 g) was obtained. Furthermore, the evaluation of spiked food samples (chicken, turkey, egg products), completely matched the expected results, as denoted by the index kappa of concordance (value of 1.00). The results obtained for the relative sensitivity, specificity and accuracy were of 100% as well as the positive and negative predictive values.

Keywords: microfluidics, gold nanoparticles, *Salmonella* spp., LAMP, *invA*

INTRODUCTION

Salmonella spp. continues to be a major health issue, not only in Europe where 94625 cases were reported in 2015, but worldwide. It has been estimated that *Salmonella enterica* causes approximately 1.2 million illnesses and 450 deaths per year in the United States (Carroll et al., 2017; Efsa and Ecdc, 2017). These figures highlight that additional improvement in the current analysis methodologies are still needed.

When talking about bacterial foodborne pathogens, official methods are based on classical microbiology which require several days for bacterial isolation and identification. These methods present the additional drawback of not being able of detecting viable but non-culturable bacteria (VBNC). Fast and reliable analytical methods are needed by both, the industry and control laboratories. The main objectives of such methods are to ensure the health of consumers, to easily determine whether a food product has been contaminated, and if possible, identify how

and when this contamination occurred. This is needed in order to establish the proper corrective measures (Prado et al., 2016a). Molecular methods have, now for long, demonstrated capability of overcoming the limitations associated with culture based methods (Garrido-Maestu et al., 2014; Chapela et al., 2015; Valderrama et al., 2016), being polymerase chain reaction (PCR) the most widely used and accepted (Garrido et al., 2013; Ceuppens et al., 2014). Furthermore, there is an increase interest on the development of miniaturized devices that would allow *in situ* and/or economic monitoring of food products. However, miniaturization of PCR instrumentation, although possible and feasible (Jia et al., 2007; Wang et al., 2009; Ha and Lee, 2015), requires the capacity of accurate thermal cycling of the sample, since the speed of temperature transitions and the thermal homogeneity throughout the PCR mixture are essential for the run time, efficiency and specificity of the amplification reactions (Wittwer et al., 1990). Such demands concerning accurate thermal control, limit the number of materials that can be used for the fabrication of miniaturized devices (Jia et al., 2007), and consequently increase fabrication costs. Therefore, novel isothermal amplification techniques have emerged in recent years with the goal of providing an analytical solution to some of the drawbacks associated with PCR/qPCR, specially due to their simplicity and reduced thermal budget (Kaprou et al., 2015). Among them, loop-mediated isothermal amplification (LAMP) has become the most popular technique (Mori and Notomi, 2009; Fang et al., 2010b; Rafati and Gill, 2015).

It has been reported that in LAMP, along with specific DNA amplification, an insoluble by product is formed ($Mg_2P_2O_7$), what allows to directly determine if a particular reaction is positive or negative by naked-eye observation (Mori et al., 2001). However, several different approaches have been published, what indicates that it may not be as straightforward as expected, and thus requiring for trained analysts (Goto et al., 2009; Wan et al., 2012; Wang et al., 2012; Birmpa et al., 2015; Tanner et al., 2015). An alternative approach relies on the use of gold nanoparticles (AuNPs), which exhibit a characteristic localized surface plasmon resonance absorption band (LSPR) in the visible light region, being dependent on the interparticle distance (aggregation causes a red shift originating a red-to-purple color change) (Wong et al., 2014; Prado et al., 2016b). Wong et al. (2014), described a methodology based on the functionalization of AuNPs with 11-mercaptoundecanoic acid (MUA), which conferred negative charge to the surface of the particles. This, allowed to control aggregation/separation based on the presence/absence of Mg^{2+} in the LAMP reaction buffer (Wong et al., 2014), provided a visible color change and a more objective result assessment than turbidity.

The potential of microfluidics to create an integrated miniaturized and stand-alone laboratory has long been a dream of the community. This capability is particularly pertinent in low-resource settings where functional laboratories are simply not available (Chiu et al., 2017). Microfluidic technology is an enabling technology for Lab-On-a-Chip (LOC) tests as it allows to reduce sample/reagent consumption, integrated components and functions, and high portability and flexibility. One major challenge is that an easy-to-operate chip inevitably requires complicated fluid circuits and even microfabricated valves or pumps. Microfluidics provide a higher surface to volume ratio, a faster rate of mass and heat transfer, and the ability to precisely handle very small volumes of reagents (Foudeh et al., 2012; Sun et al., 2014). Microfluidic systems allow precise control of mixing in reduced sample volumes, integration with sensing elements, and provide the ideal conditions to develop a LOC systems for molecular detection of bacteria.

The aim of the current study was to determine the possibility of combining a miniaturized device for LAMP based DNA amplification, with functionalized AuNPs for naked-eye detection of *Salmonella* spp. in food samples. Additionally, its application in real food samples was also assessed, to determine its applicability in the food industry as a simple, inexpensive and fast analytical approach.

MATERIALS AND METHODS

Sample Contamination

In order to evaluate the performance of the methodology, food samples were artificially contaminated with *S.* Typhimurium CECT 4594, purchased from the Spanish Type Culture Collection. A pure culture was prepared by inoculating 4 mL of Buffered Peptone Water (BPW, Biokar Diagnostics S.A., France) with a single colony, and incubated overnight at 37°C. After incubation, this culture was 10-fold serially diluted in BPW and used for the inoculation of the food samples (1 mL of the corresponding dilution was added to the mixture of 25 g of food sample and 225 mL of diluent), as well as plated on Tryptic Soy Broth (TSB, Biokar Diagnostics S.A., France) with 15 g/L of agar, to get viable bacterial counts. The plates were incubated at 37°C overnight.

Food Sample Treatment and DNA Extraction

To reach regulatory limits for most foodstuffs, regarding *Salmonella* spp. (absence/25 g, see European Regulation 2073/2005) a pre-enrichment step was included in the protocol. To this end, 25 g of sample were weighted and mixed with 225 mL of BPW. Positive samples were inoculated with 1 mL of the corresponding dilution of *S.* Typhimurium prepared as detailed above, homogenized for 30 s and incubated at 37°C for 18–24 h (negative samples were directly homogenized and placed in the incubator). After incubation, 1 mL was taken for DNA extraction. Chicken was selected as the reference food type for the determination of the Limit of Detection (LoD), and in addition to this, turkey, and omelet prepared with eggs were also included in the evaluation of the method. Food samples were obtained from local suppliers. A detailed list of the samples analyzed is provided in **Table 1**.

DNA extraction was performed as previously described by Garrido-Maestu et al. (2017). Briefly, the aliquot from the enriched matrix was centrifuged at 2,000 rpm for 2 min, to eliminate large food debris. The supernatant was centrifuged at 13,000 rpm for 5 min, the pellet was rinsed with 1 mL of PBS and centrifuged again. The clean pellet was resuspended in 300 μL of

TABLE 1 | Samples inoculated with one single strain.

Sample	*N*	Inoculum (cfu/25 g)	Result
Chicken breast	10*	<10	+
Chicken breast	10	–	–
Chicken breast	2	<10	+
Chicken breast	1	10^3–10^4	+
Chicken breast	2	10^4–10^5	+
Chicken breast	1	10^7–10^8	+
Turkey	2	–	–
Turkey	1	<10	+
Turkey	2	10–10^2	+
Turkey	1	10^3–10^4	+
Omelet (egg)	2	–	–
Omelet (egg)	2	10^2–10^3	+
Omelet (egg)	2	10^7–10^8	+

*N is the number of samples. *These samples were used for the determination of the LOD.*

6% Chelex®100 (w/v) (Bio-Rad Laboratories, Inc., United States), incubated at 56°C for 15 min at 1,000 rpm in a Thermomixer comfort (Eppendorf AG, Germany), and finally the bacteria were lysed by heating at 99°C for 10 min. After thermal lysis, the samples were centrifuged at 13,000 rpm for 5 min at 4°C, and the supernatant containing the DNA was transferred to a clean tube which was stored at 4°C until analysis.

Microfluidic Device

The fabrication of the microfluidic device was achieved through the combination of computer-numerical-control (CNC) polymer machining and polydimethylsiloxane (PDMS) replica molding. The CNC mold was designed using AutoCAD software, and fabricated in poly(methyl methacrylate) (PMMA) material using a CNC miller (FlexiCAM Viper 606). The PDMS prepolymer (Sylgard 184 silicone elastomer kit), was prepared by mixing the base and curing agent at a weight ratio of 10: 1, then was poured onto the PMMA mold, placed under vacuum for 15–20 min to remove the air bubbles and cured in the oven at 65°C for 1 h. After cooling down, the PDMS replica was peeled off from the mold and microfluidic channels were irreversibly sealed against a glass slide using oxygen plasma bonding. The final dimensions of the microfluidic device were 4 mm (thickness) × 76 mm (length) × 26 mm (width) and its pattern included a total of 8 capillarity-driven microchannels with a geometry of 40 mm (length) × 800 μm (depth) × 600 μm (width). Each microchannel had one inlet and one outlet, incorporated in the PMMA mold, and a volume capacity of 20 μL.

Gold Nanoparticle Synthesis and Characterization

For the synthesis of spherical AuNPs the well-known Turkevich method was followed (Turkevich et al., 1951). Briefly, 5 mL of a 1% solution of trisodium citrate ($Na_3C_6H_5O_7$, Sigma-Aldrich, St. Louis, MO, United States) was added to a boiling solution of gold chloride ($HAuCl_4 \cdot {}_3H_2O$, Sigma-Aldrich, St. Louis, MO, United States) (95 mL, 0.5 mM) under vigorous magnetic stirring. After 5 min, the color of the solution turned from pale yellow to intense red.

The AuNPs were characterized by UV-Vis spectra using a NanoDrop 2000c (Thermo Fisher Scientific, Inc., Waltham, MA, United States). Additionally, the morphological characterization of the citrate-stabilized nanoparticles was performed by transmission electron microscopy (TEM). The images were acquired in a JEOL JEM-2100 electron microscope, operated at 200 kV. TEM samples were prepared by dropping ca. 12 μL of the nanoparticles dispersion onto a formvar/carbon-coated Cu grid (400 mesh) placed on a filter paper followed by the evaporation of the solvent at room temperature.

Gold Nanoparticle Functionalization

The functionalization of the AuNP was performed as described by Wong et al. (2014). Briefly, 20 nM AuNPs and 2 mM MUA (freshly prepared in DMSO) were mixed, and incubated for 24 h at room temperature with constant agitation (1400 rpm, Thermomixer comfort).

Salmonella spp. DNA Amplification and Detection by Microfluidic-AuNP

The detection of *Salmonella* spp. was assessed targeting *invA* gene. To this end, the primers designed by Hara-Kudo et al. (2005) were selected: FIP: GACGACTGGTA CTGATCGATAGTTTTTCAACGTTTCCTGCGG, BIP: CCGG TGAAATTATCGCCACACAAAACCCACCGCCAGG, F3: GG CGATATTGGTGTTTATGGGG, B3: AACGATAAACTGGACC ACGG.

A final reaction volume of 25 μL was prepared with 3 μL template DNA, 2.5 μL of 10X Isothermal Amplification Buffer (New England BioLabs, Inc., Ipswich, MA, United States), 1 M betaine (Sigma-Aldrich, St. Louis, MO, United States), 0.35 mM dNTP mix (Thermo Fisher Scientific, Inc., Waltham, MA, United States), and 8 U *Bst* 2.0 WarmStart® DNA Polymerase (New England BioLabs, Inc., Ipswich, MA, United States). The primer concentration was 700 and 100 nM for FIP/BIP and F3/B3 respectively. Out of the master mix prepared, 20 μL were loaded in one of the microfluidic channels, which was carefully sealed with a glass slide and few clamps to avoid evaporation, and placed in a conventional laboratory incubator (Memmert GmbH, Schwabach, Germany) with the temperature set at 65°C for 1 h.

After incubation, 4 μL of the LAMP product were diluted with sterile milli-Q water (8.5 μL) and mixed with the functionalized AuNP (final concentration 6 nM), making a final volume of 15 μL. The results (positive-red, negative-purple) were directly assessed by naked-eye observation. For comparison purposes, gel electrophoresis was also performed; to do so a 2% agarose gel was prepared (NzyTech, Lisbon, Portugal) in Sodium Borate buffer prepared as previously described (SB, Brody and Kern, 2004). The gel was stained with 4 μL of Midori Green (Nippon Genetics Europe GmbH, Düren, Germany). One μL of the LAMP-AuNP product was mixed with 5 μL of 6X DNA loading dye (Thermo Fisher Scientific, Inc., Waltham, MA, United States), and loaded in the gel. The samples were separated for 20 min

at 300 V, and finally visualized in a GelDoc™ EZ Imager (Bio-Rad Laboratories, Inc., United States). Finally, UV-vis was also measured for positive and negative samples in a NanoDrop 2000c.

Fitness-for-Purpose of the Method

Following the protocol described above, the complete method was evaluated taking into account the LoD, relative sensitivity (SE), specificity (SP) and accuracy (AC), positive and negative predictive values (PPV, NPV) and the kappa index of concordance (*k*), as previously described (Tomas et al., 2009; Anderson et al., 2011; Garrido et al., 2013). To do so, every sample was classified as being in Positive, or Negative, Agreement (PA and NA) and Positive, or Negative, Deviation respect to the expected results (if the samples were, or not, inoculated with the target microorganism). Then the previously mentioned parameters were calculated based on the following formulas:

$SE = [PA/(PA + ND)] \times 100$

$SP = [NA/(PD + NA)] \times 100$

$AC = [(PA + NA)/N] \times 100$;

where "N" number of analyzed samples.

$PPV = [PA/(PA + PD)] \times 100$

$NPV = [NA/(NA + ND)] \times 100$

$k = 2 \times (PA \times NA - ND \times PD)/ [(PA + PD) \times (PD + NA) + (PA + ND) \times (ND + NA)]$.

The LoD was determined to be the lowest, reproducible, detectable concentration. The rest of the parameters were calculated based on the obtained and expected results.

RESULTS

Microfluidics

The microfluidic device, with eight channels was successfully designed and fabricated as can be observed in **Figures 1a,b**. The capillary-driven chip can perfectly hold 20 μL of reaction mixture in each channel, and was carefully sealed by placing a second glass slide was placed on top, which was held with few clamps. This simple setup avoided evaporation of reagents as well as bubble formation in the channels, when performing the DNA amplification. The enzymatic reaction was not affected by the setup and run as expected.

AuNP Synthesis and Characterization

The correct synthesis of AuNPs was confirmed by UV-vis, showing a peak at ≈520 nm, which was still present after MUA functionalization, as depicted in **Figure 2**. In addition to this, the AuNPs were visualized by TEM, confirming spherical morphology and an average size of 13.3 ± 1.2 nm (see **Figures 3a,b**).

Salmonella spp. Detection

Successful DNA amplification was obtained in the microfluidic chip, as demonstrated by the typical LAMP banding pattern after gel electrophoresis (**Figure 4a**). Clear color differences

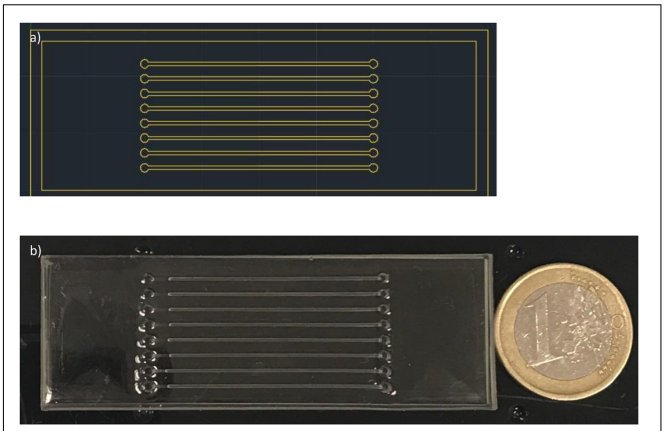


FIGURE 1 | Microfluidic device design and fabrication. **(a)** Schematic microfluidic chip designed with AutoCad. **(b)** Final PDMS microfluidic device (PDMS replica bonded against a glass slide).

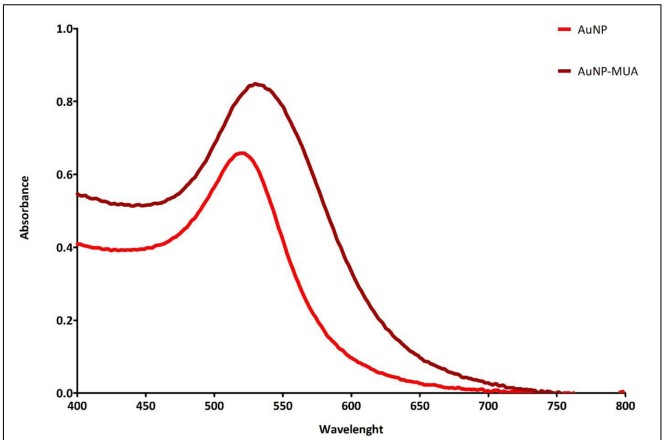


FIGURE 2 | UV-Vis of AuNPs before and after MUA functionalization.

[red (+) vs. purple (−)] can be observed after combination of functionalized AuNPs with the diluted LAMP amplification product, as shown in **Figure 4b**. These differences are also clearly visualized by UV-Vis, see **Figure 4c**.

Method Evaluation

The evaluation of the microfluidic-AuNP method demonstrated a LoD of 10 cfu/ 25 g. In addition to this, all performance parameters evaluated (SE, SP, AC, PPV, NPV, and *k*) obtained excellent results when compared to the expected ones (100%), further details are provided in **Table 2**.

DISCUSSION

The development and implementation of novel techniques, which allow *in situ*, fast and accurate detection of foodborne pathogens is highly desirable in the food industry. In the current study, the combination of microfluidic-LAMP DNA amplification with AuNP detection was assessed.

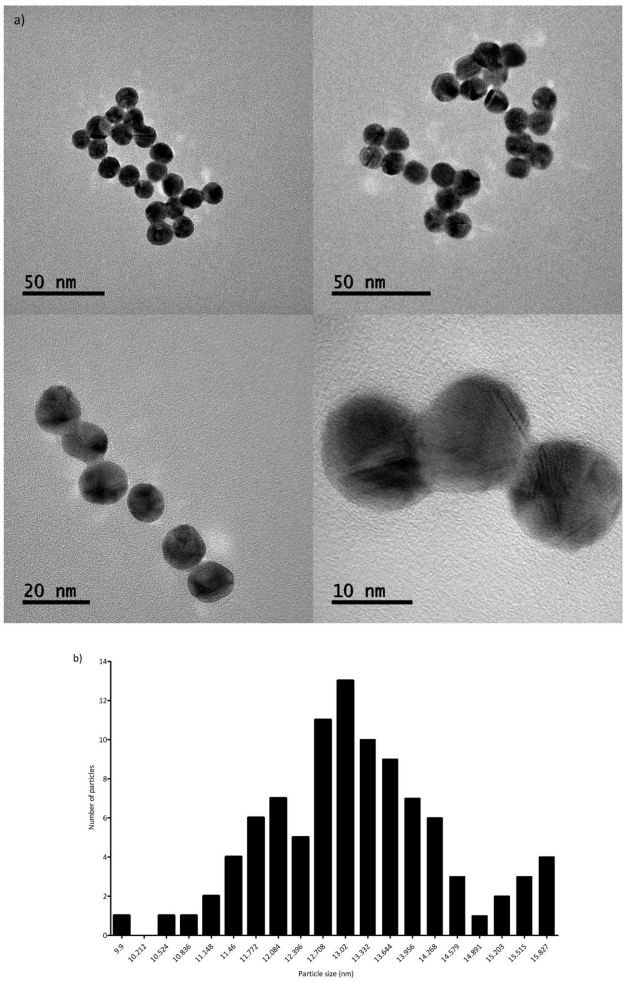


FIGURE 3 | (a) AuNP TEM images at different magnifications. **(b)** AuNPs size distribution, based on 96 measurements.

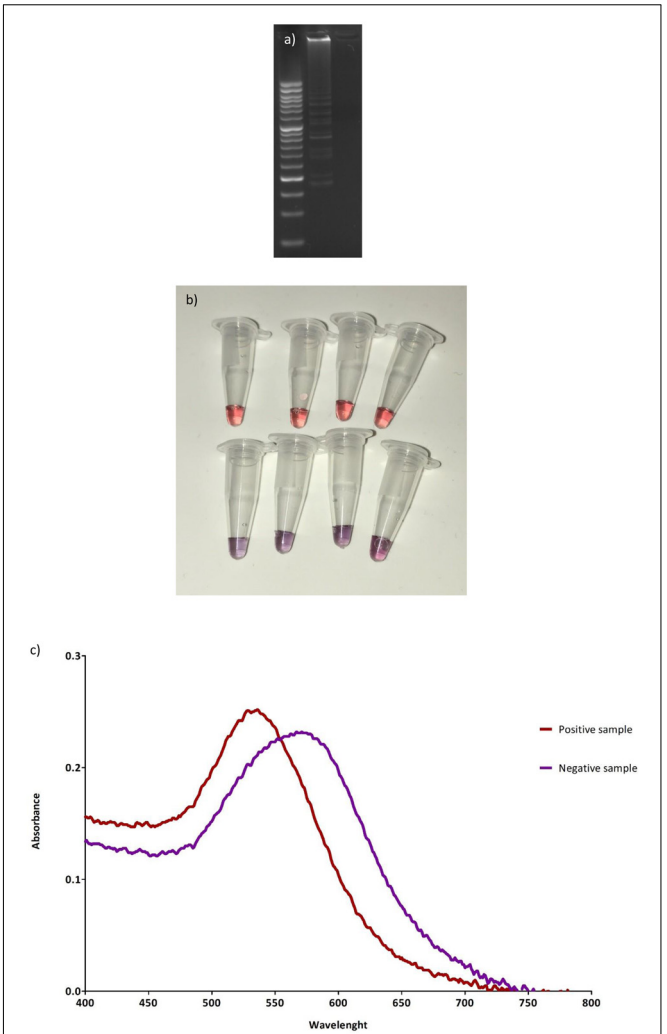


FIGURE 4 | Results of microfluidic-LAMP amplification and MUA-AuNPs detection. **(a)** Gel electrophoresis of a positive and negative sample after LAMP DNA amplification in the microfluidic chip and combination with AuNPs. **(b)** Positive (top) and negative (bottom) samples for *Salmonella* spp. after LAMP DNA amplification in the microfluidic chip, and the addition of AuNPs. **(c)** Typical UV-Vis spectra obtained for positive and negative samples after the addition of AuNPs.

The construction of a microfluidic device was successfully accomplished and demonstrated to be suitable for LAMP-based DNA amplification. This approach allowed an increased specificity in bacteria detection, as it was demonstrated in a set of parallel experiments (data not shown). In these experiments, negative samples that were reacted with AuNPs in regular PCR tubes (DNase, RNase and pyrogen free, Nippon Genetics Europe GmbH, Duren, Germany) were determined to be positive; while when the microfluidic chip was used resulted negative, as expected. This may be explained by enhanced performance of the positive reactions due to the advantages of microfluidics previously commented (better heat transfer, controlled mixing and no gravity that prevented NP aggregation, etc.).

Wong et al. (2014) reported for the first time the combination of MUA and AuNPs as a naked-eye approach to asses

TABLE 2 | Method evaluation summary.

Bacterium	Gen	N	PA	PD	NA	ND	SE	SP	AC	PPV	NPV	κ
S. Typhimurium CECT 4594	*invA*	38	24	0	14	0	100	100	100	100	100	1.00

N, total number of samples; PA, positive agreement; PD, positive deviation; NA, negative agreement; ND, negative deviation; SE, relative sensitivity; SP, relative specificity; AC, relative accuracy; PPV, positive predictive value; NPV, negative predictive value; k, kappa index of concordance, interpretation: 0.61–0.8 substantial agreement; 0.81–1.00 almost complete concordance according to Altman (1991) and Anderson et al. (2011).

LAMP DNA amplification. In Wong et al. (2014) study, the functionalized AuNPs were added before the LAMP reaction, what caused particle aggregation. After DNA amplification, in order to see color differences ultrasounds had to be applied to re-disperse the particles in positive samples. This simple treatment, may be problematic when thinking on *in situ* analyses. In the current study, the addition of the AuNPs after DNA amplification, allowed to clearly observe the color changes without applying ultrasounds.

Recently, many studies were published combining LAMP amplification with DNA-functionalized AuNPs (Jaroenram et al., 2012; Arunrut et al., 2013, 2016; Seetang-Nun et al., 2013; Kumvongpin et al., 2016). Even though this approach is expected to present higher specificity, due to the incorporation of the DNA probe, the use of MUA is more economic, the functionalization protocol is easier, and these particles can be incorporated to any LAMP assay, thus expanding their direct applicability (we have tested the sample protocol targeting *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes* with comparable results in terms of LoD, SE, SP, AC, PPV, NPV and *k*, by just modifying the primers selected, data not shown).

The application of this methodology to *Salmonella*-spiked food samples allowed the reliable detection of the pathogen, even at low concentration (10 cfu/25 g), what is compatible with the needs of the food industry. These results are similar to those obtained with other conventional LAMP methods (Ohtsuka et al., 2005; D'Agostino et al., 2016; Garrido-Maestu et al., 2017), or even with other DNA amplification techniques (González-Escalona et al., 2012; Maurischat et al., 2015; Zhang et al., 2015).

To the best of our knowledge this is the first study combining AuNPs, LAMP and microfluidics, which has undergone an extensive evaluation in food samples (LoD, SE, SP, AC, PPV, NPV, *k*), being critical for future implementation in the food industry. Previous studies have been just focused on the development of the methodology, but a thorough evaluation of its applicability in food products was missing (Fang et al., 2010a,b; Wong et al., 2014; Sayad et al., 2016; Park et al., 2017).

To summarize, in the current study we report the successful combination of microfluidic isothermal DNA amplification with AuNPs-based detection, allowing naked-eye discrimination, for the specific detection of *Salmonella* spp. In food samples. The results obtained indicate the suitability of the methodology for its implementation in the food industry. It is a simple, fast and inexpensive analytical approach for foodborne pathogen detection although an inter-laboratory validation is needed.

AUTHOR CONTRIBUTIONS

AG-M designed the experiments and wrote the manuscript. SA performed the DNA amplification experiments and AuNPs evaluation. LD and JC designed the microfluidic system and helped in the optimization of the DNA amplification within the system. SA-C, MP, and YK worked on the synthesis and functionalization of the AuNPs. EC-A and SA-C performed the characterization of the AuNPs by TEM. MP collaborated in the design of the experiments and evaluation of the results. All the co-authors collaborated in the proofreading of the final manuscript.

FUNDING

This work was supported by a Marie Curie COFUND Action (Project No: 600375. NanoTRAINforGrowth – INL Fellowship programme in nanotechnologies for biomedical, environment and food applications), by the EU Framework Programme for Research and Innovation H2020 COFUND, Grant Agreement 713640, by the project Nanotechnology Based Functional Solutions (NORTE-01-0145-FEDER-000019), supported by Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

ACKNOWLEDGMENT

Authors would like to thank Dr. Antonio Lozano-León, director of the Institute of Applied Microbiology-ASMECRUZ, for kindly providing the bacterial strains used in the current study, and the Microbiology and Bioassays Laboratory of ANFACO-CECOPESCA for technical assistance.

REFERENCES

Altman, D. G. (1991). *Practical Statistics for Medical Research*. New York, NY: Chapman and Hall.

Anderson, A., Pietsch, K., Zucker, R., Mayr, A., Muller-Hohe, E., Messelhausser, U., et al. (2011). Validation of a duplex real-time PCR for the detection of *Salmonella* spp. in different food products. *Food Anal. Methods* 4, 259–267. doi: 10.1016/j.ijfoodmicro.2013.05.012

Arunrut, N., Kampeera, J., Sirithammajak, S., Sanguanrut, P., Proespraiwong, P., Suebsing, R., et al. (2016). Sensitive visual detection of AHPND bacteria using loop-mediated isothermal amplification combined with DNA-functionalized gold nanoparticles as probes. *PLOS ONE* 11:e0151769. doi: 10.1371/journal.pone.0151769

Arunrut, N., Kampeera, J., Suebsing, R., and Kiatpathomchai, W. (2013). Rapid and sensitive detection of shrimp infectious myonecrosis virus using a reverse transcription loop-mediated isothermal amplification and visual colorogenic nanogold hybridization probe assay. *J. Virol. Methods* 193, 542–547. doi: 10.1016/j.jviromet.2013.07.017

Birmpa, A., Kalogeropoulos, K., Kokkinos, P., and Vantarakis, A. (2015). Evaluation of two loop-mediated isothermal amplification methods for the detection of *Salmonella* enteritidis and *Listeria monocytogenes* in artificially contaminated ready-to-eat fresh products. *Ital. J. Food Saf.* 4, doi: 10.4081/ijfs.2015.5383

Brody, J. R., and Kern, S. E. (2004). Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36, 214–216.

Carroll, L. M., Wiedmann, M., den Bakker, H., Siler, J., Warchocki, S., Kent, D., et al. (2017). Whole-genome sequencing of drug-resistant *Salmonella enterica* isolated from dairy cattle and humans in New York and Washington states reveals source and geographic associations. *Appl. Environ. Microbiol.* 83:e140-17. doi: 10.1128/AEM.00140-17

Ceuppens, S., Li, D., Uyttendaele, M., Renault, P., Ross, P., Van Ranst, M., et al. (2014). Molecular methods in food safety microbiology: Interpretation and

implications of nucleic acid detection. *Compr. Rev. Food Sci. Food Saf.* 13, 551–577. doi: 10.1111/1541-4337.12072

Chapela, M., Garrido-Maestu, A., and Cabado, A. G. (2015). Detection of foodborne pathogens by qPCR: a practical approach for food industry applications. *Cogent Food Agric.* 1, 1–19. doi: 10.1080/23311932.2015.1013771

Chiu, D. T., Di Carlo, D., Doyle, P. S., Hansen, C., Maceiczyk, R. M., and Wootton, R. C. R. (2017). Small but perfectly formed? Successes, challenges, and opportunities for microfluidics in the chemical and biological sciences. *Chem* 2, 201–223. doi: 10.1016/j.chempr.2017.01.009

D'Agostino, M., Robles, S., Hansen, F., Ntafis, V., Ikonomopoulos, J., Kokkinos, P., et al. (2016). Validation of a loop-mediated amplification/ISO 6579-based method for analysing soya meal for the presence of *Salmonella enterica*. *Food Anal. Methods* 9, 2979–2985. doi: 10.1007/s12161-016-0602-7

Efsa, and Ecdc. (2017). The Euroean Union summary report on trends and sources of zoonsoes, zoonotic agents and food-borne outbreaks in 2015. *EFSA J.* 13, 4329.

Fang, X., Chen, H., Yu, S., Jiang, X., and Kong, J. (2010a). Predicting viruses accurately by a multiplex microfluidic loop-mediated isothermal amplification chip. *Anal. Chem.* 83, 690–695. doi: 10.1021/ac102858j

Fang, X., Liu, Y., Kong, J., and Jiang, X. (2010b). Loop-mediated isothermal amplification integrated on microfluidic chips for point-of-care quantitative detection of pathogens. *Anal. Chem.* 82, 3002–3006. doi: 10.1021/ac1000652

Foudeh, A. M., Didar, T. F., Veres, T., and Tabrizian, M. (2012). Microfluidic designs and techniques using lab-on-a-chip devices for pathogen detection for point-of-care diagnostics. *Lab Chip* 12, 3249–3266. doi: 10.1039/c2lc40630f

Garrido, A., Chapela, M. J., Román, B., Fajardo, P., Lago, J., Vieites, J. M., et al. (2013). A new multiplex real-time PCR developed method for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Control* 30, 76–85. doi: 10.1016/j.foodcont.2012.06.029

Garrido-Maestu, A., Chapela, M. J., Peñaranda, E., Vieites, J. M., and Cabado, A. G. (2014). In-house validation of novel multiplex real-time PCR gene combination for the simultaneous detection of the main human pathogenic vibrios (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*). *Food Control* 37, 371–379. doi: 10.1016/j.foodcont.2013.09.026

Garrido-Maestu, A., Fuciños, P., Azinheiro, S., Carvalho, J., and Prado, M. (2017). Systematic loop-mediated isothermal amplification assays for rapid detection and characterization of *Salmonella* spp., Enteritidis and Typhimurium in food samples. *Food Control* 80, 297–306. doi: 10.1016/j.foodcont.2017.05.011

González-Escalona, N., Brown, E. W., and Zhang, G. (2012). Development and evaluation of a multiplex real-time PCR (qPCR) assay targeting ttrRSBCA locus and *invA* gene for accurate detection of *Salmonella* spp. in fresh produce and eggs. *Food Res. Int.* 48, 202–20. doi: 10.1016/j.foodres.2012.03.009

Goto, M., Honda, E., Ogura, A., Nomoto, A., and Hanaki, K. I. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46, 167–172. doi: 10.2144/000113072

Ha, M. L., and Lee, N. Y. (2015). Miniaturized polymerase chain reaction device for rapid identification of genetically modified organisms. *Food Control* 57, 238–245. doi: 10.1016/j.foodcont.2015.04.014

Hara-Kudo, Y., Yoshino, M., Kojima, T., and Ikedo, M. (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS Microbiol. Lett.* 253, 155–161. doi: 10.1016/j.femsle.2005.09.032

Jaroenram, W., Arunrut, N., and Kiatpathomchai, W. (2012). Rapid and sensitive detection of shrimp yellow head virus using loop-mediated isothermal amplification and a colorogenic nanogold hybridization probe. *J. Virol. Methods* 186, 36–42. doi: 10.1016/j.jviromet.2012.08.013

Jia, G., Siegrist, J., Deng, C., Zoval, J. V., Stewart, G., Peytavi, R., et al. (2007). A low-cost, disposable card for rapid polymerase chain reaction. *Colloids Surf. B Biointerfaces* 58, 52–60. doi: 10.1016/j.colsurfb.2007.03.007

Kaprou, G. D., Papadakis, G., Papageorgiou, D. P., Kokkoris, G., Papadopoulos, V., Kefala, I., et al. (2015). Miniaturized devices for isothermal DNA amplification addressing DNA diagnostics. *Microsyst. Technol.* 22, 1–6. doi: 10.1007/s00542-015-2750-x

Kumvongpin, R., Jearanaikool, P., Wilailuckana, C., Sae-ung, N., Prasongdee, P., Daduang, S., et al. (2016). High sensitivity, loop-mediated isothermal amplification combined with colorimetric gold-nanoparticle probes for visual detection of high risk human papillomavirus genotypes 16 and 18. *J. Virol. Methods* 234, 90–95. doi: 10.1016/j.jviromet.2016.04.008

Maurischat, S., Baumann, B., Martin, A., and Malorny, B. (2015). Rapid detection and specific differentiation of *Salmonella enterica* subsp. enterica Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i:- by real-time multiplex PCR. *Int. J. Food Microbiol.* 193, 8–14. doi: 10.1016/j.ijfoodmicro.2014.10.004

Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.* 289, 150–154. doi: 10.1006/bbrc.2001.5921

Mori, Y., and Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J. Infect. Chemother.* 15, 62–69. doi: 10.1007/s10156-009-0669-9

Ohtsuka, K., Ohtsuka, K., Yanagawa, K., Yanagawa, K., Takatori, K., Takatori, K., et al. (2005). Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. *Appl. Environ. Microbiol.* 71, 6730–6735. doi: 10.1128/AEM.71.11.6730

Park, B. H., Oh, S. J., Jung, J. H., Choi, G., Seo, J. H., Kim, D. H., et al. (2017). An integrated rotary microfluidic system with DNA extraction, loop-mediated isothermal amplification, and lateral flow strip based detection for point-of-care pathogen diagnostics. *Biosens. Bioelectron.* 91, 334–340. doi: 10.1016/j.bios.2016.11.063

Prado, M., Espiña, B., Fernandez-Argüelles, M. T., Diéguez, L., Fuciños, P., Vial, S., et al. (2016a). Detection of foodborne pathogens using nanoparticles. Advantages and Trends. *Antimicrob. Food Packag.* 183–201. doi: 10.1016/B978-0-12-800723-5.00014-0

Prado, M., Ortea, I., Vial, S., Rivas, J., Calo-Mata, P., and Barros-Velázquez, J. (2016b). Advanced DNA-and protein-based methods for the detection and investigation of food allergens. *Crit. Rev. Food Sci. Nutr.* 56, 2511–2542.

Rafati, A., and Gill, P. (2015). Microfluidic method for rapid turbidimetric detection of the DNA of *Mycobacterium tuberculosis* using loop-mediated isothermal amplification in capillary tubes. *Microchim. Acta* 182, 523–530. doi: 10.1007/s00604-014-1354-y

Sayad, A. A., Ibrahim, F., Uddin, S. M., Pei, K. X., Mohktar, M. S., Madou, M., et al. (2016). A microfluidic lab-on-a-disc integrated loop mediated isothermal amplification for foodborne pathogen detection. *Sens. Actuators B Chem.* 227, 600–609. doi: 10.1016/j.snb.2015.10.116

Seetang-Nun, Y., Jaroenram, W., Sriurairatana, S., Suebsing, R., and Kiatpathomchai, W. (2013). Visual detection of white spot syndrome virus using DNA-functionalized gold nanoparticles as probes combined with loop-mediated isothermal amplification. *Mol. Cell. Probes* 27, 71–79. doi: 10.1016/j.mcp.2012.11.005

Sun, J., Xianyu, Y., and Jiang, X. (2014). Point-of-care biochemical assays using gold nanoparticle-implemented microfluidics. *Chem. Soc. Rev.* 43, 6239. doi: 10.1039/C4CS00125G

Tanner, N. A., Zhang, Y., and Evans, T. C. (2015). Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques* 58, 59–68. doi: 10.2144/000114253

Tomas, D., Rodrigo, A., Hernandez, M., and Ferrus, M. A. (2009). Validation of real-time PCR and enzyme-linked fluorescent assay-based methods for detection of *Salmonella* spp. in chicken feces samples. *Food Anal. Methods* 2, 180–189. doi: 10.1007/s12161-009-9082-3

Turkevich, J., Stevenson, P. C., and Hillier, J. (1951). A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discuss. Faraday Soc.* 11, 55–75. doi: 10.1039/df9511100055

Valderrama, W. B., Dudley, E. G., Doores, S., and Cutter, C. N. (2016). Commercially available rapid methods for detection of selected food-borne pathogens. *Crit. Rev. Food Sci. Nutr.* 56, 1519–1531. doi: 10.1080/10408398.2013.775567

Wan, C., Yang, Y., Xu, H., Aguilar, Z. P., Liu, C., Lai, W., et al. (2012). Development of a propidium monoazide treatment combined with loop-mediated isothermal amplification (PMA-LAMP) assay for rapid detection of viable *Listeria monocytogenes*. *Int. J. Food Sci. Technol.* 47, 2460–2467. doi: 10.1111/j.1365-2621.2012.03123.x

Wang, J.-H., Chien, L.-J., Hsieh, T.-M., Luo, C.-H., Chou, W.-P., Chen, P.-H., et al. (2009). A miniaturized quantitative polymerase chain reaction system for DNA amplification and detection. *Sens. Actuators B Chem.* 141, 329–337. doi: 10.1016/j.snb.2009.06.034

Wang, L., Li, Y., Chu, J., Xu, Z., and Zhong, Q. (2012). Development and application of a simple loop-mediated isothermal amplification method on rapid detection of *Listeria monocytogenes* strains. *Mol. Biol. Rep*. 39, 445–449. doi: 10.1007/s11033-011-0757-7

Wittwer, C. T., Fillmore, G. C., and Garling, D. J. (1990). Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal. Biochem*. 186, 328–331. doi: 10.1016/0003-2697(90)90090-V

Wong, J. K. F., Yip, S. P., and Lee, T. M. H. (2014). Ultrasensitive and closed-tube colorimetric loop-mediated isothermal amplification assay using carboxyl-modified gold nanoparticles. *Small* 10, 1495–1499. doi: 10.1002/smll.201302348

Zhang, X., Du, X. J., Guan, C., Li, P., Zheng, W. J., and Wang, S. (2015). Detection of *Vibrio cholerae* by isothermal cross-priming amplification combined with nucleic acid detection strip analysis. *Mol. Cell. Probes* 29, 208–214. doi: 10.1016/j.mcp.2015.05.001

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Garrido-Maestu, Azinheiro, Carvalho, Abalde-Cela, Carbó-Argibay, Diéguez, Piotrowski, Kolen'ko and Prado. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 November 2017
doi: 10.3389/fmicb.2017.02120

Risk of Transmission of Antimicrobial Resistant *Escherichia coli* from Commercial Broiler and Free-Range Retail Chicken in India

*Arif Hussain[1], Sabiha Shaik[2], Amit Ranjan[2], Nishant Nandanwar[2], Sumeet K. Tiwari[2], Mohammad Majid[2], Ramani Baddam[2,3], Insaf A. Qureshi[4], Torsten Semmler[5], Lothar H. Wieler[5], Mohammad A. Islam[3], Dipshikha Chakravortty[1] and Niyaz Ahmed[2,3]**

[1] Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, India, [2] Pathogen Biology Laboratory, Department of Biotechnology and Bioinformatics, University of Hyderabad, Hyderabad, India, [3] International Centre for Diarrhoeal Disease Research Bangladesh (icddr,b), Dhaka, Bangladesh, [4] Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, India, [5] Robert Koch Institute, Berlin, Germany

Multidrug-resistant *Escherichia coli* infections are a growing public health concern. This study analyzed the possibility of contamination of commercial poultry meat (broiler and free-range) with pathogenic and or multi-resistant *E. coli* in retail chain poultry meat markets in India. We analyzed 168 *E. coli* isolates from broiler and free-range retail poultry (meat/ceca) sampled over a wide geographical area, for their antimicrobial sensitivity, phylogenetic groupings, virulence determinants, extended-spectrum-β-lactamase (ESBL) genotypes, fingerprinting by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR and genetic relatedness to human pathogenic *E. coli* using whole genome sequencing (WGS). The prevalence rates of ESBL producing *E. coli* among broiler chicken were: meat 46%; ceca 40%. Whereas, those for free range chicken were: meat 15%; ceca 30%. *E. coli* from broiler and free-range chicken exhibited varied prevalence rates for multi-drug resistance (meat 68%; ceca 64% and meat 8%; ceca 26%, respectively) and extraintestinal pathogenic *E. coli* (ExPEC) contamination (5 and 0%, respectively). WGS analysis confirmed two globally emergent human pathogenic lineages of *E. coli*, namely the ST131 (*H*30-Rx subclone) and ST117 among our poultry *E. coli* isolates. These results suggest that commercial poultry meat is not only an indirect public health risk by being a possible carrier of non-pathogenic multi-drug resistant (MDR)-*E. coli*, but could as well be the carrier of human *E. coli* pathotypes. Further, the free-range chicken appears to carry low risk of contamination with antimicrobial resistant and extraintestinal pathogenic *E. coli* (ExPEC). Overall, these observations reinforce the understanding that poultry meat in the retail chain could possibly be contaminated by MDR and/or pathogenic *E. coli.*

Keywords: food borne pathogens, poultry, antibiotic resistance, zoonosis, whole genome sequencing

OPEN ACCESS

Edited by:
Rosanna Tofalo,
Università di Teramo, Italy

Reviewed by:
Zhao Chen,
University of California, Davis, United States
Shu-Wei Marcia Su,
Jackson Laboratory, United States

****Correspondence:***
Niyaz Ahmed
ahmed.nizi@gmail.com;
niyaz.ahmed@icddrb.org

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 July 2017*
Accepted: *18 October 2017*
Published: *13 November 2017*

Citation:
Hussain A, Shaik S, Ranjan A, Nandanwar N, Tiwari SK, Majid M, Baddam R, Qureshi IA, Semmler T, Wieler LH, Islam MA, Chakravortty D and Ahmed N (2017) Risk of Transmission of Antimicrobial Resistant Escherichia coli from Commercial Broiler and Free-Range Retail Chicken in India. Front. Microbiol. 8:2120. doi: 10.3389/fmicb.2017.02120

INTRODUCTION

The rapid global rise of *Escherichia coli* infections that are resistant to therapeutically important antimicrobials, including first-line drugs such as cephalosporins and fluoroquinolones, is of serious concern, as it hampers treatment of infections leading to significant morbidity, mortality, medical costs as well as production losses in livestock (de Been et al., 2014). *E. coli* is responsible for

infections in humans and animals; these could be nosocomial and/or community-acquired (Jadhav et al., 2011). Being part of the endogenous microbiota, *E. coli* can easily acquire resistance against antimicrobials consumed by humans and animals (van den Bogaard et al., 2001).

Poultry are recognized as important source for dissemination of antimicrobial resistant *E. coli* in the community and environment (van den Bogaard et al., 2001). Pathogenic *E. coli* in poultry are a direct threat to both poultry industry and human health as they may result in hard-to-treat infections (Kaper et al., 2004). Extraintestinal pathogenic *E. coli* (ExPEC), the causative agent of colibacillosis in chickens inflict severe losses due to morbidity and condemnations (Kaper et al., 2004). ExPEC can also cause several extraintestinal diseases in humans, including urinary tract infections, neonatal meningitis, and sepsis (Avasthi et al., 2011; Hussain et al., 2012; Nandanwar et al., 2016; Ranjan et al., 2017). Apart from ExPEC, the poultry gut could also harbor other variants of intestinal pathogenic *E. coli* (Lutful Kabir, 2010). Recent studies in different parts of India have reported antimicrobial residues in food animal products such as milk and chicken meat, indicating that antimicrobial usage is widespread in food animal production (Laxminarayan and Chaudhury, 2016). Such practices lead to high proportion of antibiotic resistant bacteria in their fecal microbiota (Chen and Jiang, 2014). Consequently, meat at slaughtering operations can be extensively contaminated with fecal *E. coli* of poultry origin (Lutful Kabir, 2010).

The ExPEC pathotype that is isolated from poultry with clinical signs of extraintestinal infections is known as avian pathogenic *E. coli* (APEC) (Kaper et al., 2004). Although research has been mainly focused on infections caused by APEC pathotype, little is known about the reservoirs of these bacteria (Bélanger et al., 2011). Also, many human and animal ExPEC isolates share virulence genes and clonal backgrounds and the human health risk posed by such bacteria from poultry is still largely undefined (Dziva et al., 2013). Moreover, the pathotype APEC itself is ill-defined (Collingwood et al., 2014). One study even suggested that an APEC strain showed high genome similarity to human enterotoxigenic *E. coli* (ETEC) (Dziva et al., 2013). Therefore, the question of whether fecal isolates of poultry serve as a source of infection for extraintestinal and intestinal infections in humans remains unanswered. Recent investigations compared *E. coli* from symptomatic poultry and human ExPEC by virulence genotyping, serotyping and *in vitro* assays (Nandanwar et al., 2014; Mitchell et al., 2015). However, the application of high-resolution, whole genome sequencing methods appears to be superior and is likely to provide accurate insights into the phylogenetic backgrounds of poultry *E. coli* and would likely highlight the similarities between isolates of different human pathotypes (de Been et al., 2014).

The presence of antimicrobial resistant bacteria in food has been attributed to the widespread use of antimicrobials in farming practices (Landers et al., 2012; Qumar et al., 2017). Currently, few data are available regarding the contamination of retail foods with *E. coli*, especially those that are multi-resistant and pathogenic (Zhang et al., 2011). Also, little is known about the frequency of antibiotic-resistant microorganisms in poultry that were raised by free-range farming as methods of livestock production differ in antibiotic usage practices (Brower et al., 2017). Therefore, the main aim of this study was to estimate the frequencies of contamination with pathogenic and/or multi resistant *E. coli* among broiler and free-range chicken specimens (ceca and meat) and to characterize the *E. coli* isolates recovered from them in relation to the human *E. coli* pathotypes. Results of this study reinforces the importance of One Health approach in addressing the spread of antimicrobial resistance and emerging infections.

MATERIALS AND METHODS

Specimen Collection

Between February, 2015 to September, 2015, 22 poultry retail outlets were sampled from four cities representatives of four different states of India: Karnataka ($n = 35$), Telangana ($n = 59$), Andhra Pradesh ($n = 15$) and Maharashtra ($n = 11$); this resulted in a total of 120 samples. A total of 75 poultry ceca, entailing broiler and free-range chicken (39 and 36, respectively) together with 45 raw meat samples, representing broiler and free-range chicken (32 and 13, respectively) were obtained from retail poultry outlets. From each shop, multiple samples (different birds) were procured. Samples were transported to laboratory in cooled boxes (4–8°C) and upon arrival were stored at 4°C and processed within 24 h.

Broiler represented commercial broiler chickens that were conventionally raised in farms and fed with commercial feeds; free-range poultry birds were country (native) chickens that were raised in households and small backyard farms that grew by free-ranging.

Culture Methods

All cecal samples were surface sterilized with 70% ethanol and a portion (∼25 g) was incised and placed in a 1.5 ml micro-centrifuge tube containing Luria Bartani (LB) broth. Similarly, around 25 g of raw meat was excised from each sample and rinsed with 1X PBS and the sample was placed in a Petri dish containing 1 ml LB broth and minced into small pieces. Each cecum and chicken sample was taken out of the LB broth and swabbed on the edge of the plate and then spread with a loop, this was done on two separate agar plates; one on unsupplemented Eosin Methylene Blue (EMB) agar plate, and another on EMB agar supplemented with 10 μg/ml ciprofloxacin together with 4 μg/ml cefotaxime (this was used to increase the chance of ST131 *E. coli* isolation). All plates were incubated at 37°C for 12 h. One putative *E. coli* was selected from each culture plate, which was then confirmed by standard biochemical methods. All *E. coli* isolates were preserved in 20% glycerol-supplemented Luria-Bertani broth at −80°C.

Antimicrobial Susceptibility, ESBL Confirmation, and ESBL Gene Detection

Antimicrobial susceptibility toward fosfomycin, gentamicin, ciprofloxacin, co-trimoxazole, tetracycline, and chloramphenicol

belonging to six different antibiotic classes was assessed by standard disk diffusion method as per CLSI guidelines (Schissler et al., 2009; Hussain et al., 2014). ESBL production was determined using double-disk synergy test following CLSI guidelines (CLSI, 2013). Phenotypically confirmed ESBL-producers were analyzed for the presence of genes encoding CTX-M-15 and that of bla_{SHV}, and bla_{TEM} genes by three gene specific PCRs (Ranjan et al., 2015).

As the EMB agar plates (i.e., with or without antibiotic supplement) were streaked using an identical inoculum, the percentage of resistance/susceptibility to different antibiotics was determined in relation to the total *E. coli* population.

Phylogenetic Groups, Virulence Genotyping, ST131 Detection, and Enterobacterial Intergenic Repetitive Element Sequences (ERIC)-PCR

Phylogenetic groups of *E. coli* isolates were determined by quadruplex PCR and by employing the criteria as described by Clermont et al. (2013). All isolates were screened for the presence of the following five ExPEC genes (Hussain et al., 2014); *papC* (P fimbriae), *sfa* (s fimbriae), *afa* (afimbrial adhesin), *aer* (siderophore related protein), and *cvaC* (protectin). The criteria elaborated by Johnson et al. (2003) were used to classify *E. coli* isolates as ExPEC with some modifications. All *E. coli* isolates were screened for ST131 gene specific PCR as described elsewhere (Hussain et al., 2014). ERIC-PCR based fingerprint analysis was performed as described in our earlier study (Hussain et al., 2014). The ERIC-PCR bands obtained were analyzed using BioNumerics software (Applied Maths, Belgium) (Hussain et al., 2014).

Whole Genome Sequencing (WGS) and Comparison with Human *E. coli* Pathotypes

Paired end sequencing of the 10 genetically distinct (based on ERIC bands), and randomly selected MDR-ESBL producing poultry *E. coli* (seven cecum and three meat) isolates was carried out using Illumina MiSeq. The accession numbers of 10 poultry *E. coli* genomes including their genomic features are represented in Supplementary Tables S1 and S4. Paired end read data were filtered for high quality reads followed by *de novo* assembly using NGS QC Toolkit (v2.3.3) (Patel and Jain, 2012) and SPAdes Genome Assembler (v3.6.1) (Bankevich et al., 2012), respectively. The *de novo* contigs thus generated were ordered and scaffolded using Contig-Layout-Authenticator (Shaik et al., 2016). Final draft genomes were obtained by merging the scaffolds using a series of N's and then submitted to the RAST (Overbeek et al., 2014) server for annotation. The genome statistics were gleaned using ARTEMIS (Rutherford et al., 2000). The sequence type (ST) of each of these strains were determined by *in silico* MLST as used/described elsewhere (Ranjan et al., 2015). Mobile genetic elements as reported in the ExPEC strain EC958 (Totsika et al., 2011) were used as reference to compare the 6 *E. coli* genomes using BRIG (Alikhan et al., 2011). A core-genome-based phylogenetic tree of 50 *E. coli* strains (10 in house poultry *E. coli* genomes, 10 healthy broiler chicken *E. coli* genomes from public sources, 10 APEC, 10 ExPEC and 10 enteric pathogens) (Supplementary Tables S1 and S3) was constructed using Harvest (Treangen et al., 2014) and the resulting tree was visualized using iTol[1]. Virulence and Resistance profiles of these 50 strains were generated and hierarchical clustering was performed using the gplot package of R as described/used by us before (Ranjan et al., 2016).

Statistical Analysis

Statistical analysis for prevalence of antimicrobial resistance, phylogenetic groups, ESBL and virulence genes were carried out using Fisher's exact two-tailed test. Statistical analyses for aggregate resistance and virulence scores were carried out using the non-parametric Mann–Whitney *U*-test. Both the tests were implemented in the Statistical Package for the Social Sciences (SPSS, version 10.0). *P*-values ≤ 0.05 were considered as statistically significant.

RESULTS

Over Half of the Chicken Meat Samples Were Contaminated with *E. coli*

A total of 168 *E. coli* isolates were recovered from 120 poultry samples using both unsupplemented and antibiotic supplemented (ciprofloxacin-plus-cefotaxime) EMB agar, encompassing 105 and 63 isolates, respectively (Supplementary Table S2). Out of the 32 and 13 raw meat samples from broiler and free-range chicken, 29 (91%) and 11 (84%) were contaminated with *E. coli,* respectively. Further, compared to broiler chicken meat (78%), free-range chicken meat demonstrated lower contamination (15%) by *E. coli* when screened on dual antibiotic supplemented plates (ciprofloxacin-plus-cefotaxime) (Supplementary Table S2).

Resistance to Empirically Used Antibiotics Was Rampant in Poultry *E. coli*

Across the entire dataset of poultry *E. coli* isolates, resistance to tetracycline was most prevalent (84%) followed by ciprofloxacin (70%), co-trimoxazole (45%), and gentamicin (32%) whereas a small fraction of total *E. coli* were found to be resistant to chloramphenicol (8%) and fosfomycin (4%) (**Table 1**). Within each category of chicken samples (broiler and free-range), the isolation sources (ceca and raw meat) did not vary with respect to antimicrobial resistance profiles ($P > 0.05$ for all variables between groups 1 and 3; and groups 2 and 4) (**Table 1**). However, the two categories of chicken *E. coli* isolates exhibited significant difference in prevalence of resistance (MDR and aggregate resistance scores). Strains of *E. coli* samples in the free-range category tended to be resistant to fewer antimicrobial agents (**Table 1**). Overall, the rates of ESBL producers in broiler and free-range chicken were as follows: meat 46%; ceca 40% and meat

[1]http://itol.embl.de/

TABLE 1 | Antimicrobial susceptibility profiles and aggregate resistance scores for 168 *Escherichia coli* isolates together with molecular characteristics of 63 extended-spectrum-β-lactamase (ESBL)-producing *E. coli*.

Specific trait		**No. (%) of isolates resistant**				**[a]*P*-value***	
	Total (*n* = 168)	**Broiler chicken meat isolates (group 1; *n* = 54)**	**Free-range chicken meat isolates (group 2; *n* = 13)**	**Broiler chicken ceca isolates (group 3; *n* = 55)**	**Free-range chicken ceca isolates (group 4; *n* = 46)**	**Group 1 vs. 2**	**Group 3 vs. 4**
ABST[e]							
Tetracycline	141 (84)	50 (93)	12 (92)	54 (98)	25 (54)	ns	0.000
Ciprofloxacin	118 (70)	52 (96)	2 (15)	40 (73)	24 (52)	0.000	0.033
Co-trimoxazole	76 (45)	33 (61)	1 (8)	26 (47)	16 (35)	0.001	ns
Gentamicin	54 (32)	23 (43)	1 (8)	21 (38)	9 (20)	0.018	0.041
Chloramphenicol	13 (8)	5 (9)	0	8 (14)	0	ns	0.007
Fosfomycin	6 (4)	3 (6)	0	3 (5)	0	ns	ns
ESBL phenotype[c]	63 (37)	25 (46)	2 (15)	22 (40)	14 (30)	0.013	ns
MDR Phenotype[d]	85 (51)	37 (68)	1 (8)	35 (64)	12 (26)	0.000	0.000
ESBL positives/genes	***n* = 63**	***n* = 25**	***n* = 2**	***n* = 22**	***n* = 14**	**[a]*P*-value***	
$bla_{CTX-M-15}$	50 (79)	21 (84)	2 (100)	17 (77)	10 (71)	ns	ns
bla_{TEM}	40 (63)	17 (68)	2 (100)	11 (50)	10 (71)	ns	ns
bla_{SHV}	20 (32)	8 (32)	1 (50)	6 (27)	5 (36)	ns	ns
Aggregate antimicrobial resistance scores		**Score median (range)**				**[b]*P*-value***	
	3 (0–6)	3 (0–5)	1 (0–4)	3 (0–6)	2 (0–4)	0.000	0.000

**All comparisons between the groups 1 and 3, and groups 2 and 4 were non-significant (ns) ($P > 0.05$).*

[a]*P-values by Fisher's exact test, two-tailed.*

[b]*P-values (Mann–Whitney U-test) are shown between aggregate resistance scores (sum of resistance genes detected).*

[c]*Extended-spectrum-β-lactamase.*

[d]*Multidrug-resistant.*

[e]*Antibiotic susceptibility tests.*

TABLE 2 | Overall prevalence of phylogenetic group and virulence gene distribution in 168 *Escherichia coli* isolates recovered from both broiler and free-range chicken with aggregate virulence scores.

Specific trait		No. (%) of isolates with trait				[a]*P*-value			
	Total ($n = 168$)	Broiler chicken meat isolates (group1; $n = 54$)	Free-range chicken meat isolates (group2; $n = 13$)	Broiler chicken ceca isolates (group 3; $n = 55$)	Free-range chicken ceca isolates (group 4; $n = 46$)	Group 1 vs. 2	Group 3 vs. 4	Group 1 vs. 3	Group 2 vs. 4
Phylogenetic group									
A	61 (36)	19 (35)	7 (54)	10 (18)	25 (54)	ns	0.000	0.045	ns
B1	60 (36)	22 (41)	1 (8)	22 (40)	15 (33)	0.024	ns	ns	ns
B2	3 (2)	1 (2)	0	0	2 (4)	ns	ns	ns	ns
C	13 (8)	3 (6)	0	6 (11)	4 (9)	ns	ns	ns	ns
D	15 (9)	5 (9)	2 (15)	8 (15)	0	ns	0.007	ns	0.007
E	3 (2)	1 (2)	1 (8)	1 (2)	0	ns	ns	ns	ns
F	11 (7)	3 (6)	2 (15)	6 (11)	0	ns	0.021	ns	0.007
Virulence genes									
aer	51 (30)	26 (48)	0	25 (45)	0	0.001	0.000	ns	–
afa	17 (10)	8 (15)	1 (8)	6 (11)	2 (4)	ns	ns	ns	ns
papC	6 (4)	4 (7)	0	2 (4)	0	ns	ns	ns	–
cvaC	29 (17)	6 (11)	5 (38)	13 (24)	5 (11)	0.017	ns	ns	0.019
sfa		0	0	0	0				
ExPEC[c]	5 (3)	4 (7)	0	1 (2)	0	ns	ns	ns	–
Aggregate virulence scores		**Score median (range)**				[b]*P*-value			
		1 (0–3)	0 (0–2)	1 (0–3)	0 (0–2)	ns	0.000	ns	0.010

[a]*P-values by Fischer's exact test, two-tailed.*
[b]*P-values (Mann–Whitney U-test) are shown between aggregate virulence scores (sum of virulence markers detected).*
[c]*ExPEC status is defined if ≥ 3 of the virulence genes are detected in an organism.*
ns, non-significant (P-values > 0.05).

15%; ceca 30%, respectively (**Table 1**). The prevalence of ESBL genes differed insignificantly among the four ESBL positive *E. coli* groups (**Table 1**).

Broiler Chicken Was a Potential Source of Pathogenic *E. coli* Variants Than the Free-Range Chicken

The distribution of poultry *E. coli* isolates with respect to the seven phylogenetic groups is shown in **Table 2**. Overall, the majority of poultry *E. coli* isolates were affiliated to group A and B1 (36% each), followed by group D (9%), C (8%), F (7%), E and B2 (2%, each). The prevalence of virulence genes among broiler and free-range chicken *E. coli* isolates was stratified, but the significant prevalence difference ($P = 0.000$) was only observed between the ceca isolates of the above two categories (**Table 2**). Overall, 5% (5/109) of broiler *E. coli* isolates were identified to be extraintestinal pathogenic *E. coli* (ExPEC) based on the detection of three or more ExPEC virulence markers. However, none of the free-range chicken *E. coli* isolates was suspected to be belonging to ExPEC. Similarly, we found one putative ST131 *E. coli* in broiler category, which was later confirmed by WGS and *in silico* MLST, whereas none (0/59) of the free-range *E. coli* isolates belonged to ST131 clone.

Multiple Chicken Samples from Same Shops Harbored Identical *E. coli* Clones

We investigated the genetic relationships among the *E. coli* isolates obtained from broiler and free-range chicken samples (**Figure 1**). A total of 60 isolates that were multidrug resistant and originating from different geographical locations were analyzed that represented 38 broiler (22 ceca and 16 raw meat) and 22 free-range chicken isolates (12 ceca and 10 raw meat). Out of 60 isolates, 52 distinct ERIC profiles were obtained. Overall, the broiler and free-range chicken *E. coli* isolates demonstrated promiscuous genetic fingerprints as the two categories did not form distinct clusters. A total of 39 out of 60 isolates demonstrated a close genetic relatedness (similarity coefficient of $\leq$80%) which were represented in the form of several small sub-clusters. Six out of eight identical clades corresponded consistently with the geographic origin, isolation source and ESBL status but not the sample origin; this possibly hints at cross contamination during rearing, slaughtering and/or processing.

Poultry *E. coli* Isolates Shared Remarkable Similarities with Human and Avian *E. coli* Pathotypes

The general features of 10 in-house whole genome sequenced poultry *E. coli* isolates were shown in Supplementary Tables S1 and S4. We generated a phylogenetic tree (**Figure 2**) of 10 in-house isolates to study their relationship with 40 other publicly available genomes comprising of 20 human disease-associated *E. coli* (10 ExPEC and 10 *enteric* pathogens), 10 Avian pathogenic *E. coli* (APEC) and 10 *E. coli* genomes from healthy broiler chickens (Supplementary Table S3). Despite genetic diversity, the phylogenetic tree was able to largely cluster isolates of different pathotypes. The clade 1 was dominated with avian strains and clade 2 mainly comprised genomes of enteric strains together with poultry *E. coli*. The clade 3 comprised strains from all five pathotypes in which the in-house poultry *E. coli* was found to be co-clustered with APEC, poultry and ExPEC strains but not with enteric strains. Out of 10 poultry genomes analyzed, one was represented in clade 1, four genomes in clade 2 and five genomes in clade 3. Overall, we observed that the core genome content of poultry *E. coli* genomes failed to demonstrate unambiguous distinction with other human *E. coli* pathotypes.

Multiple genome comparison was carried out using BRIG tool for one in-house poultry *E. coli* isolate (NAEC1 or NAPEC_15) belonging to the ST131 lineage with five other ST131 *E. coli* genomes to determine the status of 22 mobile genetic elements as reported in EC958 (**Figure 3**). Out of six genomes, three strains (JJ1886, NA114, and NA097) including the NAEC1 shared significant genetic similarities with respect to composition of mobile genetic elements. However, the other avian genome IHT25637 and the commensal ST131 genome SE15 contained only few complete mobile elements. The presence of mobile genomic islands in the poultry *E. coli* resembling to that of human *E. coli* pathotypes reinforces the understanding that these isolates could possibly cross infect humans and poultry.

In order to understand the genetic relationships of virulence and resistance of poultry *E. coli* in juxtaposition with the genetic landscapes of different human *E. coli* pathotypes, we compared virulence and resistance profiles of 10 sequenced poultry *E. coli* genotypes with 40 publicly *E. coli* genomes (Supplementary Table S3). Hierarchical clustering of resistance profiles (**Figure 4B**) demonstrated that the two sister clusters 1a and 1b represented isolates from all pathotypes including the five in-house poultry genomes. However, cluster 2 was clearly defined or dominated by poultry *E. coli* isolates that also contained the remaining five in-house poultry genomes. The cluster obtained by the virulence gene profiles (**Figure 4A**) grouped strains into two categories; one composed of mixed pathotypes (sister clusters 1a and 1b) and the other cluster 2 was dominated with enteric isolates (8/10 enteric genomes). The 10 in-house poultry *E. coli* isolates were represented in the mixed clusters (1a and 1b) indicating closer genetic identity with some pathotypes (ExPEC, APEC and poultry *E. coli*), than with others (IPEC or enteric pathotypes). Overall, these observations hint at the zoonotic potential of poultry *E. coli.*

DISCUSSION

In this molecular-epidemiological study, we assessed *E. coli* isolates obtained from retail poultry (meat/carcass) from a wide geographical region extending across three south Indian and one west-central Indian states (four states) by using both conventional typing and WGS methods. Our findings provide evidence that the raw retail poultry meat could indeed be contaminated with antimicrobial-resistant and potentially pathogenic *E. coli.* This is particularly alarming for countries such as India given high disease burden, emergence of resistance traits, and the confluence of prevailing socio-economic, demographic and environmental factors (Jadhav et al., 2011; Dikid et al., 2013).

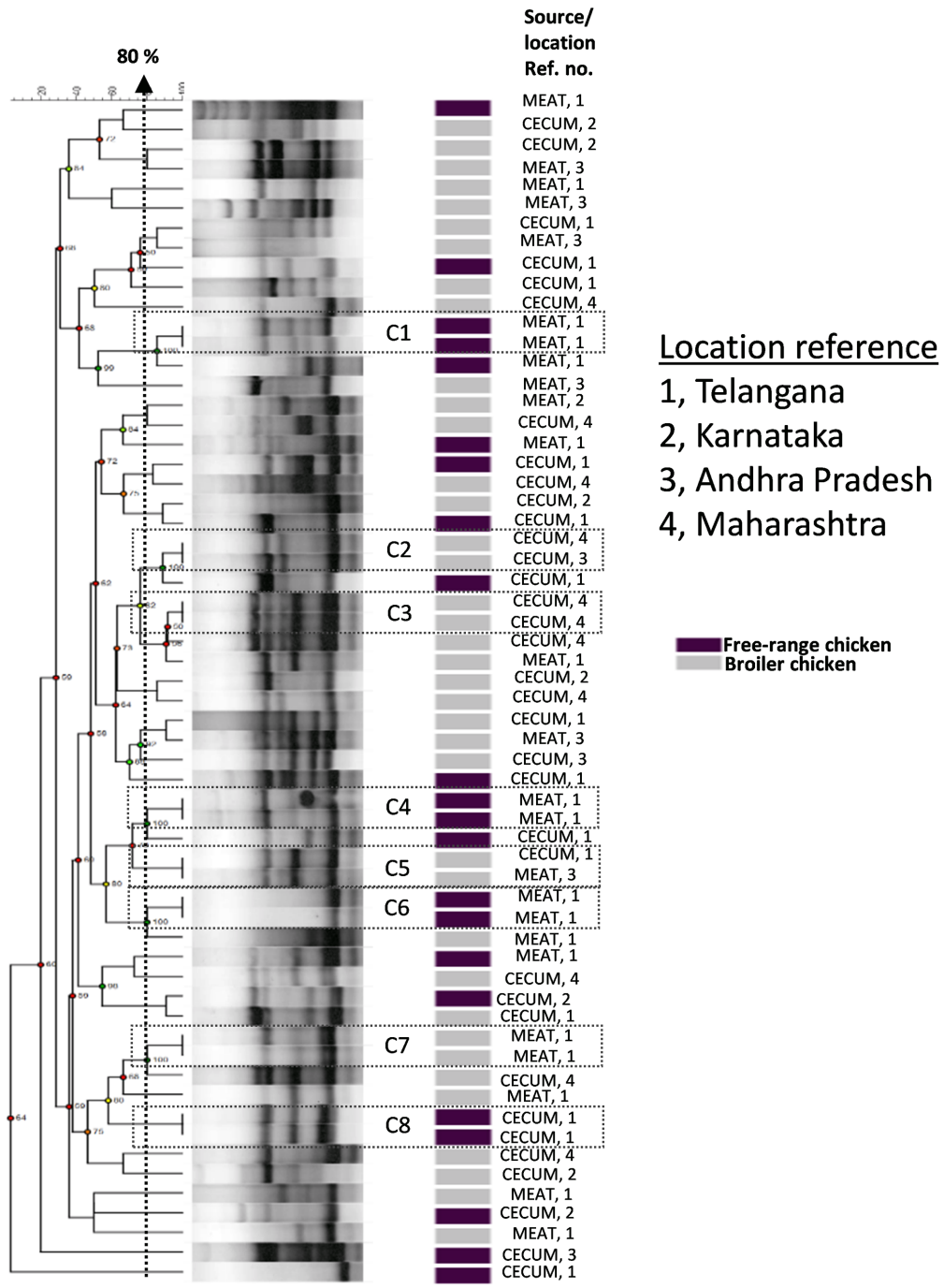


FIGURE 1 | Dendrogram representing genetic relationships of 38 broiler and 22 free-range chicken *Escherichia coli* isolates based on ERIC-PCR fingerprints. The clustering was performed by UPGMA algorithm based on Dice similarity coefficients. The genotypic patterns generated by ERIC-PCR were analyzed at 80% cutoff. Among the eight identical clades (C1 to C8), six clades corresponded consistently to geographic and isolation source.

Pathogenic *E. coli* (ExPEC and diarrheagenic) are the leading cause of infections in both humans and poultry (Ewers et al., 2007). Increased antimicrobial usage in the poultry industry due to the growing demand might have contributed significantly to the emergence and dissemination of multiresistant and pathogenic *E. coli* variants, which could be a serious public health threat (Laxminarayan and Chaudhury, 2016). Hence, constant surveillance and knowledge of their transmission and epidemiology with respect to their genetic backgrounds, antimicrobial resistance patterns and specific virulence attributes is pertinent (Mellata, 2013; Laxminarayan and Chaudhury, 2016). Our findings therefore have potential

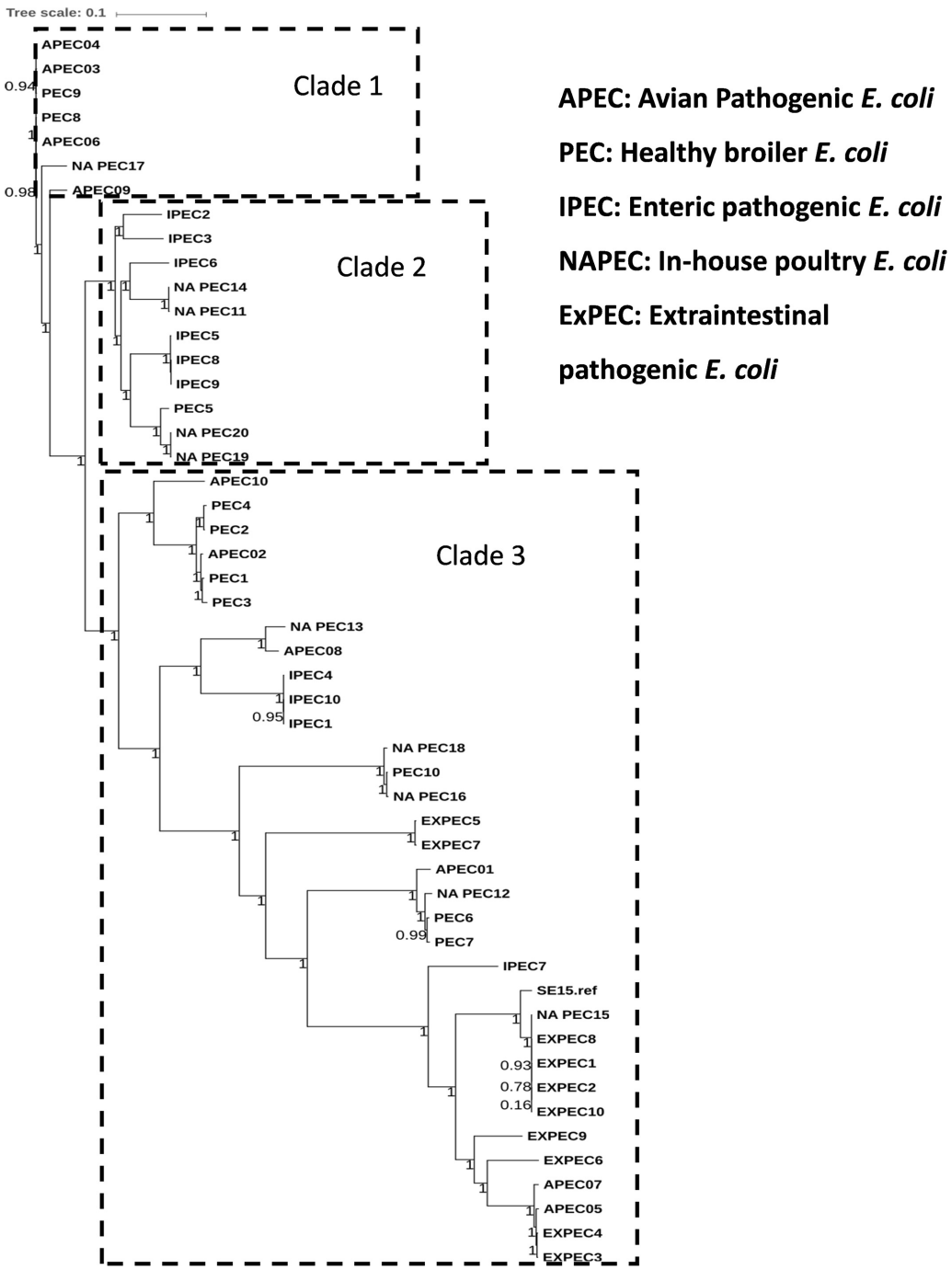


FIGURE 2 | Phylogenetic tree of 50 *E. coli* strains: the core genome based consensus Maximum Likelihood phylogenetic tree of 10 in-house poultry *E. coli* isolates together with 40 other publicly available human and animal disease associated *E. coli* isolates generated using Harvest. The output of Harvest was visualized using iTOL tool (http://itol.embl.de/). Despite genetic diversity, the phylogenetic tree on the whole was able to diffusely group isolates belonging to different pathotypes.

implications for public health policies entailing antibiotic usage regulation.

Poultry industries use antibiotics both for therapeutic purposes and for growth promotion (Singer and Hofacre, 2006). Recent studies in different parts of India have reported antimicrobial residues in food animal products such as chicken meat suggesting large-scale unregulated use of antibiotics by the poultry industry (Laxminarayan and Chaudhury, 2016; Brower et al., 2017). This is consistent with our observations as we also found a marked predominance of antibiotic resistance among *E. coli* isolates obtained from conventionally raised (broiler) chicken. In contrast, free-range chicken meat were

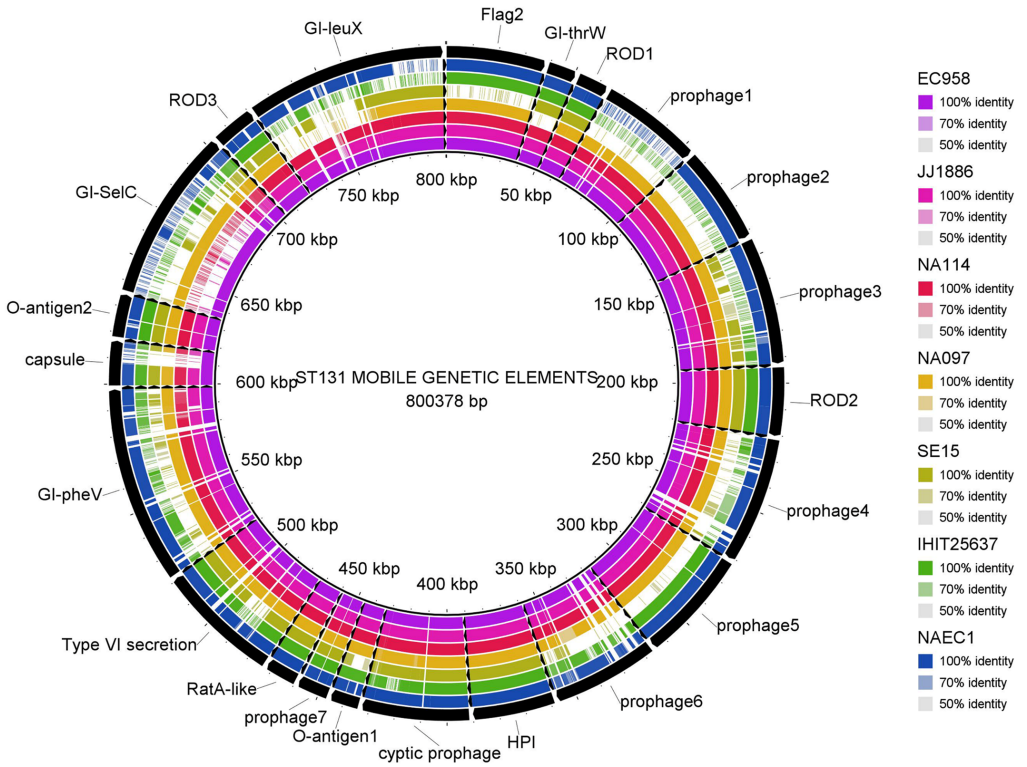


FIGURE 3 | Distribution of EC958 genomic islands (GIs) in 6 ST131 *E. coli* genomes: results show that the 22 GIs of EC958 are well-conserved among three human and one in-house ST131 poultry *E. coli* genome (NAEC1) but partially present in the commensal ST131 (SE15) and the poultry *E. coli* genome from Germany (IHIT25637). Detail description of the genomes analyzed are as follows- *JJ1886*; CP006784.1 (Human ExPEC, United States), *NA114*; GCA_000214765.3 (Human ExPEC, India), *NA097*; GCA_001029415.1 (Human ExPEC, India), *SE15*; GCA_000010485.1 (Human commensal, Japan), *IHIT25637*; GCA_001676995.1 (Avian ExPEC, Germany).

comparatively less contaminated with multidrug resistant *E. coli*; this observation echoes previously reported studies (Koga et al., 2015; Rugumisa et al., 2016). The above observation complements the cross contamination hypothesis of poultry carcasses with the host's fecal flora during slaughter and processing also given that we found comparable antimicrobial resistance prevalences in *E. coli* isolates entailing ceca and raw meat samples of broiler chicken. Such observations were also evidenced by others (Rasschaert et al., 2008; Firildak et al., 2015). The reason for higher prevalence of antimicrobial resistance in *E. coli* isolates of broiler chicken could be due to the high antibiotic selection pressure.

Similar to other studies (Klimienė et al., 2017) the predominant ESBL genotype detected in chicken *E. coli* isolates was $bla_{\mathrm{CTX-M-15}}$ gene which is also the most frequently reported genotype in human health care settings (Chen et al., 2014). The pan-genome based resistance gene profiling revealed that 50% of poultry *E. coli* shared resistance genes with human *E. coli* pathotypes (**Figure 4B**). The formation of a separate cluster of the remaining 50% of the poultry genomes indicates difference in resistance determinants with that of human *E. coli* resistomes which could be reflective of differences in antimicrobial usage practices in human medicine and livestock rearing.

Although only a fraction of total poultry isolates did belong to two well-established pathogenic phylogroups B2 and D (2%, 9%, respectively), a majority of them (36% each) belonged to phylogroups A and B1 (**Table 2**), as also reported by others (Jakobsen et al., 2010; Klimienė et al., 2017). Nonetheless, these two phylogroups have also been previously described to harbor isolates with a high pathogenic potential for birds and humans (Rodriguez-Siek et al., 2005; Ewers et al., 2007; Kobayashi et al., 2011). Virulence gene (Franz et al., 2015) screening demonstrated that the broiler *E. coli* isolates differed from free-range chicken *E. coli* isolates by their higher prevalence of all the virulence genes. Furthermore, five broiler *E. coli* isolates were identified to be ExPEC with none from free-range chicken *E. coli* (**Table 2**). The overall low prevalence of poultry *E. coli* isolates from ExPEC category was expected because all samples were collected from healthy birds. This corroborates also with the low prevalence of phylogenetic group B2.

The phylogenetic tree, based on core genomes demonstrated that, the poultry *E. coli* isolates are not a homogeneous group, because some of the poultry *E. coli* isolates revealed similar genetic backgrounds with human ExPEC and enteric isolates, thus pointing to the role of chickens as reservoir of potentially pathogenic *E. coli*. Only a few studies have investigated the presence of *E. coli* ST131 in food animals; *E. coli* ST131 producing

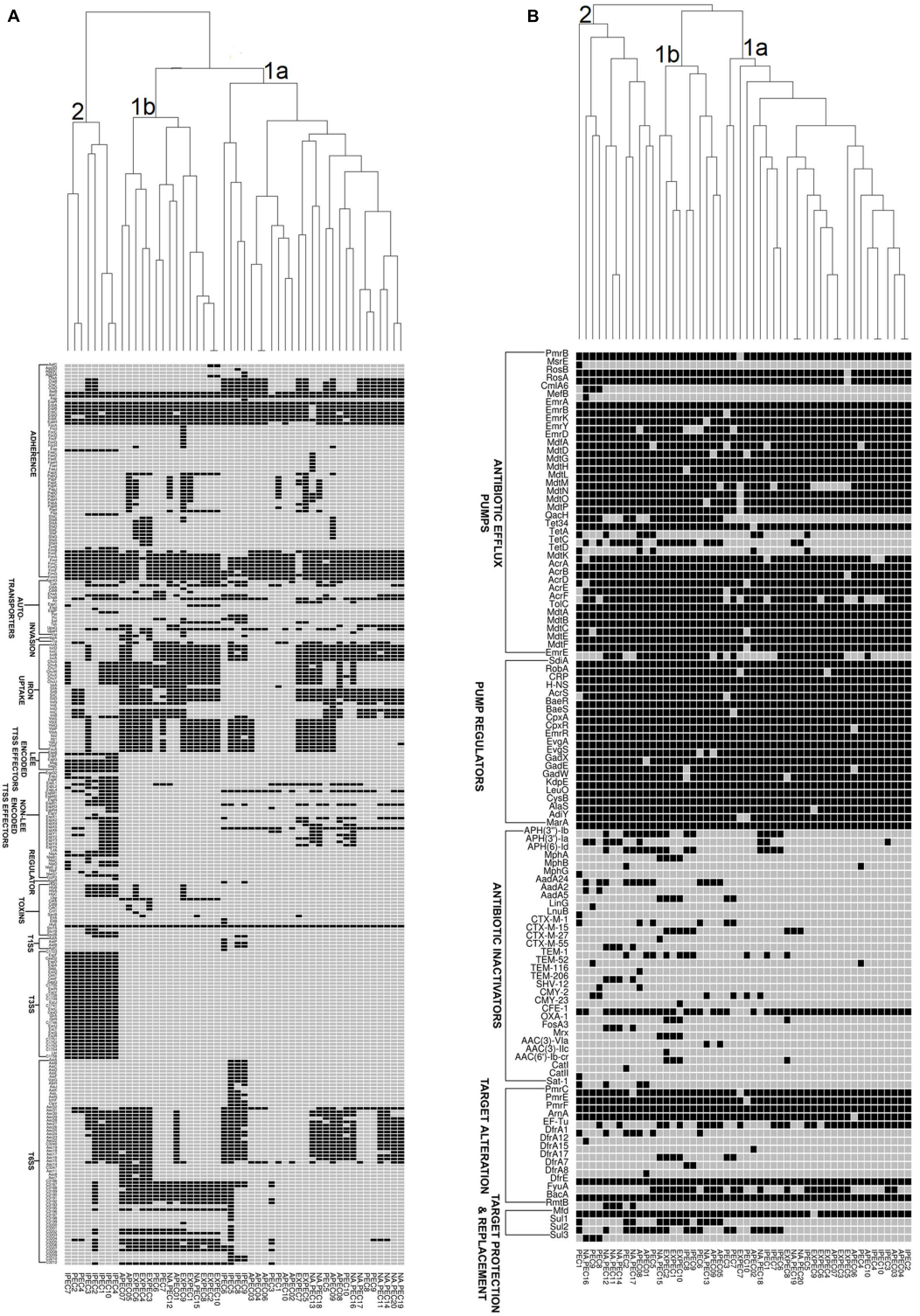


FIGURE 4 | Gene cluster results for 50 *E. coli* isolates: the presence (Black Square) and the absence (Gray Square) of virulence genes **(A)** and resistance genes **(B)** are represented in the image. Gene names are listed on the left. *E. coli* pathotypes are listed below the image (APEC: avian pathogenic *E. coli*, ExPEC: extraintestinal pathogenic *E. coli*, IPEC: intestinal pathogenic *E. coli*, PEC: healthy poultry *E. coli*, NA_PEC: in-house poultry *E. coli*). Results of resistance clustering indicated that only 50% of our poultry *E. coli* shared resistance genes with other *E. coli* pathotypes (distributed in mixed clusters 1a and 1b) and the rest formed a separate group (cluster 2, **B**). However, the virulence based cluster diagram showed that the in-house poultry (NAPEC) shared more virulence similarity with ExPEC and APEC genomes compared to intestinal pathogenic *E. coli* (IPEC), as cluster 2 of **(A)** was dominated by enteric *E. coli* genomes without any poultry *E. coli* genome.

CTX-M-15 also appears to be very rare in foodstuffs of animal origin (Nicolas-Chanoine et al., 2014). This study confirmed the presence of two globally emerging pathogenic lineages of *E. coli*; ST131 (*H*30-Rx subclone) and ST117. The other commonly identified sequence types among the 10 poultry *E. coli* genomes were; ST115, ST155, and ST1640 which are reported to be mostly associated with ESBL phenotypes (Borjesson et al., 2013). Moreover, the identified ST131 *E. coli* contained more pathogenicity islands than the other poultry genomes from Europe and the commensal ST131 genome-SE15 (**Figure 3**). The presence of such a strain in poultry meat belonging to the epidemiologically successful clonal lineage ST131 (*H*30-Rx subclone) warrants attention.

Corroborating with phylogeny results, virulence gene profiling revealed that the poultry *E. coli* are not a homogenous group and showed mixed clustering. However, it provided better resolution between ExPEC and enteric pathotypes (**Figure 4A**). Nonetheless, a majority of poultry *E. coli* isolates and human ExPEC were clustered together with respect to the virulence gene content suggesting that they share remarkable similarities with human ExPEC pathotypes (**Figure 4A**). The ERIC fingerprinting of broiler and free-range chicken *E. coli* isolates demonstrated that the poultry *E. coli* are diverse. The identical clones that we observed mostly corresponded to the geographic (abattoir) and host (broiler and free-range) origins (**Figure 1**).

This geographically wide report on molecular and genomic characterization of *E. coli* from broiler and free-range chickens is the first from India. Herein, we found that the raw retail poultry meat was frequently contaminated with antimicrobial-resistant *E. coli* and/or potentially pathogenic *E. coli* variants. However, free-range chickens represented a low risk of contamination with pathogenic and or resistant *E. coli*; this is particularly important as India is an agricultural country with around 70% of the population living in rural areas and many of the rural households engage in backyard poultry raising (Singh, 2014). The comparative genomic analysis suggests that the poultry *E. coli* isolates share closer genetic identity to human *E. coli* as regards to core genome phylogeny, antimicrobial genes and virulence gene content and thus represent potential zoonotic risks. The possibility that multi-drug resistant and /or pathogenic *E. coli* could be potentially transmitted from food sources such as chicken meat raises serious public health concerns regarding food preparations where chicken meat may not be properly cooked or could only be pickled raw.

AUTHOR CONTRIBUTIONS

AH, DC, and NA designed and conducted the study. AR, NN, TS, LW, MI, MM, and IQ helped in analysis of data and preparation of the manuscript. SS, ST, and RB helped in analyzing genome sequence data.

FUNDING

The financial support of UGC, New Delhi through its project No. F.4-2/2006 (BSR)/BL/14-15/0273 to AH as a UGC's Dr. D. S. Kothari post-doctoral fellowship is thankfully acknowledged.

ACKNOWLEDGMENTS

We are thankful to the members of the molecular pathogenesis lab at Indian Institute of Science for their help and support. We would also like to acknowledge the 'Microsoft Azure for Research Award' from Microsoft Corporation to NA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02120/full#supplementary-material

REFERENCES

Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Avasthi, T. S., Kumar, N., Baddam, R., Hussain, A., Nandanwar, N., Jadhav, S., et al. (2011). Genome of multidrug-resistant uropathogenic *Escherichia coli* strain NA114 from India. *J. Bacteriol.* 193, 4272–4273. doi: 10.1128/JB.05413-11

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., and Dozois, C. M. (2011). *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. *FEMS Immunol. Med. Microbiol.* 62, 1–10. doi: 10.1111/j.1574-695X.2011.00797.x

Borjesson, S., Egervarn, M., Lindblad, M., and Englund, S. (2013). Frequent occurrence of extended-spectrum beta-lactamase- and transferable AmpC Beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. *Appl. Environ. Microbiol.* 79, 2463–2466. doi: 10.1128/AEM.03893-12

Brower, C. H., Mandal, S., Hayer, S., Sran, M., Zehra, A., Patel, S. J., et al. (2017). The prevalence of extended-spectrum Beta-lactamase-producing multidrug-resistant *Escherichia Coli* in poultry chickens and variation according to farming practices in Punjab, India. *Environ. Health Perspect.* 125, 1–10. doi: 10.1289/EHP292

Chen, L. F., Freeman, J. T., Nicholson, B., Keiger, A., Lancaster, S., Joyce, M., et al. (2014). Widespread dissemination of CTX-M-15 genotype extended-spectrum-β-lactamase-producing *enterobacteriaceae* among patients presenting to community hospitals in the southeastern United States. *Antimicrob. Agents Chemother.* 58, 1200–1202. doi: 10.1128/AAC.01099-13

Chen, Z., and Jiang, X. (2014). Microbiological safety of chicken litter or chicken litter-based organic fertilizers: a review. *Agriculture* 4, 1–29. doi: 10.3390/agriculture4010001

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* 5, 58–65. doi: 10.1111/1758-2229.12019

CLSI (2013). *VET01-A4 Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals*; Approved Standard, Fourth Edn. Wayne, PA: CLSI.

Collingwood, C., Kemmett, K., Williams, N., and Wigley, P. (2014). Is the concept of avian pathogenic *Escherichia coli* as a single pathotype fundamentally flawed? *Front. Vet. Sci.* 1:5. doi: 10.3389/fvets.2014.00005

de Been, M., Lanza, V. F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., et al. (2014). Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. *PLOS Genet.* 10:e1004776. doi: 10.1371/journal.pgen.1004776

Dikid, T., Jain, S. K., Sharma, A., Kumar, A., and Narain, J. P. (2013). Emerging & re-emerging infections in India: an overview. *Indian J. Med. Res.* 138, 19–31.

Dziva, F., Hauser, H., Connor, T. R., van Diemen, P. M., Prescott, G., Langridge, G. C., et al. (2013). Sequencing and functional annotation of avian pathogenic *Escherichia coli* serogroup O78 strains reveal the evolution of *E. coli* lineages pathogenic for poultry via distinct mechanisms. *Infect. Immun.* 81, 838–849. doi: 10.1128/IAI.00585-12

Ewers, C., Li, G., Wilking, H., Kiessling, S., Alt, K., Antáo, E.-M., et al. (2007). Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? *Int. J. Med. Microbiol.* 297, 163–176. doi: 10.1016/j.ijmm.2007.01.003

Firildak, G., Asan, A., and Goren, E. (2015). Chicken carcasses bacterial concentration at poultry slaughtering facilities. *Asian J. Biol. Sci.* 8, 16–29. doi: 10.3923/ajbs.2015.16.29

Franz, E., Veenman, C., van Hoek, A. H. A. M., de Roda Husman, A., and Blaak, H. (2015). Pathogenic *Escherichia coli* producing extended-spectrum β-Lactamases isolated from surface water and wastewater. *Sci. Rep.* 5:14372. doi: 10.1038/srep14372

Hussain, A., Ewers, C., Nandanwar, N., Guenther, S., Jadhav, S., Wieler, L. H., et al. (2012). Multiresistant uropathogenic *Escherichia coli* from a region in India where urinary tract infections are endemic: genotypic and phenotypic characteristics of sequence type 131 isolates of the CTX-M-15 extended-spectrum-β-lactamase-producing lineage. *Antimicrob. Agents Chemother.* 56, 6358–6365. doi: 10.1128/AAC.01099-12

Hussain, A., Ranjan, A., Nandanwar, N., Babbar, A., Jadhav, S., and Ahmed, N. (2014). Genotypic and phenotypic profiles of *Escherichia coli* isolates belonging to clinical sequence type 131 (ST131), clinical non-ST131, and fecal non-ST131 lineages from India. *Antimicrob. Agents Chemother.* 58, 7240–7249. doi: 10.1128/AAC.03320-14

Jadhav, S., Hussain, A., Devi, S., Kumar, A., Parveen, S., Gandham, N., et al. (2011). Virulence characteristics and genetic affinities of multiple drug resistant uropathogenic *Escherichia coli* from a semi urban locality in India. *PLOS ONE* 6:e18063. doi: 10.1371/journal.pone.0018063

Jakobsen, L., Kurbasic, A., Skjøt-Rasmussen, L., Ejrnæs, K., Porsbo, L. J., Pedersen, K., et al. (2010). *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. *Foodborne Pathog. Dis.* 7, 537–547. doi: 10.1089/fpd.2009.0409

Johnson, J. R., Murray, A. C., Gajewski, A., Sullivan, M., Snippes, P., Kuskowski, M. A., et al. (2003). Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli*. *Antimicrob. Agents Chemother.* 47, 2161–2168. doi: 10.1128/AAC.47.7.2161

Kaper, J. B., Nataro, J. P., and Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2, 123–140. doi: 10.1038/nrmicro818

Klimienė, I., Virgailis, M., Kerzienė, S., Šiugždinienė, R., Mockeliūnas, R., and Ružauskas, M. (2017). Evaluation of genotypical antimicrobial resistance in *ESBL* producing *Escherichia coli* phylogenetic groups isolated from retail poultry meat. *J. Food Saf.* e12370. doi: 10.1111/jfs.12370 [Epub ahead of print].

Kobayashi, R. K. T., Aquino, I., Ferreira, A. L., da, S., and Vidotto, M. C. (2011). EcoR phylogenetic analysis and virulence genotyping of avian pathogenic *Escherichia coli* strains and *Escherichia coli* isolates from commercial chicken carcasses in Southern Brazil. *Foodborne Pathog. Dis.* 8, 631–634. doi: 10.1089/fpd.2010.0726

Koga, V. L., Scandorieiro, S., Vespero, E. C., Oba, A., de Brito, B. G., de Brito, K. C. T., et al. (2015). Comparison of antibiotic resistance and virulence factors among *Escherichia coli* isolated from conventional and free-range poultry. *Biomed. Res. Int.* 2015:618752. doi: 10.1155/2015/618752

Landers, T. F., Cohen, B., Wittum, T. E., and Larson, E. L. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *Public Health Rep.* 127, 4–22. doi: 10.1177/003335491212700103

Laxminarayan, R., and Chaudhury, R. R. (2016). Antibiotic resistance in India: drivers and opportunities for action. *PLOS Med.* 13:e1001974. doi: 10.1371/journal.pmed.1001974

Lutful Kabir, S. M. (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int. J. Environ. Res. Public Health* 7, 89–114. doi: 10.3390/ijerph7010089

Mellata, M. (2013). Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathog. Dis.* 10, 916–932. doi: 10.1089/fpd.2013.1533

Mitchell, N. M., Johnson, J. R., Johnston, B., Curtiss Iii, R., and Mellata, M. (2015). Zoonotic potential of *Escherichia coli* isolates from retail chicken meat products and eggs. *Appl. Environ. Microbiol.* 81, 1177–1187. doi: 10.1128/AEM.03524-14

Nandanwar, N., Hussain, A., Ranjan, A., Jadhav, S., and Ahmed, N. (2016). Population structure and molecular epidemiology of human clinical multi-drug resistant (MDR) *Escherichia coli* strains from Pune, India. *Int. J. Infect. Dis.* 45, 343–344. doi: 10.1016/j.ijid.2016.02.741

Nandanwar, N., Janssen, T., Kühl, M., Ahmed, N., Ewers, C., and Wieler, L. H. (2014). Extraintestinal pathogenic *Escherichia coli* (ExPEC) of human and avian origin belonging to sequence type complex 95 (STC95) portray indistinguishable virulence features. *Int. J. Med. Microbiol.* 95, 6–13. doi: 10.1016/j.ijmm.2014.06.009

Nicolas-Chanoine, M. H., Bertrand, X., and Madec, J. Y. (2014). *Escherichia coli* st131, an intriguing clonal group. *Clin. Microbiol. Rev.* 27, 543–574. doi: 10.1128/CMR.00125-13

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Patel, R. K., and Jain, M. (2012). NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PLOS ONE* 7:e30619. doi: 10.1371/journal.pone.0030619

Qumar, S., Majid, M., Kumar, N., Tiwari, S. K., Semmler, T., Devi, S., et al. (2017). Genome dynamics and molecular infection epidemiology of multidrug-resistant *Helicobacter pullorum* isolates obtained from broiler and free-range chickens in India. *Appl. Environ. Microbiol.* 83, e2305–e2316. doi: 10.1128/AEM.02305-16

Ranjan, A., Shaik, S., Hussain, A., Nandanwar, N., Semmler, T., Jadhav, S., et al. (2015). Genomic and functional portrait of a highly virulent, CTX-M-15-producing H30- Rx subclone of *Escherichia coli* sequence type (ST) 131. *Antimicrob. Agents Chemother.* 59, 6087–6095. doi: 10.1128/AAC.01447-15

Ranjan, A., Shaik, S., Mondal, A., Nandanwar, N., Hussain, A., Semmler, T., et al. (2016). Molecular epidemiology and genome dynamics of New Delhi metallo-beta-lactamase (NDM) producing extraintestinal pathogenic *E. coli* (ExPEC) strains from India. *Antimicrob. Agents Chemother.* 60, 6795–6805. doi: 10.1128/AAC.01345-16

Ranjan, A., Shaik, S., Nandanwar, N., Hussain, A., Tiwari, S. K., Semmler, T., et al. (2017). Comparative genomics of *Escherichia coli* isolated from skin and soft tissue and other extraintestinal infections. *MBio* 8:e01070–17. doi: 10.1128/mBio.01070-17

Rasschaert, G., Houf, K., Godard, C., Wildemauwe, C., Pastuszczak-Frak, M., and De Zutter, L. (2008). Contamination of carcasses with *Salmonella* during poultry slaughter. *J. Food Prot.* 71, 146–152. doi: 10.4315/0362-028X-71.1.146

Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., Fakhr, M. K., and Nolan, L. K. (2005). Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. *Microbiology* 151, 2097–2110. doi: 10.1099/mic.0.27499-0

Rugumisa, B. T., Call, D. R., Mwanyika, G. O., Subbiah, M., and Buza, J. (2016). African journal of microbiology research comparison of the prevalence of antibiotic-resistant *Escherichia coli* isolates from commercial-layer and free-range chickens in Arusha district. *Tanzania* 10, 1422–1429. doi: 10.5897/AJMR2016.8251

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M. A., et al. (2000). Artemis: sequence visualization and annotation. *Bioinformatics* 16, 944–945. doi: 10.1093/bioinformatics/16.10.944

Schissler, J. R., Hillier, A., Daniels, J. B., Cole, L. K., and Gebreyes, W. A. (2009). Evaluation of Clinical Laboratory Standards Institute interpretive criteria for methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs. *J. Vet. Diagn. Invest.* 21, 684–688. doi: 10.1177/104063870902100514

Shaik, S., Kumar, N., Lankapalli, A. K., Tiwari, S. K., Baddam, R., and Ahmed, N. (2016). Contig-Layout-Authenticator (CLA): a combinatorial approach to ordering and scaffolding of bacterial contigs for comparative genomics and molecular epidemiology. *PLOS ONE* 11:e0155459. doi: 10.1371/journal.pone.0155459

Singer, R. S., and Hofacre, C. L. (2006). Potential impacts of antibiotic use in poultry production. *Avian. Dis.* 50, 161–172. doi: 10.1637/7569-033106R.1

Singh, P. (2014). Population and agro climatic zones in India: an analytical analysis. *Procedia Soc. Behav. Sci.* 120, 268–278. doi: 10.1016/j.sbspro.2014.02.104

Totsika, M., Beatson, S. A., Sarkar, S., Phan, M. D., Petty, N. K., Bachmann, N., et al. (2011). Insights into a multidrug resistant *Escherichia coli* pathogen of the globally disseminated ST131 lineage: genome analysis and virulence mechanisms. *PLOS ONE* 6:e26578. doi: 10.1371/journal.pone.0026578

Treangen, T. J., Ondov, B. D., Koren, S., and Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 15:524. doi: 10.1186/PREACCEPT-2573980311437212

van den Bogaard, A. E., London, N., Driessen, C., and Stobberingh, E. E. (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.* 47, 763–771. doi: 10.1093/JAC/47.6.763

Zhang, J., Massow, A., Stanley, M., Papariella, M., Chen, X., Kraft, B., et al. (2011). Contamination rates and antimicrobial resistance in Enterococcus spp., *Escherichia coli*, and Salmonella isolated from "no antibiotics added"-labeled chicken products. *Foodborne Pathog. Dis.* 8, 1147–1152. doi: 10.1089/fpd.2011.0852

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Hussain, Shaik, Ranjan, Nandanwar, Tiwari, Majid, Baddam, Qureshi, Semmler, Wieler, Islam, Chakravortty and Ahmed. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 November 2017
doi: 10.3389/fmicb.2017.02221

Increased Adhesion of *Listeria monocytogenes* Strains to Abiotic Surfaces under Cold Stress

Bo-Hyung Lee[1,2], Michel Hébraud[3] and Thierry Bernardi[1]*

[1] BioFilm Control, Biopôle Clermont Limagne, Saint-Beauzire, France, [2] Université Clermont Auvergne, Clermont-Ferrand, France, [3] Institut National de la Recherche Agronomique, Université Clermont Auvergne, UMR MEDiS, Saint-Genès-Champanelle, France

Food contamination by *Listeria monocytogenes* remains a major concern for some food processing chains, particularly for ready-to-eat foods, including processed foods. Bacterial adhesion on both biotic and abiotic surfaces is a source of contamination by pathogens that have become more tolerant or even persistent in food processing environments, including in the presence of adverse conditions such as cold and dehydration. The most distinct challenge that bacteria confront upon entry into food processing environments is the sudden downshift in temperature, and the resulting phenotypic effects are of interest. Crystal violet staining and the BioFilm Ring Test® were applied to assess the adhesion and biofilm formation of 22 listerial strains from different serogroups and origins under cold-stressed and cold-adapted conditions. The physicochemical properties of the bacterial surface were studied using the microbial adhesion to solvent technique. Scanning electron microscopy was performed to visualize cell morphology and biofilm structure. The results showed that adhesion to stainless-steel and polystyrene was increased by cold stress, whereas cold-adapted cells remained primarily in planktonic form. Bacterial cell surfaces exhibited electron-donating properties regardless of incubation temperature and became more hydrophilic as temperature decreased from 37 to 4°C. Moreover, the adhesion of cells grown at 4°C correlated with affinity for ethyl acetate, indicating the role of cell surface properties in adhesion.

Keywords: ***Listeria monocytogenes*, cold stress, adhesion, biofilm, BRT®, crystal violet staining, MATS, SEM**

OPEN ACCESS

Edited by:
Pierina Visciano,
Università di Teramo, Italy

Reviewed by:
Efstathios D. Giaouris,
University of the Aegean, Greece
Even Heir,
Nofima, Norway

****Correspondence:***
Bo-Hyung Lee
2bohyung@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 August 2017*
Accepted: *30 October 2017*
Published: *14 November 2017*

Citation:
Lee B-H, Hébraud M and Bernardi T (2017) Increased Adhesion of Listeria monocytogenes Strains to Abiotic Surfaces under Cold Stress. Front. Microbiol. 8:2221. doi: 10.3389/fmicb.2017.02221

INTRODUCTION

In recent decades, the foodborne pathogen *Listeria monocytogenes* has become a notable threat to food manufacturers, particularly those making ready-to-eat (RTE) foods (Jofré et al., 2016; Vongkamjan et al., 2016). Infection with this saprophytic and psychrotrophic gram-positive pathogen results in a high mortality rate, especially in susceptible groups such as pregnant women or senior populations (Orsi et al., 2011).

Listeria monocytogenes efficiently survives under extreme conditions, such as 40% w/v NaCl or pH 3.0 (Liu et al., 2005). The risk of listeriosis has increased with growing consumption of RTE foods or frozen foods requiring minimal heat treatment before consumption because food processing plants often utilize adverse conditions such as refrigeration, high salt concentration,

or low pH to preserve foods. Moreover, *L. monocytogenes* persists by adhering to food contact surfaces, causing the contamination of final food products (Ferreira et al., 2014). A biofilm is a sessile community of bacterial cells embedded in a matrix of self-produced extracellular polymeric substances (EPS), including proteins, polysaccharides, and extracellular DNA. According to recent studies, the composition of EPS in the *L. monocytogenes* biofilm matrix is dominated by teichoic acids (Brauge et al., 2016; Colagiorgi et al., 2016). Biofilms are often multi-species in nature, and interactions with other bacteria may benefit biofilm formation by *L. monocytogenes* (Giaouris et al., 2015). Although maturity may vary depending on environmental conditions, fundamental biofilm growth involves bacterial adhesion to surfaces (Garrett et al., 2008).

Given increasing concerns that biofilms in food premises lead to food contamination with *L. monocytogenes*, studies have primarily compared the effects of temperature on biofilm formation by growing bacteria at different temperatures, most frequently ranging from 4 to 37°C, and demonstrated that bacteria survive and form biofilms at low temperatures (Di Bonaventura et al., 2008; Nilsson et al., 2011). In general, total biomass production by *L. monocytogenes* strains is augmented with increased incubation temperature, regardless of the adhesion surface, including hydrophilic stainless-steel coupons and hydrophobic polystyrene culture plates. One study showed that storage of *L. monocytogenes* strains at –20°C for 6 and 24 months increased adhesion and biofilm formation on various surfaces, including polystyrene microtiter plates and stainless-steel (Slama et al., 2012). Given its exceptional adaptive ability to mitigate and survive harsh environments, biofilm formation by *L. monocytogenes* may be an adaptation response to stress (Tasara and Stephan, 2006). However, direct observation of cold stress-induced biofilm production by *L. monocytogenes* has not been reported to date.

In this study, a total of 22 *L. monocytogenes* strains of diverse origins and serogroups were investigated to elucidate the impact of cold on phenotypic changes. Cells acclimatized at 37 and 4°C were exposed to cold to evaluate the effects of cold stress on bacterial adhesion and biofilm formation on polystyrene and stainless-steel surfaces using the BioFilm Ring Test® (BRT®), crystal violet (CV) staining, and scanning electron microscopy (SEM). Furthermore, microbial affinity to solvents (MATS) analysis was performed to assess cell surface physicochemical properties and their relationship to surface adhesion characteristics.

MATERIALS AND METHODS

Listeria monocytogenes Isolates and Culture Conditions

A panel of 22 isolates of *L. monocytogenes* from human listeriosis cases, animals, foods and food-related premises were used in this study (**Table 1**). All strains were analyzed by the Institut Pasteur (Paris, France) for serogrouping using a multiplex PCR assay (Doumith et al., 2004). Serogroup IVb includes serovars 4b, 4d, and 4e; serogroup IIb includes serovars 1/2b, 3b and 7; serogroup IIa includes 1/2a and 3a; and serogroup IIc includes serovars 1/2c and 3c.

Strains were stored in Brain–Heart Infusion (BHI) broth (Laboratorios Conda, Spain) with 8.3% glycerol at –20°C, and each set of experiments was conducted with freshly recovered isolates on BHI agar (Laboratorios Conda, Spain). Strains were maintained on BHI agar for at least 2 days at 37°C by sub-culturing daily onto a fresh agar plate.

Sample Preparation

Several colonies were harvested using a sterile inoculating loop, suspended in 20 ml of BHI broth and grown at 37°C with shaking at 100 rpm to reach stationary phase. After incubation for 15 h, stationary cells were pelleted by centrifugation at 5,000 × *g* for 10 min at room temperature, dispersed in 5 ml of fresh BHI broth by vortexing, and utilized for different experiments. Some cells were incubated at 37 and 4°C and were denoted positive control and cold-stressed cells, respectively (**Figure 1**, box A). A portion of the culture was diluted in 20 ml of BHI medium pre-cooled to 4°C to obtain an optical density at 600 nm (OD_{600}) of 0.1 and brought to 4°C to grow under shaking at 100 rpm for 4 to 7 days until the cells reached stationary phase. Stationary cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and suspended by vortexing in fresh pre-cooled BHI broth for use as cold-adapted samples (**Figure 1**, box B). Some cold-adapted cells were streaked onto BHI agar with a sterile inoculating loop and incubated at 37°C overnight. This culture was then exposed to sudden cold stress as described above and designated cold-stressed cells[2] (**Figure 1**, box C).

Viable Cell Counts

Portions of the positive control (precultured at 37°C) and cold-adapted cells (precultured at 4°C) were used to obtain viable cell counts. After measuring OD_{600} with a spectrophotometer (Biomate3, Thermo Scientific, United States), 50 µl of culture was transferred to tryptone salt (TS) solution containing 0.1% w/v tryptone (Conda, Spain) and 0.85% w/v NaCl (Sigma–Aldrich, France) and 10-fold serially diluted in TS solution. From each dilution, 100 µl was spread on a BHI agar plate using sterile glass beads in triplicate. After overnight incubation at 37°C, colonies were counted to calculate colony forming units (CFU) per ml. Total CFU of 30 to 300 per plate were considered valid data.

Two experiments were performed for each condition.

BRT®

The BRT® assay was performed in a polystyrene 96-well microplate (BRT kit C004, BioFilm Control, France) as described by Chavant et al. (2007).

A freshly prepared culture was measured at OD_{600} to obtain a final OD_{600} of 0.5 (approximately 1.2×10^9 CFU/ml) in BHI broth kept at two temperatures: 4°C for cold-stressed and cold-adapted conditions, and room temperature for a positive control. A portion of the suspension was used to perform a threefold dilution to obtain an OD_{600} of 0.17 (approximately 3.8×10^8 CFU/ml). Toner 4 (magnetic beads) was added to

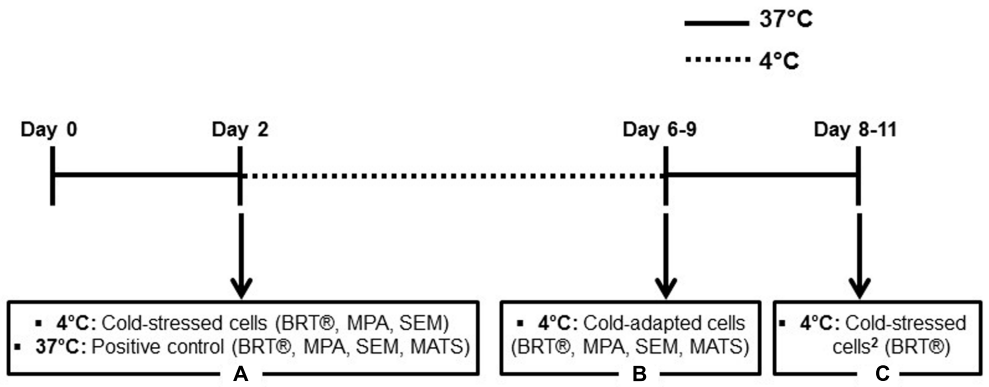


FIGURE 1 | Experimental scheme. Time frame for incubation at 37 or 4°C is shown, with arrows indicating cells in stationary phase used for experiments. Each box (A–C) contains the temperatures and names used for sample conditions, with a list of experiments in parentheses.

a final concentration of 10 µl ml^{-1}. After homogenization of these mixtures, 200 µl of each bacterial suspension was deposited in each well of the microplate in triplicate. Wells containing only BHI broth with magnetic beads were added as negative controls.

Microplates were kept static for 24 h at 4°C for cold-stressed (precultured at 37°C) and cold-adapted (precultured at 4°C) conditions and at 37°C for the positive control. After incubation, wells were covered with a few drops of Liquid Contrast (inert and non-toxic oil), and the plates were placed on a magnetic block with 96 mini-magnets centered under the 96 wells of the microplate for 1 min to apply magnetic fields that attracted mobile beads, creating a quantifiable spot above each mini-magnet. The bottoms of the plates were scanned with a plate reader and analyzed using BFC elements 3® software (BioFilm Control, France) to obtain a numerical value termed the BioFilm Index (BFI) for each well, which ranged from 0 to 20 depending on the size and intensity of the spot. A BFI of approximately 20 corresponded to high magnetic bead mobility, implying no or very few sessile cells, while a lower BFI or a value of zero resulted from the immobilization of beads by sessile cells.

At least four experiments were performed, with triplicate wells for each condition.

TABLE 1 | *Listeria monocytogenes* strains used in this study.

Strain	Lineage	Serogroup	Origin	Reference
1	I	IVb	Human, epidemic (pasteurized milk)	ScottA
2	I	IVb	Human, epidemic (hot dog)	
3	I	IVb	Meat (sausage)	
4	I	IVb	FCS[a] in FPE[b]	
5	I	IIb	Cow	
6	I	IIb	Human, sporadic	
7	I	IIb	Chocolate milk, epidemic	
8	I	IIb	FCS in FPE	
9	I	IIb	Lean meat	
10	II	IIa	Not known	
11	II	IIa	Cow	
12	II	IIa	Hot dog, sporadic	
13	II	IIa	Human, sporadic (hot dog)	
14	II	IIa	Meat (batter)	
15	II	IIa	Meat (sausage)	
16	II	IIa	FCS in FPE	
17	II	IIa	FCS in FPE	
18	II	IIa	Meat (cured ham)	
19	II	IIa	Meat (batter)	
20	II	IIc	Rabbit	EGDe
21	II	IIc	FCS in FPE	
22	II	IIc	Human	LO28

[a]*FCS: food contact surface* [b]*FPE: food processing environment The contaminated food sources of certain epidemic and sporadic cases are specified.*

Microtiter Plate Assay (MPA)

The procedure to prepare a 96-well microplate for microtiter plate assay (MPA) was the same as that described above (BRT®), except that no Toner 4 was added. The assay was performed as previously described by Doijad et al. (2015), with slight modifications. After static incubation for 24 h, the absorbance values of negative controls and total cell densities, including sessile and planktonic cells, were measured using a microplate reader (EL800, BioTek, United States) at OD_{600}.

Plates were inverted, and the media and planktonic cells were discarded via gentle tapping. To remove loosely attached bacteria, wells were washed twice with 300 µl of sterile saline solution (8.5 g of NaCl per l). Sessile cells were fixed with 300 µl of 96% ethanol (Sigma–Aldrich, France) for 20 min and air-dried for 2–3 h at room temperature after removal of the ethanol until no standing moisture was visible. To stain the bacterial biomass, a 0.1% w/v CV (Merck KGaA, Germany) solution was filtered (0.22-µm filter, Millipore, France), and 220 µl was added to each well. After static incubation for 30 min, CV solution was removed by sharply flicking the plates while upside down. Wells were washed three times with 300 µl of saline, followed by tapping them upside down on paper towels. Plates were air-dried for 3–4 h, filled with 150 µl of 33% v/v acetic acid

TABLE 2 | Viable cell counts of six strains grown at stationary phase at two temperatures.

Strain	37°C	4°C
1	9.36 ± 0.03	9.46±0.05
4	9.33 ± 0.03	9.50±0.05
6	9.30 ± 0.03	9.45±0.03
11	9.27 ± 0.06	9.43±0.01
14	9.24 ± 0.01	9.33±0.02
20	9.22 ± 0.04	9.35±0.20

Data are presented as the mean log CFU/ml ± standard deviation at an OD_{600} of 1.

(Sigma–Aldrich, France) and placed on a plate shaker with slight agitation for 10 min to completely destain CV and obtain a homogenized solution. Destained CV levels were determined using a microplate reader at OD_{600}.

At least four experiments were performed, with triplicate wells for each condition.

Physicochemical Experiments

The MATS partitioning method (Bellon-Fontaine et al., 1996) was performed to define bacterial cell surface properties. This method involves comparing the affinities of microbial cells for pairs of monopolar and nonpolar solvents, which have similar van der Waals surface tension components. In this study, the following sets of solvents were used: (i) chloroform, an acidic solvent (electron acceptor), and hexadecane, a nonpolar *n*-alkane, and (ii) ethyl acetate, a basic solvent (strong electron donor), and decane, a nonpolar *n*-alkane (Sigma–Aldrich, France).

Cultures grown until stationary phase at 37 and 4°C were pelleted by centrifugation at 5,000 × g for 10 min at room temperature and 4°C, respectively. Sterile 0.15 M NaCl (Sigma–Aldrich, France) solutions pre-incubated at 37 and 4°C were used to wash pellets, in compliance with the original culture temperatures, followed by centrifugation. Bacterial suspensions were prepared to obtain an OD at 400 nm (OD_{400}) of approximately 0.6 to 0.7, and the initial OD_{400} was measured as [A_0]. A suspension in a volume of 2.4 ml was vortexed for 60 s with 0.4 ml of each solvent in a glass tube. The mixture was allowed to stand static for 15 min to ensure the complete separation of both phases. The absorbance of the aqueous phase was measured at OD_{400} [A]. The percentage of cells in each solvent was calculated using the following equation: percent affinity = $[1–(A/A_0)] \times 100$.

Each experiment was performed in quadruplicate with independently grown bacterial cultures.

SEM

Biofilms were grown on sterile stainless-steel coupons to visualize adhesion patterns, biofilm architecture, and cell morphologies via SEM with positive control, cold-stressed, and cold-adapted cells.

Fresh cultures in BHI broth were prepared to obtain an OD_{600} of 0.5, and 7 ml of each bacterial suspension was poured into a petri dish (55-mm diameter) containing a sterile stainless-steel coupon (AISI 304, mean roughness = 0.064) and statically incubated for 24 h at 37°C for a positive control and at 4°C for the cold-stressed and cold-adapted conditions. After removing the cultures using a pipette, the coupons were gently washed twice by filling the petri dishes with sterile saline solution to remove nonadherent cells. Sessile cells and biofilms were fixed on each coupon in 10 ml of a solution containing 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) in a 50-ml glass beaker at 4°C for a minimum of 1 h to overnight. Coupons were washed three times for 15 min each via immersion in cacodylate buffer, followed by dehydration using a graded ethanol series (70, 90, and 100%) three times for 15 min each. Further dehydration was performed in a 50:50 mixture of ethanol:hexamethyldisilazane (HMDS) three times for 10 min each. Samples were immersed in pure

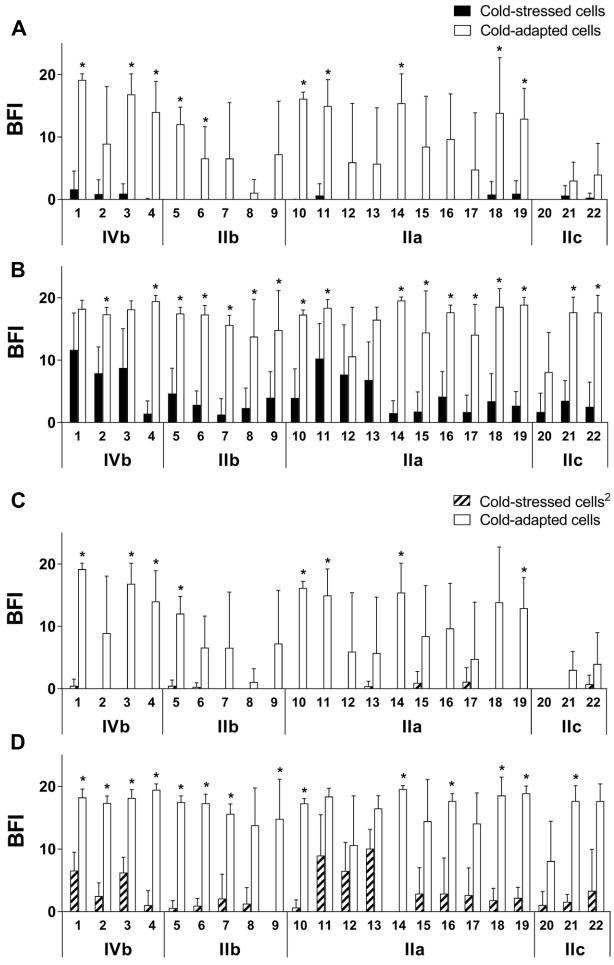


FIGURE 2 | Increased adhesion of cold-stressed cells compared to cold-adapted cells, measured by BRT®. Sudden exposure to cold for the first time was denoted as cold-stressed cells **(A,B)**, and exposure for a second time was denoted as cold-stressed cells^2 **(C,D)**; initial inocula were at an OD_{600} of 0.5 **(A,C)** and 0.17 **(B,D)**. Strains and serogroups are indicated on the X-axis, and data are presented as the mean ± standard deviation of the BFI. A BFI of 0 represents full blockage of the magnetic beads. *$p < 0.05$.

HMDS (Delta Microscopies, France) twice for 10 min, followed by air-drying overnight at room temperature. Coupons were mounted on stubs using adhesive carbon tabs, sputter-coated with gold-palladium (JFC-1300, JEOL, Japan) and observed with a scanning electron microscope (JEOL 6060-LV, JEOL, Japan) at 5 kV in high-vacuum mode.

Statistical Analysis

A *t*-test was performed on data comparing cold-stressed and cold-adapted cells or positive control and cold-adapted cells to test for statistically significant differences. Correlations were evaluated to identify any effects of cell surface properties on bacterial adhesion and biofilm formation by calculating the Pearson's correlation coefficient. All data were analyzed using Prism 7 software (Graphpad software Inc., United States), and significance was assigned at $p < 0.05$.

RESULTS

Viable Cell Counts

Six stains composed of four different serogroups with diverse origins were selected for viable cell count tests to verify the relationship between OD_{600} values and viable cell numbers. As shown in **Table 2**, the two stationary cultures acclimated to 37 and 4°C resulted in comparable numbers of viable cells, with no significant differences.

Evaluation of Adhesion and Biofilm Formation on a Polystyrene Surface

BRT®

The ability of 22 *L. monocytogenes* strains to adhere to an abiotic surface was analyzed using BRT®. BFI, with a value ranging from 20 to 0, is associated with the extent of blockage of magnetic beads (Toner 4) by sessile bacterial cells at the bottom of polystyrene microplate wells. Therefore, differences in BFI are caused by varying abilities to adhere. All strains exhibited higher adhesion ability when exposed to cold shock by demonstrating lower BFI scores (**Figure 2**). Statistical differences ($p < 0.05$) in BFI values between cold-stressed and cold-adapted cells were observed for 19 strains in either of the two inocula (**Figures 2A,B**). Further tests were performed to verify whether this adhesion phenomenon was reversible. Cold-adapted cells were cultured at 37°C and re-exposed to a sudden temperature downshift (cold-stressed cells^2). Interestingly, the same response was observed for adhesion profiles (**Figures 2C,D**). No significant difference was observed for any strain between cells that were exposed to sudden cold shock for the first time (cold-stressed cells) and the second time (cold-stressed cells^2), demonstrating that enhanced adhesion upon cold exposure represents a transient phenotype switch (Supplementary Figure 1).

When positive control cells were incubated at 37°C, all strains completely blocked the beads, resulting in a BFI of 0 for all inocula (data not shown).

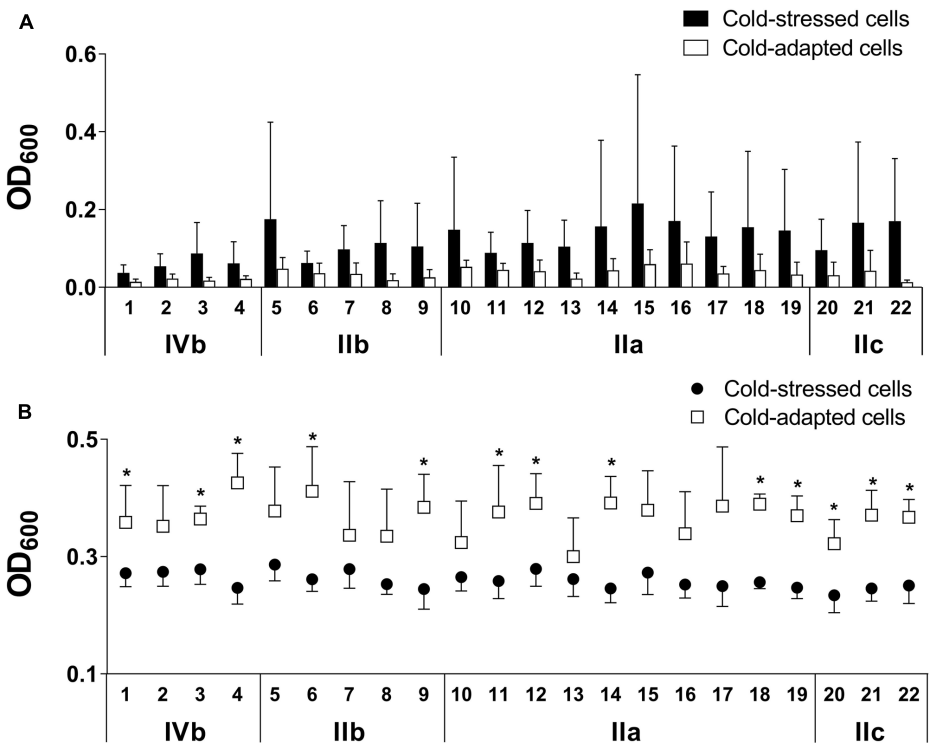


FIGURE 3 | Cold-stressed cells form more biomass than cold-adapted cells **(A)**, while cold-adapted cells exceed total cell densities **(B)** as assessed using microtiter plate assay (MPA). Adherent cells are quantified by CV staining **(A)**, and total cell densities combining planktonic and sessile cells are measured based on the turbidity of wells **(B)**. Strains and serogroups are indicated on the X-axis, and data are presented as the mean ± standard deviation. *$p < 0.05$.

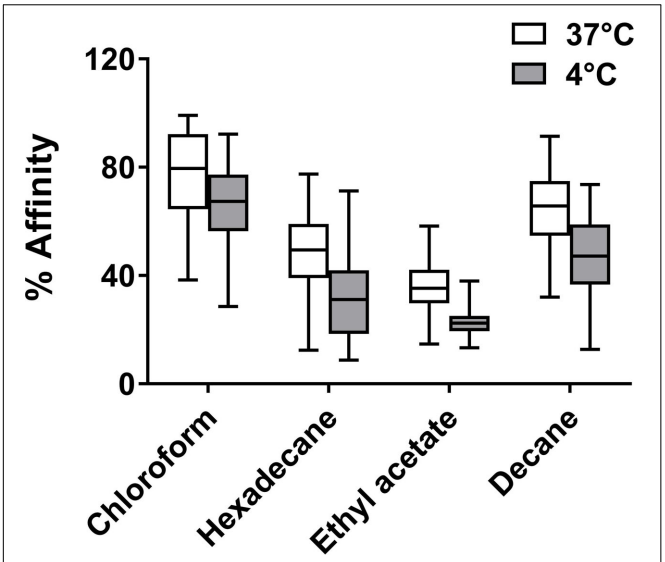


FIGURE 4 | Solvent affinities (%) of all 22 *Listeria monocytogenes* strains in stationary phase at 37 and 4°C. Box plot whiskers indicate minimum and maximum values, and the line in the middle of the box is plotted at the median. The affinity for each solvent under two temperatures differs significantly ($p < 0.01$).

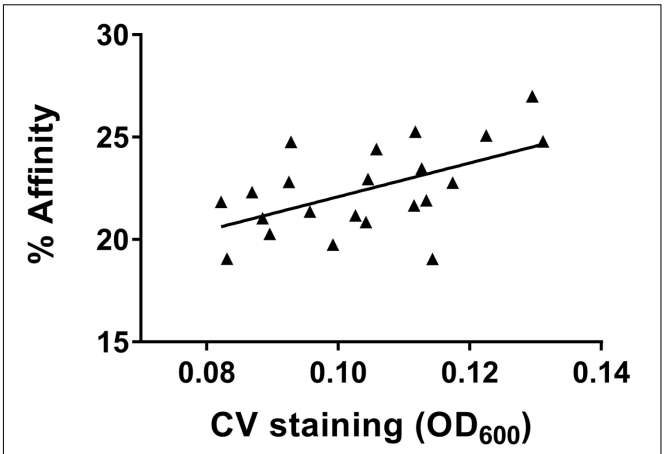


FIGURE 5 | Correlation ($r^2 = 0.3055$, $p < 0.01$) between affinity for ethyl acetate and quantification of adherent cells, measured by CV staining of cold-adapted cells.

MPA

Microtiter plate assay was performed to assess sessile biomasses and total cell densities after incubation for 24 h at 4°C for cold-stressed and cold-adapted conditions and at 37°C for positive control. Much greater biomass quantities were obtained by CV staining for the positive control than for cold-stressed or cold-adapted cells (Supplementary Figure 2).

Cold-adapted cells showed overall higher total cell densities than cold-stressed cells; 13 of the 22 strains were statistically significant ($p < 0.05$, **Figure 3B**). Cold-stressed cells, however, resulted in more sessile bacterial communities than cold-adapted cells (no significance found) as quantified by CV staining (**Figure 3A**). The higher total cell densities obtained for cold-adapted cells were primarily attributable to planktonic cells. Based on these findings, increased cell numbers in cultures did not result in bacterial adhesion, indicating that enhanced adhesion is a distinct feature of cold-stressed cells.

Cell Surface Physicochemical Properties

Positive control and cold-adapted cells, grown at 37 and 4°C until stationary phase, respectively, were prepared for the MATS test to compare the surface physicochemical properties of cells that acclimated to different temperatures. The MATS results obtained for all 22 *L. monocytogenes* grown at 37 and 4°C until stationary phase in BHI media are shown in Supplementary Figure 3.

As shown in **Figure 4**, the general affinity of the 22 *L. monocytogenes* for chloroform (an electron-acceptor solvent) was higher than the affinity for hexadecane (a non-polar solvent), regardless of the culture temperature, indicating the strong electron donor nature of these bacteria. Likewise, bacterial affinity for ethyl acetate (an electron-donating solvent) was lower than for decane (a nonpolar solvent), indicating that the electron-accepting nature of the bacteria grown at either temperature was weak. Affinity for hexadecane decreased from 49 ± 14% at 37°C to 32 ± 15% at 4°C ($p < 0.01$), demonstrating that cell surfaces became relatively more hydrophilic as cells adapted to cold temperature.

Relationships between the affinities for the four solvents and adhesion data obtained via two methods, CV staining and BRT, were evaluated using Pearson's correlation coefficient (r^2). A positive correlation was identified between the adhesion results obtained from CV staining and the affinity for ethyl acetate obtained from the MATS test for cold-adapted cells grown at 4°C ($r^2 = 0.3055$, $p < 0.01$) (**Figure 5**).

SEM Observation

Scanning electron microscopy images of cells grown under all three tested conditions (positive control, cold-stressed, and cold-adapted cells) were obtained to analyze surface colonization patterns and biofilm structures as well as the morphologies of individual cells. Low to high magnifications were applied over several zones. There was greater variance in the maturity of biofilms among strains grown under positive control conditions (**Figure 6**, left column), showing that the biofilm-forming capability of *L. monocytogenes* is strain-dependent. Conversely, under cold-stressed and cold-adapted conditions, the variance among strains was less obvious, primarily because no homogeneous mature biofilms were produced. However, cold-stressed cells underwent surface colonization with cell aggregates (arrowhead) resulting from sessile cell division (**Figure 6**, middle column), while analysis of cold-adapted cells revealed the attachment of individual cells in the absence of noticeable cell clusters (**Figure 6**, right column). Extracellular matrix was observed at high magnification (X 9,000 and higher) among individual cells and between cells and the stainless-steel surface (**Figure 7**, red circle). Irregular cell sizes were observed under all conditions, but significant cell elongation was more

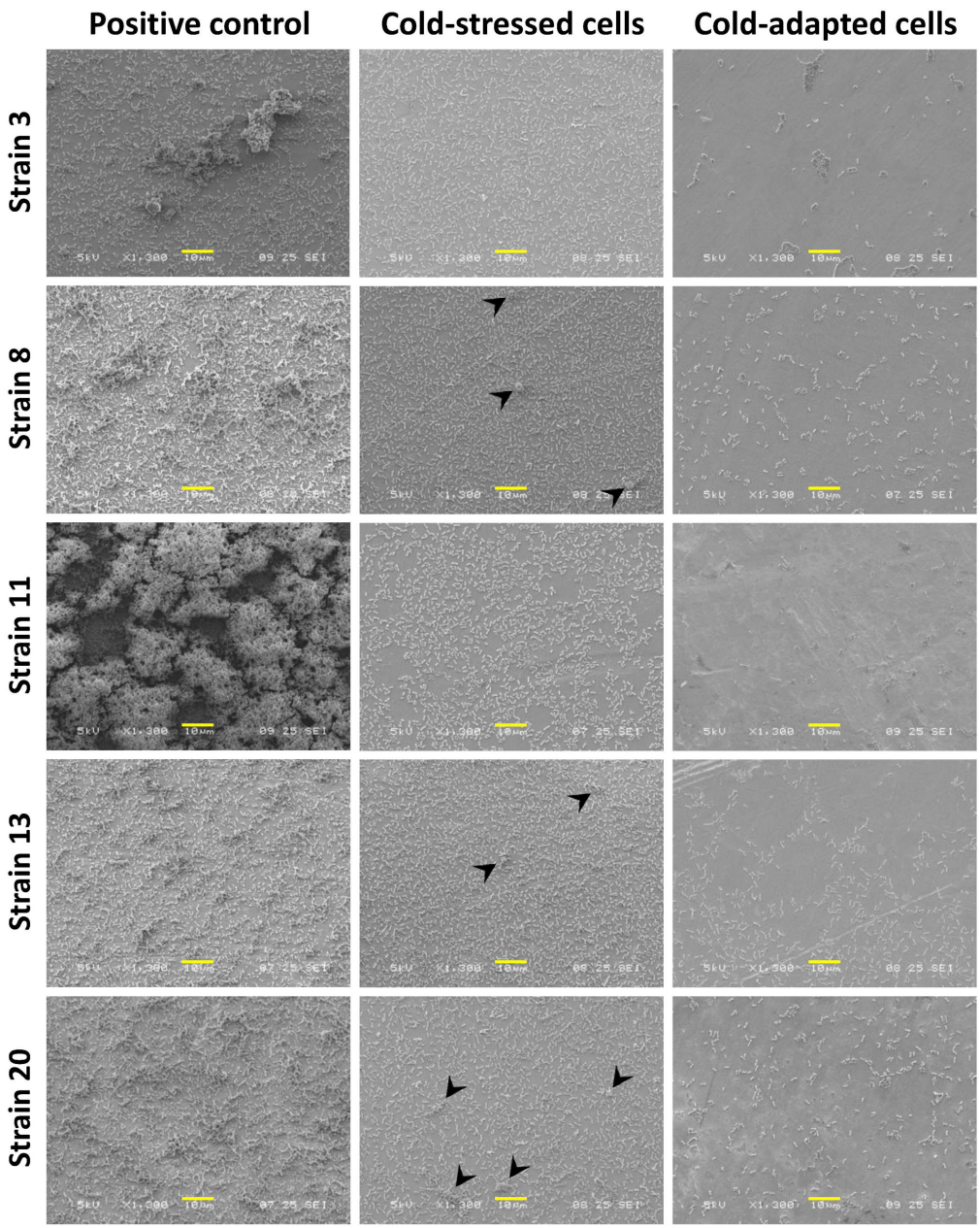


FIGURE 6 | Comparison of *L. monocytogenes* biofilm formation under different conditions. Positive control cells demonstrated great variance in terms of interstrain biofilm formation (left column), while cold-stressed cells formed a single biofilm layer with cell aggregates (arrowhead) (middle column), and cold-adapted cells adhered sparsely on the surface (right column). Scale bars: 10 μm.

frequently noted among cold-stressed and cold-adapted cells (**Figure 7**, arrow). This result may be because positive control cells formed more complex biofilm structures that limited the distinction of individual cell morphologies. Similar to a previous report by Harvey et al., spatial colonization was observed, constituting a network of microcolonies (**Figures 7E,I**) (Harvey et al., 2007). Cells were often found in indented substrate surfaces resulting from scratches on the coupons (**Figure 7**, arrowhead).

DISCUSSION

Certain bacteria adapt to inhabit environments by assuming different forms that are favorable to survival, such as planktonic cells, sessile biofilm communities or spore formation. Biofilms of *L. monocytogenes* in food processing environments are of great concern for food contamination. *L. monocytogenes* adapts to the harsh environments employed by food processing facilities, such as antibacterial agents or refrigeration, and reports have

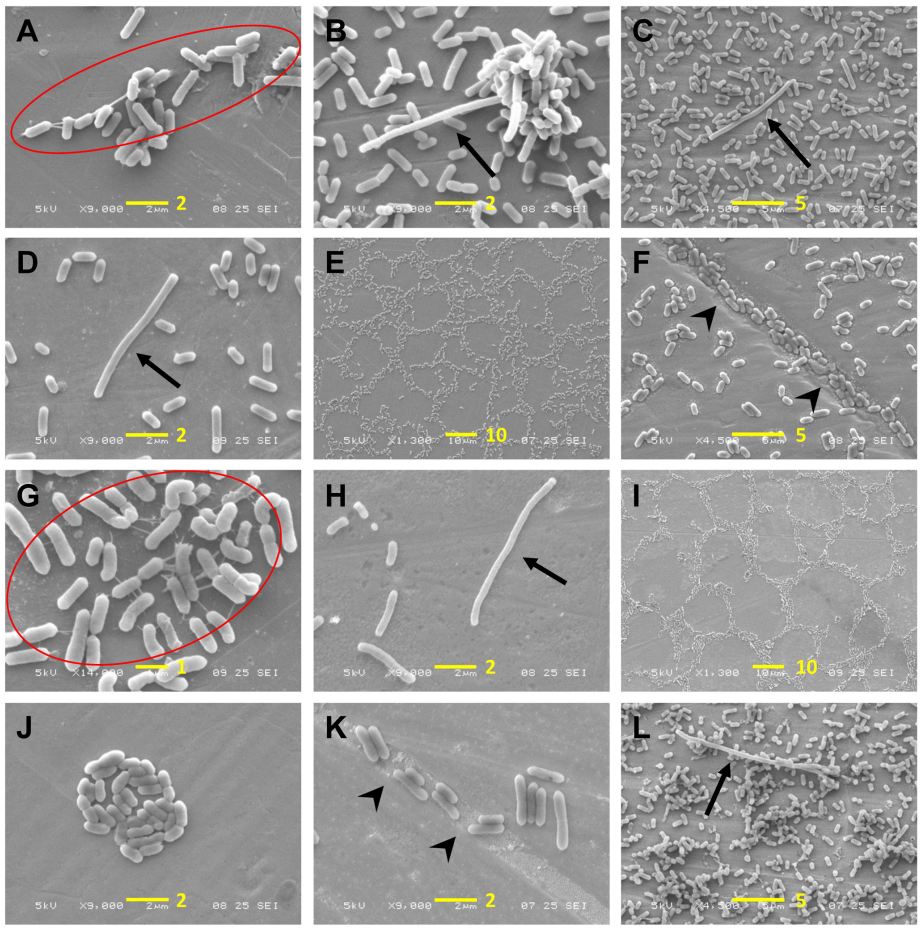


FIGURE 7 | Observation of *L. monocytogenes* adhesion patterns and cellular morphology. **(A–F)** Cold-stressed cells; **(G–K)** cold-adapted cells; **(L)** positive control cells. EPS are marked in red circles, arrows indicate elongated cells and arrowheads indicate cells in crevices. A scale bar (length in μm) is indicated in yellow in each figure.

demonstrated that exposing bacteria to sublethal stress leads to cross-protection or cross-adaptation to various stresses and lethal factors (Lou and Yousef, 1997; Lundén et al., 2003). Biofilm production by *L. monocytogenes* is stimulated to protect against various stressful conditions, making bacterial elimination a serious challenge at food processing facilities (Giaouris et al., 2014).

Significant variation in biofilm production under various conditions was observed for one *L. monocytogenes* strain, indicating that intra-strain phenotype changes are dependent on experimental settings (Nowak et al., 2015). Moreover, the interstrain variability of biofilm formation has been extensively studied with a focus on its relationships to serogroups or persistence in the food industry. However, a study employing 143 *L. monocytogenes* strains indicated that experimental settings such as temperature and culture media affect the comprehension of biofilm formation and its relationship to serotype or origin (Kadam et al., 2013). Recently, a study of 98 *L. monocytogenes* strains revealed no correlation between serological groups and biofilm production (Doijad et al., 2015).

Numerous methods and devices have been developed to detect or quantify biofilms, including staining-based quantification methods, visual identification by microscopy, viable and culturable cell counts, and devices to test bacterial adhesion (Azeredo et al., 2017). BRT® is a microbial adhesion test that is primarily used to assess the simultaneous phenotype switch from planktonic cells into sessile cells. In BRT®, microorganisms are added to microplate wells in planktonic form, some of which adhere to the bottoms of the wells during incubation and switch to a sessile form that hinders the magnetic beads attracted to the magnetic block. Results vary with experimental conditions that affect the process, including planktonic cell growth and adhesion to polystyrene microplates, as well as sessile cell growth. Recently, a new approach to BRT®, designated cBRT®, was developed using serial 10-fold dilutions of bacterial suspensions to better discriminate biofilm-forming abilities among strains (Di Domenico et al., 2016). However, in the current study, all 22 *L. monocytogenes* strains revealed homogenous adhesion behavior that was not discernible by cBRT® under the same experimental conditions, i.e., cold-adapted or cold-stressed

conditions or incubation at 37°C. Nevertheless, cBRT® was sensitive for discriminating between cold-stressed and cold-adapted cells in terms of bacterial adhesion.

Cells undergo cold shock when subjected to a sudden downshift in temperature. Such a rapid environmental change induces modifications in bacterial cell surface proteins and lipid composition to maintain membrane fluidity homeostasis, which presumably facilitates adhesion as an adaptation strategy against adverse conditions. This behavior may be advantageous for bacterial survival in FPEs where cells might be exposed to sudden cold shock. The current study employed preculture temperatures of 37 and 4°C to compare differences in adhesion characteristics upon further exposure to 4°C. BRT® and MPA results revealed that cold-stressed cells (precultured at 37°C) are more efficient at forming biofilms, while cold-adapted cells (precultured at 4°C) favor growth in the planktonic state. *L. monocytogenes* cells entering the food processing chain are exposed to temperature downshifts, such as ambient temperature in outdoor food materials or optimal temperature in infected animals to refrigeration temperatures used during food processing or storage. When introduced to the food processing chain, *L. monocytogenes* adhesion to food contact surfaces is potentially fortified by cold shock, which will increase the chance of food product contamination. Once adapted to the cold, the bacteria in final food products will proliferate to hazardous levels during distribution and storage.

The heterogeneity of a population contributes to the adaptation of *L. monocytogenes* to sublethal conditions, accompanied by phenotypic and genetic changes (Abee et al., 2016). When cold-adapted cells were returned to 37°C and re-exposed to cold, they exhibited the same enhanced adhesion, which was indistinguishable by BRT® (Supplementary Figure 1), demonstrating that this transient trait was reacquired when cold stress was removed.

All 22 strains retained basic (electron-donating) properties (a higher affinity for chloroform than hexadecane), regardless of growth temperature, and became more hydrophilic with decreased temperature, as previously described (Chavant et al., 2002). The adhesion data obtained from CV staining of all 22 *L. monocytogenes* correlated best with cell affinity for ethyl acetate under cold-adapted conditions (**Figure 5**). This finding aligned with that of Briandet et al., who observed a linear correlation between listerial adhesion to stainless-steel and an affinity for ethyl acetate at different temperatures (37, 20, 15, and 8°C) in the presence of NaCl (Briandet et al., 1999). Physicochemical properties, including the hydrophobicity of bacterial cells versus that of a substratum, affect the interfacial interactions involved in bacterial attachment to abiotic surfaces. Nonetheless, this trait is negligible in the context of building a mature biofilm structure that is highly dependent on bacterial growth kinetics and EPS production, thus explaining the absence of an obvious relationship between cellular surface properties and biofilm formation at 37°C in the current study and in the literature (Cunliffe et al., 1999; Chmielewski and Frank, 2003).

Scanning electron microscopy observations supported the quantitative results obtained with CV staining and BRT®. **Figure 6** shows variable biofilm maturity among the strains. This variability may be attributable to differential biofilm production capabilities, although divergent biofilm kinetics among strains are unable to be excluded. Some strains may have already begun to disperse, while others were still in the process of structuring mature biofilms. SEM observation confirmed the higher bacterial adhesion of cold-stressed cells, along with the formation of cellular aggregates, while cold-adapted cells were only able to form a single sparse layer of adherent cells. This finding aligns with the BRT® results, demonstrating that BRT® is applicable for testing the early step of biofilm formation. Moreover, our observations of cells that densely accumulated in the crevices and scratches of stainless-steel surfaces strongly support the thorough cleaning of food contact surfaces to eliminate bacteria, although this may also further damage surfaces and create additional niches for bacterial adhesion.

In the current study, enhanced adhesion of sudden cold-stressed *L. monocytogenes* cells was observed for the first time. CV staining and SEM observation revealed that *L. monocytogenes* possesses dramatic interstrain variances in biofilm production, independent of origin or serotype. BRT® is shown to be a sensitive tool to discern the first layer of biofilm formation, facilitating the detection of increased adhesion of cold-stressed cells in the current study. Interestingly, the adhesion of cold-adapted cells correlated with an affinity for ethyl acetate. Further study of cold-stressed cells during cross-adaptation to other stress factors, such as dehydration or antimicrobial agents, will add to our understanding of the behavior of *L. monocytogenes* in the food processing industry.

AUTHOR CONTRIBUTIONS

B-HL conceived, designed, and conducted experiments, analyzed the results, and drafted the manuscript. All authors contributed to the experimental design and reviewed and approved the final manuscript.

FUNDING

This project received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant agreement N° 641984.

ACKNOWLEDGMENTS

Our thanks to Brigitte Gaillard-Martinie for SEM sample preparation at INRA, Saint-Genès-Champanelle, and Christelle Blavignac for her assistance with SEM technologies at the Centre Imagerie Cellulaire Santé (Université Clermont Auvergne).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02221/full#supplementary-material

REFERENCES

Abee, T., Koomen, J., Metselaar, K. I., Zwietering, M. H., and den Besten, H. M. W. (2016). Impact of pathogen population heterogeneity and stress-resistant variants on food safety. *Annu. Rev. Food Sci. Technol.* 7, 439–456. doi: 10.1146/annurev-food-041715-033128

Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., et al. (2017). Critical review on biofilm methods. *Crit. Rev. Microbiol.* 43, 313–351. doi: 10.1080/1040841X.2016.1208146

Bellon-Fontaine, M.-N., Rault, J., and van Oss, C. J. (1996). Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids Surf. B Biointerfaces* 7, 47–53. doi: 10.1016/0927-7765(96)01272-6

Brauge, T., Sadovskaya, I., Faille, C., Benezech, T., Maes, E., Guerardel, Y., et al. (2016). Teichoic acid is the major polysaccharide present in the *Listeria monocytogenes* biofilm matrix. *FEMS Microbiol. Lett.* 363:fnv229. doi: 10.1093/femsle/fnv229

Briandet, R., Meylheuc, T., Maher, C., and Bellon-Fontaine, M. N. (1999). *Listeria monocytogenes* Scott A: cell surface charge, hydrophobicity, and electron donor and acceptor characteristics under different environmental growth conditions. *Appl. Environ. Microbiol.* 65, 5328–5333.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M., and Bernardi, T. (2007). A new device for rapid evaluation of biofilm formation potential by bacteria. *J. Microbiol. Methods* 68, 605–612. doi: 10.1016/j.mimet.2006.11.010

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.-N., and Hebraud, M. (2002). *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. *Appl. Environ. Microbiol.* 68, 728–737. doi: 10.1128/AEM.68.2.728-737.2002

Chmielewski, R. A. N., and Frank, J. F. (2003). Biofilm formation and control in food processing facilities. *Compr. Rev. Food Sci. Food Saf.* 2, 22–32. doi: 10.1111/j.1541-4337.2003.tb00012.x

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., and Ianieri, A. (2016). A look inside the *Listeria monocytogenes* biofilms extracellular matrix. *Microorganisms* 4:22. doi: 10.3390/microorganisms4030022

Cunliffe, D., Smart, C. A., Alexander, C., and Vulfson, E. N. (1999). Bacterial adhesion at synthetic surfaces. *Appl. Environ. Microbiol.* 65, 4995–5002.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., et al. (2008). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *J. Appl. Microbiol.* 104, 1552–1561. doi: 10.1111/j.1365-2672.2007.03688.x

Di Domenico, E. G., Toma, L., Provot, C., Ascenzioni, F., Sperduti, I., Prignano, G., et al. (2016). Development of an in vitro assay, based on the biofilm ring test®, for rapid profiling of biofilm-growing bacteria. *Front. Microbiol.* 7:1429. doi: 10.3389/fmicb.2016.01429

Doijad, S. P., Barbuddhe, S. B., Garg, S., Poharkar, K. V., Kalorey, D. R., Kurkure, N. V., et al. (2015). Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. *PLOS ONE* 10:e0137046. doi: 10.1371/journal.pone.0137046

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., and Martin, P. (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.* 42, 3819–3822. doi: 10.1128/JCM.42.8.3819-3822.2004

Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J. Food Prot.* 77, 150–170. doi: 10.1128/JCM.42.8.3819-3822.2004

Garrett, T. R., Bhakoo, M., and Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Prog. Nat. Sci.* 18, 1049–1056. doi: 10.1016/j.pnsc.2008.04.001

Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., et al. (2015). Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. *Front. Microbiol.* 6:841. doi: 10.3389/fmicb.2015.00841

Giaouris, E., Heir, E., Hébraud, M., Chorianopoulos, N., Langsrud, S., Møretrø, T., et al. (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Sci.* 97, 298–309. doi: 10.1016/j.meatsci.2013.05.023

Harvey, J., Keenan, K. P., and Gilmour, A. (2007). Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiol.* 24, 380–392. doi: 10.1016/j.fm.2006.06.006

Jofré, A., Garriga, M., Aymerich, T., Pérez-Rodríguez, F., Valero, A., Carrasco, E., et al. (2016). Closing gaps for performing a risk assessment on *Listeria monocytogenes* in ready-to-eat (RTE) foods: activity 1, an extensive literature search and study selection with data extraction on *L. monocytogenes* in a wide range of RTE food. *EFSA Support. Publ.* 13:E1141. doi: 10.2903/sp.efsa.2016.EN-1141

Kadam, S. R., den Besten, H. M. W., van der Veen, S., Zwietering, M. H., Moezelaar, R., and Abee, T. (2013). Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. *Int. J. Food Microbiol.* 165, 259–264. doi: 10.1016/j.ijfoodmicro.2013.05.025

Liu, D., Lawrence, M. L., Ainsworth, A. J., and Austin, F. W. (2005). Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiol. Lett.* 243, 373–378. doi: 10.1016/j.femsle.2004.12.025

Lou, Y., and Yousef, A. E. (1997). Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Appl. Environ. Microbiol.* 63, 1252–1255.

Lundén, J., Autio, T., Markkula, A., Hellström, S., and Korkeala, H. (2003). Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. *Int. J. Food Microbiol.* 82, 265–272. doi: 10.1016/s0168-1605(02)00312-4

Nilsson, R. E., Ross, T., and Bowman, J. P. (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *Int. J. Food Microbiol.* 150, 14–24. doi: 10.1016/j.ijfoodmicro.2011.07.012

Nowak, J., Cruz, C. D., Palmer, J., Fletcher, G. C., and Flint, S. (2015). Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions. *J. Microbiol. Methods* 119, 189–195. doi: 10.1016/j.mimet.2015.10.022

Orsi, R. H., den Bakker, H. C., and Wiedmann, M. (2011). *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol.* 301, 79–96. doi: 10.1016/j.ijmm.2010.05.002

Slama, R. B., Bekir, K., Miladi, H., Noumi, A., and Bakhrouf, A. (2012). Adhesive ability and biofilm metabolic activity of *Listeria monocytogenes* strains before and after cold stress. *Afr. J. Biotechnol.* 11, 12475–12482. doi: 10.5897/AJB11.3939

Tasara, T., and Stephan, R. (2006). Cold stress tolerance of *Listeria monocytogenes*: a review of molecular adaptive mechanisms and food safety implications. *J. Food Prot.* 69, 1473–1484. doi: 10.4315/0362-028x-69.6.1473

Vongkamjan, K., Fuangpaiboon, J., Turner, M. P., and Vuddhakul, V. (2016). Various ready-to-eat products from retail stores linked to occurrence of diverse *Listeria monocytogenes* and *Listeria* spp. Isolates. *J. Food Prot.* 79, 239–245. doi: 10.4315/0362-028X.JFP-15-361

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Lee, Hébraud and Bernardi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 November 2017
doi: 10.3389/fmicb.2017.02193

Characterization of Four Novel Bacteriophages Isolated from British Columbia for Control of Non-typhoidal *Salmonella in Vitro* and on Sprouting Alfalfa Seeds

*Karen Fong[1], Brett LaBossiere[1], Andrea I. M. Switt[2], Pascal Delaquis[3], Lawrence Goodridge[4], Roger C. Levesque[5], Michelle D. Danyluk[6] and Siyun Wang[1]**

[1] Food, Nutrition, and Health, University of British Columbia, Vancouver, BC, Canada, [2] Escuela de Medicina Veterinaria, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, Santiago, Chile, [3] Agriculture and Agri-Food Canada, Summerland, BC, Canada, [4] Department of Food Science and Agricultural Chemistry, McGill University, Montreal, QC, Canada, [5] Institute for Integrative and Systems Biology, Université Laval, Québec City, QC, Canada, [6] Department of Food Science and Human Nutrition, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, United States

OPEN ACCESS

Edited by:
Maria Schirone, Università di Teramo, Italy

Reviewed by:
Sunil D. Saroj, Symbiosis International University, India
Sanna Sillankorva, University of Minho, Portugal

***Correspondence:**
Siyun Wang
siyun.wang@ubc.ca

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *10 August 2017*
Accepted: *25 October 2017*
Published: *15 November 2017*

Citation:
Fong K, LaBossiere B, Switt AIM, Delaquis P, Goodridge L, Levesque RC, Danyluk MD and Wang S (2017) Characterization of Four Novel Bacteriophages Isolated from British Columbia for Control of Non-typhoidal Salmonella in Vitro and on Sprouting Alfalfa Seeds. Front. Microbiol. 8:2193. doi: 10.3389/fmicb.2017.02193

Alfalfa sprouts have been linked to numerous North American outbreaks of *Salmonella* in recent years. Conventionally, treatments involving chlorine, heat, and irradiation are used for alfalfa seed sanitation. However, such treatments may be highly variable in their efficacy for pathogen control and/or detrimental to sprout quality, therefore negatively perceived by consumers advocating for natural alternatives. The usage of bacteriophages for pathogen control in sprouts has been previously explored, although with conflicting and inconsistent results. Lytic phages, viral predators of bacteria, represent an attractive approach as they provide several advantages compared to conventional treatments, such as their high specificity for bacterial targets and their ubiquity in nature. In this study, four *Salmonella* phages were isolated from British Columbia, Canada and characterized with respect to host range, burst size, latent period, and environmental stability to assess their potential to control *Salmonella*. Phage isolate SI1 showed the greatest host range, highest burst size and shortest latent period, greatest stability across all pH and temperatures and was the most effective in control of *S.* Enteritidis *in vitro*. Therefore, SI1 was chosen for treatment of sprouting alfalfa seeds artificially contaminated with *S.* Enteritidis with a multiplicity of infection (MOI) of ∼110 PFU/CFU. A significant ($p < 0.05$) reduction of 38.3 ± 3.0% of viable *Salmonella* cells was observed following two h of phage treatment. On days two to six of the sprouting process, reductions of *Salmonella* were also observed, but were not significant compared to the control ($p > 0.05$). It was further demonstrated that the sprout yield was not significantly ($p > 0.05$) affected by phage treatment. These results highlight the potential of phages recovered from the British Columbia environment for use as biocontrol agents against *Salmonella*, although differing efficacies *in vitro* was observed.

Moreover, the effectiveness of SI1 to significantly ($p < 0.05$) control *Salmonella* on sprouting alfalfa seeds on day 1 of treatment was demonstrated. Although promising, future work should aim to optimize this treatment to achieve more effective, and longer lasting, biocontrol of *Salmonella* in sprouting alfalfa seeds.

Keywords: *Salmonella*, bacteriophage, biocontrol, sprouts, food safety

INTRODUCTION

Foodborne diseases caused by non-typhoidal *Salmonella* represent a significant public health burden worldwide. It is estimated that 80.3 million cases arise yearly from food products contaminated by *Salmonella*, resulting in over 100, 000 deaths (Majowicz et al., 2010). Although particular foods (e.g., poultry, eggs, and swine) have been historically classified as definitive causative agents for salmonellosis, emerging disease transmission vectors (e.g., fresh produce) have been associated with numerous outbreaks in recent years (Centers for Disease Control and Prevention [CDC], 2017).

In North America, seed sprouts are becoming increasingly popular amongst consumers as they are nutrient-dense, versatile, and relatively inexpensive (Pérez-Balibrea et al., 2008). Unfortunately, an increase in the number of *Salmonella* outbreaks has been attributed to the consumption of raw seeds sprouts (Centers for Disease Control and Prevention [CDC], 2017), with the majority of outbreaks linked to alfalfa sprouts (Proctor et al., 2001; Kumar et al., 2006). A variety of *Salmonella* serotypes have been associated with sprouts-related illnesses, including common serotypes (e.g., *S.* Enteritidis, *S.* Typhimurium and *S.* 4,[5],12:i:-) and less common serotypes (e.g., *S.* Agona, *S.* Reading, and *S.* Abony) (Centers for Disease Control and Prevention [CDC], 2017).

Contamination of seeds used for sprouts production is likely to occur in the field (Food and Drug Administration [FDA], 2015). Once contaminated, sprouting seeds provide an ideal habitat for support and growth of *Salmonella* (Fahey et al., 2006). During the sprouting process, enhanced humidity, warm temperatures, and the release of nutrients from the seed itself, result in the rapid proliferation of *Salmonella* (Fahey et al., 2006); an increase of over three log CFU/g of viable *Salmonella* during the sprouting of alfalfa seeds has been reported (Charkoswski et al., 2002).

A variety of intervention strategies are available to disinfect sprouts seeds, including the usage of chemical disinfectants (e.g., hypochlorite, calcium hydrogen peroxide), heat, and irradiation (National Advisory Committee on Microbiological Criteria for Foods [NACMCF], 1999). The reliability and consistent implementation of these treatments, however, has been questioned as sprouts-related illnesses continue to rise. In Canada, seed sanitation is not required by law (Canadian Food Inspection Agency [CFIA], 2014), although it is recommended that a seed treatment capable of attaining a minimum three-log reduction be considered (Canadian Food Inspection Agency [CFIA], 2014). However, negative consumer perceptions regarding chemical and physical treatments and its potentially negative impacts on the yield and quality of sprouts may hinder the widespread adoption of such treatments (Kim et al., 2003). Additionally, the efficacy of these treatments has been shown to be highly variable (Montville and Schaffner, 2004). For instance, the reference standard for seed disinfection, 20,000 ppm calcium hypochlorite, has resulted in variable microbial reductions of 0.51 – 6.90 log CFU/g (Ding et al., 2013). Disinfection with other chemicals alternative chemicals is also highly variable regarding their microbial kill; electrolyzed oxidizing water previously achieved reductions of 1.66 log CFU/g (Kim et al., 2003), while 5% acetic acid achieved reductions of 2.40 log CFU/g (Lang et al., 2000). Lastly, physical inactivation methods are gaining interest; soaking seeds in hot water at 85°C for 10 s was reported to achieve a 3.0 log CFU/g reduction (Bari et al., 2010). High pressure for 500 MPa for two min similarly achieved a 3.5 log CFU/g reduction (Neetoo and Chen, 2010). However, physical treatments have been shown to inhibit the germination rate and may not be commercially viable methods for disinfection (Bari et al., 2009). Therefore, alternative measures are urgently needed for effective, clean-label decontamination methods, without negatively impacting seed viability.

Bacteriophages (phages) are viral predators of bacteria (Hagens and Loessner, 2007) that have attracted considerable interest as a method for pathogen control on foods. Previous research has evaluated its use on a variety of foodstuffs, including chicken skin (Goode et al., 2003), broccoli and mustard sprout seeds (Pao et al., 2004), fresh-cut produce (Leverentz et al., 2001) and cheddar cheese (Modi et al., 2001). Phages possess several properties that render them suitable for use on food because they are: (i) highly specific, not crossing species or genus barriers; (ii) designed to kill host cells only; (iii) self-replicating and self-limiting; and (iv) ubiquitously distributed in nature (Rohwer and Edwards, 2002). Despite the range of desirable attributes, however, the usage of phages as biocontrol agents for *Salmonella* in sprouts has not been widely adopted, although there have been previous reports of similar efforts (Pao et al., 2004; Kocharunchitt et al., 2009). At an initial density of approximately seven log CFU/g of *S.* Oranienburg, relatively low log reductions of approximately one log CFU/g of *Salmonella* were achieved with phage SSP6 (Kocharunchitt et al., 2009). Additionally, Pao et al. (2004) reported a 1.50 log CFU/g reduction of *Salmonella* upon application of a bacteriophage cocktail on broccoli seeds artificially contaminated with an initial density of 7–7.5 log CFU/g. The relatively low reduced efficacies may have been due to the limited number of effective phages recovered, and/or the failure to adequately characterize phages for this particular purpose. The objectives of this study were to characterize four broad-host range *Salmonella* phages on the basis of their phenotypic and genotypic determinants,

assess their infectivity against various *Salmonella* strains *in vitro*, and evaluate the efficacy of a promising phage isolate, SI1, for biocontrol of *S.* Enteritidis on alfalfa seeds throughout the sprouting process.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Salmonella enterica serotype Enteritidis FSL S5-483 was used as the bacterial host for the phages in this study. The *Salmonella* strains used in the host range study are listed in **Table 1**. All strains were maintained at −80°C in Brain-Heart-Infusion broth (BD/Difco, East Rutherford, NJ, United States) supplemented with 20% glycerol. Working stocks were maintained on tryptic soy agar (TSA; BD/Difco, East Rutherford, NJ, United States) at 4°C for a maximum of 1 month. Prior to each experiment, fresh overnight cultures were prepared by inoculating an isolated colony into 10 ml tryptic soy broth (TSB; BD/Difco, East Rutherford, NJ, United States). Cultures were then incubated for 16 h at 37°C with gentle shaking at 170 rpm.

Bacteriophage Isolation and Purification

Bacteriophages were isolated from irrigation water ($n = 15$), cattle feces ($n = 9$) and sediment obtained from the bottom of irrigation ditches ($n = 8$) from Greater Vancouver, British Columbia, Canada. *S.* Enteritidis FSL S5-483 was used as an indicator organism for phage isolation. Of the phages recovered, four broad-host range phages were isolated: SI1 (from irrigation water), SF1 (from cattle feces) and SS1 and SS4 (from sediment). Phage SI1 was isolated following direct plating by mixing 10 g of sample with 90 ml of salt-magnesium (SM) buffer (0.05 M Tris-HCl; 0.1 M NaCl and 0.01 M $MgSO_4$; pH 7.5), followed by passage through a 0.45 μm membrane (Pall Corporation, Port Washington, NY, United States). Then, 100 μl of filtrate was mixed with 300 μl of 1:10 diluted *S.* Enteritidis grown to 16 h and four ml 0.7% TSA top agar, according to the double agar overlay method (Adams, 1959).

Phages SF1, SS1, and SS4 were isolated following enrichment by mixing 10 g of water, cattle feces or sediment samples, 90 ml TSB and 1 ml of *S.* Enteritidis cells grown to 16 h, followed by incubation at 37°C for 22 ± 2 h. The enriched samples were then spun at 4,000 × *g* and the supernatant subsequently passed through a 0.45 μm filter membrane (Pall Corporation). Then, 100 μl of filtrate was mixed with 300 μl of 1:10 diluted *S.* Enteritidis grown to 16 h and four ml 0.7% TSA top agar, according to the double agar overlay method (Adams, 1959).

Plates were incubated at 37°C for 18 ± 2 h for visualization of plaques. Plaques were lifted from the agar surface using a truncated pipette tip, suspended in 200 μl SM buffer, and rested for at least 6 h at room temperature. Double agar overlays were prepared with the suspension as described previously (Adams, 1959). Three single plaque isolations were carried out to obtain a pure phage lysate. Finally, phages were concentrated and stored at 4°C for further analyses.

TABLE 1 | Host ranges of SI1, SS1, SS4 and SF1.

Salmonella strain	SI1	SF1	SS1	SS4
S. Enteritidis FSL S5-483[a] (host)	4	4	4	4
S. Thompson FSL S5-523[a]	0	0	0	0
S. Braenderup FSL S5-373[a]	0	0	0	0
S. Muenchen FSL S5-504[a]	0	0	0	0
S. Montevideo FSL S5-630[a]	0	0	0	0
S. Saintpaul FSL S5-649[a]	3	3	2	3
S. Typhimurium FSL S5-536[a]	4	2	0	3
S. Javiana FSL S5-406[a]	0	0	0	2
S. Senftenberg FSL S5-658[a]	0	0	0	0
S. Mbandaka FSL S5-451[a]	0	0	0	0
S. Agona FSL S5-517[a]	4	2	3	4
S. Newport FSL S5-436[a]	0	0	0	0
S. Typhimurium LMFS-JF-001[b]	4	4	4	4
S. Newport S2[c]	0	0	0	0
S. Newport S195[c]	0	0	0	0
S. Enteritidis S3[c]	4	4	4	4
S. Enteritidis S187[c]	3	3	4	4
S. Canada S30[c]	4	4	4	4
S. Chingola S32[c]	0	0	0	0
S. Luciana S43[c]	0	0	0	0
S. Typhimurium S189[c]	4	4	4	4
S. Heidelberg S191[c]	3	3	4	4
S. Thompson S193[c]	0	0	1	1
S. Thompson S194[c]	0	0	1	1
S. Infantis S198[c]	0	0	0	0
S. Javiana S200[c]	3	3	4	4
S. Javiana S203[c]	3	3	4	4
S. Saintpaul S204[c]	3	3	2	4
S. Saintpaul S205[c]	3	3	2	4
S. Muenchen S206[c]	0	0	0	0
S. Muenchen S207[c]	0	0	0	0
S. Agona S213[c]	2	2	0	0
S. Agona S215[c]	4	4	3	4
S. Oranienburg S216[c]	0	0	0	0
S. Hadar S219[c]	0	0	0	0
S. Mbandaka S236[c]	0	0	0	0
S. Mbandaka S238[c]	0	0	0	0
S. Montevideo S239[c]	0	0	0	0
S. Montevideo S241[c]	0	0	0	0
S. Bareilly S258[c]	0	0	0	0
S. Senftenberg S269[c]	0	0	0	0
S. Senftenberg S270[c]	0	0	0	0
S. Litchfield S272[c]	0	0	0	0
S. Litchfield S273[c]	0	0	0	0
S. Uganda S276[c]	0	0	0	0
S. Uganda S277[c]	0	0	0	0
S. Havana S286[c]	0	0	0	0
S. Poona S306[c]	0	0	0	0
S. Poona S307[c]	0	0	0	0
S. Ohio S316[c]	0	0	0	0
S. Berta S333[c]	4	1	4	4
S. Liverpool S346[c]	0	0	0	0
S. Rubislaw S348	0	0	0	0

(Continued)

TABLE 1 | Continued

Salmonella strain	SI1	SF1	SS1	SS4
S. Typhimurium S441[c]	4	4	4	4
S. Anatum S443[c]	0	0	0	0
S. Typhimurium LT2[d]	3	3	4	3
4, 5, 12:I:- FSL S5-580[a,e]	4	4	3	4
S. Typhimurium 14028S 1-5[e]	3	3	4	3
S. Typhimurium var. Copenhagen FSL S5-786[a,f]	1	1	0	0
S. Typhimurium SL1344[g]	3	4	4	3
S. Schwarzengrund FSL S5-458[a,h]	0	0	0	0

Salmonella strains susceptible to phage infection are indicated by a +3 or +4 clearing. [a]*ILSI North America Collection;* [b]*strain isolated from irrigation water in the Lower Mainland, British Columbia, Canada;* [c]*the Salmonella Foodborne SystOMICS database (SalFoS) collection;* [d]*Strain resistant to STR (Lilleengen, 1950);* [e]*strain resistant to AMC, AMP, FOX, CHL, STR, SUF, TET;* [f]*strain resistant to AMC, AMP, FOX, CRO, CHL, KAN, STR, SUF, TET;* [g]*strain resistant to STR (Hoiseth and Stocker, 1981);* [h]*strain resistant to AMP, CIP, NAL, SUF, TET, SXT.*

Phage Host Range Determination and Lysis from without

Prior to host range determination, phage lysates were standardized to a concentration of 10^9 PFU/ml as recommended by Khan Mirzaei and Nilsson (2015). The host ranges of the phages were tested by spotting 5 μl of lysate, in duplicate, on a lawn of *Salmonella* host cells grown to 16 h ($n = 61$, **Table 1**). To test for the presence of lysis from without (LO), successive 10-fold dilutions of the phage were also prepared in sterile SM buffer and 5 μl of each dilution spotted in duplicate on the *Salmonella* lawn. Plates were incubated at 37°C for 18 ± 2 h. Zones of clearing were characterized with a scaling system as described by Kutter (2009), where 0 indicated a zone with complete turbidity (no lysis) and +4 indicated a completely clear zone with no turbidity (**Table 1**). LO was detected where complete lysis (+4 ranking) was observed at low dilutions (i.e., 10^{-1} PFU/ml), yet no lysis was observed at further dilutions (Kutter, 2009).

Transmission Electron Microscopy

High titer phage lysates (10^9 – 10^{11} PFU/ml) were chosen for transmission electron microscopy (TEM) and prepared for imaging as described previously (Deveau et al., 2006), with modifications. Briefly, one ml of lysate was spun at 4°C for 1.5 h at 21,000 × g. The supernatant was subsequently discarded and the last 100 μl was saved. Consequently, 1 ml of 0.1 M ammonium acetate (Amresco, Solon, OH, United States) was added and the suspension subsequently spun again at 4°C for 1.5 h at 21,000 × g. This purification was repeated twice, with the last 100 μl reserved for TEM.

For grid preparation, 3 μl of purified lysates were placed on carbon coated copper grids (Ted Pella, Redding, CA, United States) following glow-discharge. The phage preparations were subsequently negatively stained with 2% phosphotungstic acid (Ted Pella). A Hitachi H-7600 transmission electron microscope was used for acquiring the images at the University of British Columbia Bioimaging Facility. An accelerating voltage of 80 kV was used for imaging.

Phage Genome Content Determination and Restriction Enzyme Analysis

Prior to nucleic acid extraction, DNase I (Invitrogen, Carlsbad, CA, United States) and RNAse A (Invitrogen) were added to high titer phage lysates (10^9 – 10^{11} PFU/ml) to final concentrations of 10 and 55 μg/ml, respectively, for degradation of host nucleic acid (Merabishvili et al., 2014), followed by incubation at 37°C for 30 min. Phage nucleic acid was then extracted with the PureLink Viral RNA/DNA Mini Kit (Thermo Fisher) as per the manufacturer's instructions. The concentration and quality of the extracted nucleic acid was determined with a Nanodrop spectrophotometer (Thermo Fisher), where an A260/280 ratio of ∼1.8 and A260/230 ratio of ∼2.0 were considered as pure.

Restriction enzyme analysis was conducted to confirm the unique identities of the phages. Nucleic acid was digested with *EcoR*1 (New England Biolabs, Ipswich, MA, United States) according to the manufacturer's instructions. Subsequently, 10 μl volumes of the nucleic acid digests were loaded onto a 1% agarose gel (Amresco) and electrophoresed in 1X TAE buffer (Thermo Fisher) at 80 V for approximately 1 h. Band patterns were visualized using the ChemiDoc MP System (Bio-Rad Laboratories).

Single Step Growth Curves

Single step growth curves were constructed to determine the phage burst sizes and burst times, according to Park et al. (2012), with modifications. Cultures of *S.* Enteritidis were grown for 16 h in TSB (37°C, 170 rpm). One ml of culture was then added to 9 ml of fresh TSB and incubated at 37°C at 170 rpm until an optical density at 600 nm (OD_{600}) of 1.0 (∼10^9 CFU/ml; stationary phase) was attained. Phages were then individually added at MOI of 0.01 and allowed to adsorb for 5 min at room temperature. To remove excess phage particles, the co-culture was spun at 4,000 × g at 4°C and the supernatant discarded. The pellets were resuspended in 10 ml of fresh TSB and incubated at room temperature with gentle agitation. Subsequently, 50 μl aliquots were collected every 5 min for a total duration of 60 min, immediately serially diluted in SM buffer, and spotted in duplicate on a host agar lawn of *S.* Enteritidis grown to 16 h for titer determination. Plates were incubated at 37°C for 18 ± 2 h for visualization of plaques. This experiment was independently conducted three times for each phage.

Lysogeny Analysis

Resistant colonies of *S.* Enteritidis in the centers of spot assays (n = five colonies per phage) were selected to test for lysogeny. First, isolated colonies were serially re-streaked five times on TSA to reduce phage carry-over. On the fifth streak, a random colony was selected for polymerase chain reaction (PCR) to confirm their *Salmonella* identity by using primers specific to *invA*, according to Fong and Wang (2016). Briefly, a single colony was suspended in 200 μl of sterile de-ionized water and lysed in a microwave for two min. PCR detection was then carried out with the TopTaq Master Mix Kit (Qiagen, Valencia, CA, United States) with primers specific for the *invA* gene (forward: 5′-TCA TGG CAC CGT CAA AGG AAC C-3′ and reverse: 5′-GTG AAA

TTA TCG CCA CGT TCG GGC AA-3′) (Li et al., 2012). PCR cycling conditions were as follows: initial denaturation (3 min, 94°C); three-step cycling, including denaturation (30 s, 94°C), annealing (30 s, 56°C), and extension (1 min, 72°C); followed by a final extension (10 min, 72°C). Sizes of the PCR products were confirmed with electrophoresis on 2% agarose (Amresco, Solon, OH, United States) with 1X Tris-acetate-EDTA (TAE) buffer (Thermo Fisher, Waltham, MA, United States). PCR products were visualized using the ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, United States).

Colonies arising from the fifth streak were cultured to test for phage lysogeny as previously described (Petty et al., 2006). Colonies ($n = 10$) were suspended in 10 ml TSB and incubated at 37°C at 170 rpm for 20 h. Cultures were subsequently spun at 4,000 × g at 4°C to sediment the bacteria, and the supernatant tested for the spontaneous release of phage particles by spotting 5 μl in duplicate onto prepared *S.* Enteritidis agar overlays. Plates were incubated at 37°C for 18 ± 2 h for visualization of plaques. Supernatants from *S.* Enteritidis infection by Felix-O1, a strictly lytic phage, were used as a negative control.

Temperature and pH Stability Assay

Phage lysates were diluted in TSB to an initial concentration of ∼10^7 PFU/ml and subsequently stored at a range of temperatures (−20, 4, 22, and 37°C) for determination of relative temperature stabilities. Controls (no phage) were included for each temperature. The samples stored at −20°C were prepared in single-use 15 μl aliquots to prevent multiple thawing and freezing events throughout the assay. Temperature stability experiments were conducted three times for each phage.

To test pH stability of the phages, phage lysates were diluted to ∼10^8 PFU/ml in TSB at varying pH ranges of pH 4.0, 6.0, 8.0, and 10.0 (adjusted with 6 M HCl or 6 M NaOH) and subsequently stored at room temperature for further analyses. Blank controls (no phage) were included for each pH. These pH and temperature ranges were chosen based on previously reported similar assessments (Thung et al., 2017) and reflect the various pH and temperature conditions encountered in produce production chains (Park et al., 2012; Rombouts et al., 2016). pH stability experiments were conducted three times for each phage.

Phage titers were assessed on days 2, 4, 8, 10, 14, 16, 20, 25, and 30. Briefly, 10 μl volumes were serially diluted in SM buffer and 5 μl spotted in duplicate on prepared top agar of *S.* Enteritidis grown to 16 h. Enumeration of plaques were determined after incubation at 37°C for 18 ± 2 h.

Spectrophotometric Analysis of Phage Lysis Efficacy

Cultures of *S.* Enteritidis FSL S5-483, *S.* Agona FSL S5-513 and *S.* Typhimurium LMFS-JF-001 were prepared as described previously in section "Bacterial Strains and Growth Conditions." Following incubation, cultures were spun at 4,000 × g and the cell pellets washed three times with fresh TSB. Then, the cultures were loaded into 96-well plates to a final concentration of 5 × 10^4 CFU/ml and infected with phages SI1, SF1, SS1, and SS4 at MOIs of 1, 10, and 100 PFU/CFU. Plates were placed into a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, United States) set to 25°C for determination of cell density at OD_{600} every 30 min for 36 h. Each experiment was independently conducted three times.

Phage SI1 Biocontrol of *Salmonella* on Sprouting Alfalfa Seeds

The lysate of phage SI1 was tested for its efficacy to control *S.* Enteritidis on germinating alfalfa seed over 6 days. Six days was chosen as approximately 5–7 days are required for alfalfa seeds to sprout (Kramer and Lim, 2004). Cultures of *S.* Enteritidis grown to 16 h were spun at 4,000 × g for 10 min, washed three times with sterile potable water and serially diluted to a final volume of 35 ml of sterile water. The seed was inoculated by drop-wise addition of 15 ml diluted culture to 150 g seed to achieve an initial concentration of approximately log 3.5 CFU/g. Blank controls were processed similarly, but with sterile water only. The seed was placed in a biological safety cabinet at room temperature under continuous air flow for 2 h. Finally, the seed was transferred to sterile plastic boxes lined with a layer of sterile gauze pad and stored in the dark in a 22°C incubator. Three independent replicate experiments were performed.

The lysate of phage SI1 was applied to the seed at 22 h post-inoculation. Briefly, 75 g of inoculated seed was aseptically removed and treated with phage SI1 in 35 ml sterile water to yield an MOI of approximately 110 PFU/CFU. The seeds were soaked for 2 h at room temperature with gentle agitation by shaking at 175 rpm. Inoculated seed and controls that received only sterile water were processed in tandem. A 2 h phage soak was chosen due to (i) the short latent period (25 min) and relatively high burst size (83 phages) of SI1, thereby facilitating approximately four cycles of productive infection; and (ii) the simulation of a logistically feasible decontamination step, performed within a time frame that could be adopted into commercial sprout production practices. Following the soak, the excess fluid was removed by straining through sterile filter paper. Seed samples were then aseptically transferred to sterile plastic boxes lined with a layer of sterile gauze pad. Treated seed was stored in the dark in a 22°C incubator.

The germinating seed was moistened with seven ml of sterile water every 24 h over 6 days. Simultaneously, 10 g of seeds were removed daily and mixed with 100 ml of sterile phosphate buffered saline (PBS; Amresco) in a sterile Whirlpak bag (Nasco, Fort Atkinson, WI, United States). The samples were placed in a Stomacher (Seward, Worthing, West Sussex, United Kingdom) and homogenized for 2 min at 230 rpm. Subsequently, 100 μl aliquots were serially diluted in PBS and spread over xylose-lysine deoxycholate (XLD; Amresco) agar in duplicate. XLD plates were incubated at 37°C for 22 ± 2 h for enumeration of *Salmonella* (red colonies with black centers). Phage titers were measured by spotting 5 μl in duplicate on TSA seeded with *S.* Enteritidis grown to 16 h. Plates were incubated at 37°C for 18 ± 2 h for enumeration of plaques.

To assess the impact of the phage treatment on the final sprout yield, 150 g of seed was artificially contaminated with *S.* Enteritidis and 75 g was withdrawn for phage treatment, as per the procedures described previously. Sprouting seeds were then

weighed after 6 days. Three independent replicate experiments were performed, with two technical replicates taken for each measurement.

Statistical Analysis

For the pH and heat stability assays, the final titer of the phages after 30 days of treatment was compared to the initial titer at the beginning of treatment with a Student's *t*-test ($\alpha = 0.05$). To compare the relative susceptibilities of the phages to each treatment, the log decreases in phage titer after 30 days of treatment was calculated (i.e., the difference in log PFU/ml at time zero and after 30 days of treatment). A one-way analysis of variance (ANOVA) was then implemented with a Tukey's Honest Significant Difference *post hoc* test applied to all significant ANOVA results ($\alpha = 0.05$).

For the sprouts biocontrol assay, the log differences in *Salmonella* counts at each sampling time point between untreated and treated alfalfa seed samples were analyzed with a Student's *t*-test with a significance level of $\alpha = 0.05$. The differences in weights between the control and treated alfalfa sprouts were also assessed using a Student's *t*-test ($\alpha = 0.05$).

All statistical analyses were performed using JMP version 11.1.1 (SAS Institute, Inc., Cary, NC, United States). A *P*-value of $\leq$0.05 was considered statistically significant for all analyses.

RESULTS AND DISCUSSION

Host Range Determination of *Salmonella* Phages and Detection of Lysis from without

Results of host range analysis of the four phages suggested that they were able to lyse 31–37% of the *Salmonella* strains tested as indicated by a +3 or +4 spot test (**Table 1**). Susceptible strains encompassed (i) the serotypes causing the highest rates of salmonellosis (i.e., *S.* Enteritidis and *S.* Typhimurium); (ii) serotypes involved in North American outbreaks (i.e., *S.* Agona, *S.* Saintpaul, and *S.* Heidelberg); and (iii) emerging, uncommon serotypes (i.e., *S.* Berta and *S.* Canada) (Ellis et al., 1998).

SI1, SS4, SF1, and SI1 were classified as broad host-range phages as they infected the largest proportion of the tested *Salmonella* strains, compared to other *Salmonella* phages recovered and characterized ($n = 44$). Of the 61 *Salmonella* strains tested, 23 were susceptible to infection by SI1, 22 were susceptible to SS4, 20 were susceptible to SF1 and 19 to SS1, as indicated by a +3 or +4 clearing (**Table 1**). The similarity between host ranges indicate that these four phages may recognize similar host receptors (Kalatzis et al., 2016). It should be noted that strains of *S.* Typhimurium ($n = 8$), responsible for the highest proportion of foodborne salmonellosis, demonstrated a high degree of susceptibility to these phages; seven were susceptible to infection by SI1 and SS4 and six were susceptible to SF1 and SS1. LO was not observed in any of the characterized phages, indicating the presence of obligate productive infection with all *S. enterica* strains tested.

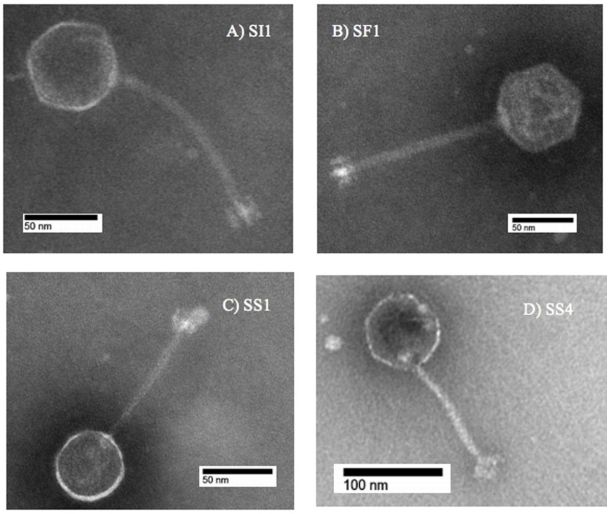


FIGURE 1 | Transmission electron microscope images of **(A)** SI1, **(B)** SF1, **(C)** SS1, and **(D)** SS4.

General Characterization

Phage TEM, Burst Size, Genotyping and Lysogeny

SI1, SF1, SS1, and SS4 formed clear plaques on their host, *S.* Enteritidis, although they differed in size. SI1 and SS1 formed 1 mm clear plaques, but SS1 plaques also possessed slightly turbid haloes. Similarly, SS4 and SF1 formed larger plaques of 1.5 mm diameter, but SS4 plaques possessed slightly turbid haloes. It is suggested that halo formation is the result of endolysin secretion upon lysis of host cells (Cornelissen et al., 2012). Structural examination with TEM revealed their distinct morphologies, with all four phages belonging to the family *Siphoviridae* (**Figure 1**), consisting of rigid, non-contractile tails and double stranded DNA. SI1 is 207 $\pm$ 5 nm in length with a spherical head and a small appendage structure located at the crown. SF1 is 215 $\pm$ 3 nm long, possessing an icosahedral-shaped head. SS1 and SS4 exhibited structural similarity; both with spherical heads and 200 $\pm$ 2 nm and 202 $\pm$ 5 nm in length, respectively.

Subsequent typing of SI1, SF1, SS1, and SS4 was accomplished by restriction enzyme analysis. Digestion with *EcoR*1 yielded four distinct banding patterns, confirming the unique identities of these phages (**Figure 2**). It was found that SI1 has an approximate genome size of 87,000 bp, SF1 with a genome size of 80,800 bp, and SS1 and SS4 with substantially smaller genome sizes of 44,150 and 65,000 bp, respectively.

Some patterns only differed by a few bands, suggesting the conservation of *EcoR*1-specific cutting sites and a familial relationship between the characterized phages. Although Siphoviruses are known to exhibit remarkable mosaicism (Santander et al., 2017), it is expected that several components would exhibit notable similarity as these phages belong to the same family and present similar, though not identical, host ranges. Indeed, *Salmonella* phages fSE1C and fSE4C previously isolated from pickle sauce and ground beef,

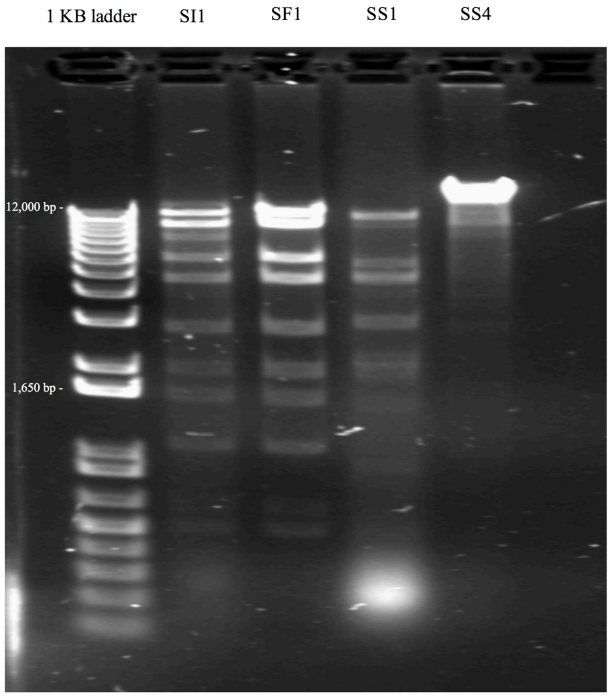


FIGURE 2 | Restriction banding patterns of phage upon digestion by endonuclease *Eco*RI.

respectively, were digested with *Eco*RI, *Hind*III, and *Hae*III restriction enzymes and showed very similar banding patterns (Santander et al., 2017). Further analysis revealed a similarity of 43.09% between the genomes, with genes involved in structure, replication, host specificity and DNA metabolism showing remarkable conservation (Santander et al., 2017).

Single step growth curves were constructed to determine the infection potential of each phage (**Figure 3**). SI1, SF1, and SS1 possess latency periods of 25 min while SS4 possesses a latency of 30 min. The burst size of SI1 is 83 phages per infected cell, whereas the burst sizes of SF1, SS1, and SS4 are 45, 20, and 31 phages per infected cell. These phage infection parameters are in the range of those observed for Siphoviridae phages (Carey-Smith et al., 2006; Silva et al., 2014; Pereira et al., 2016b). For phage therapy in the food industry, it is often desirable to possess short latent periods and high burst sizes (Kalatzis et al., 2016), therefore the infection parameters outlined here demonstrate the potential of the characterized phages, particularly SI1, for use in biocontrol efforts.

Phages were tested for harborage of lysogenic elements by culturing phage-resistant colonies of *S.* Enteritidis and testing the supernatant for spontaneous release of phage particles. Absence of lysogenic integration into the host genome is a pre-requisite for phage biocontrol of food (Levin and Bull, 2004). No phage particles were detected upon spot-testing on overlays seeded with *S.* Enteritidis, indicating a strictly lytic life cycle and their suitability for use in phage therapy (Rombouts et al., 2016). Additionally, the production of clear plaques further confirmed their lytic life cycle.

pH and Temperature Stability

At pH = 4, SF1, SS1, and SS4 were reduced to undetectable concentrations (<200 PFU/ml) by day 20 (**Figure 4**). SI1 was reduced to less than 200 PFU/ml by day 30. From day 6 onward, SS1 showed a more rapid decline than SF1 and SS4. These results suggest that of the four phages, SS1 would be the least stable biocontrol agent in acidic conditions, whereas SI1 would be the most stable.

In conditions ranging from pH = 6 – 10, no phage titer decreased by more than 0.544 ± 0.067 log PFU/ml, and only in one instance was titer (SS4 at pH 10) significantly ($p < 0.05$) reduced. On average, stability increased as the pH increased from pH 4 to pH 10, but there was some variability between phages. SI1 and SF1 were most stable at pH 10, but SS1 and SS4 were most stable at pH 8. SI1 was significantly ($p < 0.05$) less stable than each of SS1, SF1, and SS4 at pH = 8.

Our results are supported with previous findings of rapid declines in titer at pH 4.2 but only a gradual decline at pH 5.8 (Leverentz et al., 2001). However, Ahiwale et al. (2013) reported instability at pH = 10–12 of *Salmonella* phage. This inconsistency may be due to the structure of individual phages assayed. Having long flexible non-contractile tails, SF1, SS1, SS4, and SI1 belong to the family *Siphoviridae*, whereas phages assayed by Ahiwale et al. (2013) possessed short, stubby, non-contractile tails, representing *Podoviridae*. Similar to our phages, Hamdi et al. (2017) found that Siphovirus SH6 was unstable at pH 2–4 and stable at pH 5–11, whereas Myovirus SH7 was stable at pH 3–11. Differences in isoelectric points (pI) of the phages may also contribute to these differences in stability, particularly at acidic pH, as viral aggregation is common when pH ≤ pI and has previously led to decreases in titer of approximately three log PFU/ml (Langlet et al., 2007).

In the heat stability assay, nine of 16 phage titers were significantly ($p < 0.05$) reduced, but by no more than 1.0 ± 0.1 log PFU/ml over the range of temperatures tested, suggesting that SS1, SF1, SS4, and SI1 will retain the stability required for use as biocontrol agents at temperatures commonly encountered in produce production chains (Rombouts et al., 2016).

SS1, SF1, and SS4 were significantly ($p < 0.05$) more stable at −20, 4, and 22°C than at 37°C (**Figure 5**). From −20 to 22°C, there were no significant ($p < 0.05$) differences in stability between SS1, SF1, and SS4, but at 37°C, SF1 was significantly ($p < 0.05$) more stable than both SS1 and SS4. SI1 was detected to be most stable ($p < 0.05$) at a temperature of 22°C, and significantly ($p < 0.05$) more stable than SS1 at this temperature. All four phages were least stable at 37°C, and more stable at −20°C than 4°C. Aside from SI1 being most stable at 22°C, our results agree with previous reports in that Siphovirus stability decreases with an increase in temperature above 20°C (Jepson and March, 2004). Previous work has demonstrated the stability of Podoviruses to be most stable from 4 to 36°C, with highest stability retained at the lower end of the temperature spectrum (Ahiwale et al., 2013). This parallelism between phages of different families suggests that differences in

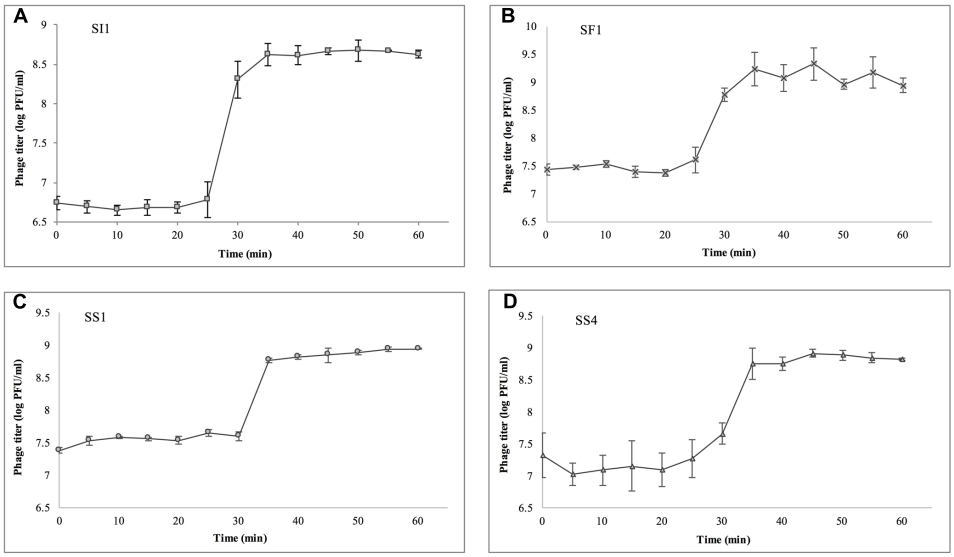


FIGURE 3 | Single step growth curves of **(A)** SI1, **(B)** SF1, **(C)** SS1, and **(D)** SS4. Data shown are the mean of three replicates ± SD.

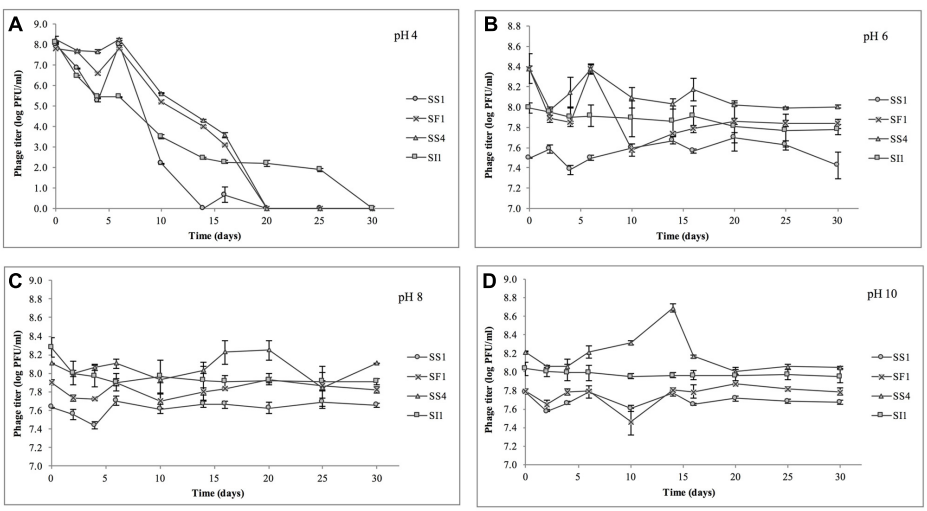


FIGURE 4 | Stability of phages SI1, SF1, SS1, and SS4 at **(A)** pH 4, **(B)** pH 6, **(C)** pH 8, and **(D)** pH 10 over a period of 30 days. Data shown are the mean of three replicates ± SD.

phage tail morphologies may not be a main contributor to the variances in phage stability at different temperatures; however, Thorne and Holt (1974) have reported a negative correlation between temperature and tail contraction, and hence loss of activity, in *Myoviridae* phages. Given the importance of assaying for stability prior to adoption into the commercial market, these results indicate that their environmental stability makes these phages good candidates for use in biocontrol.

Assessment of *in Vitro* Phage Infectivity in Tryptic Soy Broth

The relative abilities of the four phages to suppress *S.* Enteritidis FSL S5-483, *S.* Agona FSL S5-513 and *S.* Typhimurium LMFS-JF-001 in TSB were assessed at an MOI of 1, 10, and 100 PFU/CFU at 25°C. At MOI = 1, both SI1 and SS4 suppressed the growth of *S.* Enteritidis over a 36 h period. Growth of *S.* Enteritidis was also suppressed by SF1 and SS1, but growth resumed at 13 and 17 h, respectively, after initial infection (**Figure 6**). Growth of *S.* Enteritidis appeared to recover at 31 h following treatment with SS4. An MOI of 10 prolonged the suppression of *S.* Enteritidis to 19 and 25 h when infected with SF1 and SS1, respectively. Further, application of SS4 at an MOI of 10 caused complete inhibition of *S.* Enteritidis growth for 36 h. Finally, phage treatment at an MOI of 100 suppressed the growth of *S.* Enteritidis for the 36 h duration (**Figure 6**).

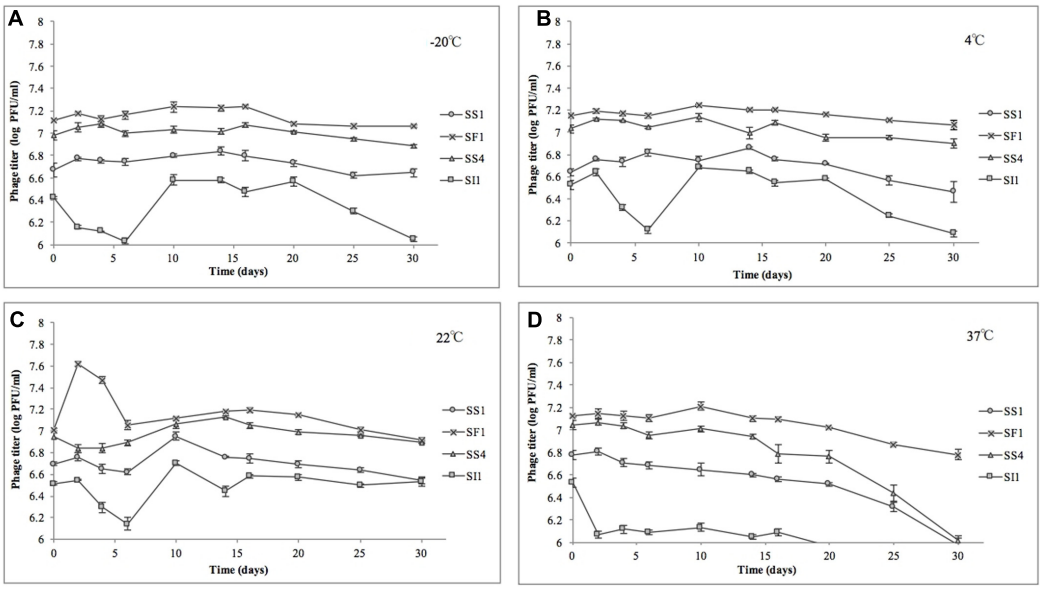


FIGURE 5 | Stability of phages SI1, SF1, SS1, and SS4 at **(A)** –20°C, **(B)** 4°C, **(C)** 22°C, and **(D)** 37°C over a period of 30 days. Data shown are the mean of three replicates ± SD.

Suppression of *S.* Agona also occurred with all MOIs tested (**Figure 7**), but the extent was not as pronounced as with the host, *S.* Enteritidis. Instead, considerable suppression did not occur until an MOI of 100 was evaluated. At this MOI, it appeared that SS1 was the least effective in controlling *S.* Agona as growth resumed 17 h after the initial infection. In contrast, SI1 and SS4 were the most effective in suppressing growth, although *S.* Agona appeared to recover at 28 and 32 h after infection with these phages, respectively.

Lastly, *S.* Typhimurium was tested for its susceptibility to the phages in TSB (**Figure 8**). Again, the extent of suppression after phage infection was not as pronounced as with *S.* Enteritidis, but did occur at all MOIs. The most dramatic reduction in growth occurred at MOI 100, although growth was not suppressed entirely for the 36 h duration. At this MOI, SI1 was the most effective in attenuating growth (**Figure 8**).

Although not all MOIs were effective in controlling growth of *Salmonella*, nor did all *Salmonella* strains show similar susceptibilities to the phages, it should be noted that phage infection at all MOIs, across all strains, resulted in an extended lag phase [defined as $OD_{600} < 0.2$ (Wang et al., 2009)], indicating that the phages had a suppressive effect on *Salmonella*. The ability of the *Salmonella* strains to recover after initial infection is likely due to the emergence of phage-resistant mutants (Guenther et al., 2012). It may be possible to prolong the duration of phage sensitivity by infection with a mixture of phages (Chan et al., 2013), though it was not evaluated in this present study.

With all strains, infection with an MOI of 100 proved to be the most effective and represents the MOI used for many food processing applications (Silva et al., 2014). However, with the phage host, *S.* Enteritidis, growth was completely suppressed at all MOIs with phage SI1, underlining its remarkable efficacy in controlling *S.* Enteritidis *in vitro*. Mechanistically, SI1 may require multiple attachment sites on the bacterial cell membrane for adsorption and/or SI1 receptor sites may be essential for cellular metabolic processes – both of which would contribute to the attenuation of phage resistance by the host (Rakhuba et al., 2010; Kong et al., 2011).

Assessment of SI1 to Control *Salmonella* on Sprouting Alfalfa Seeds

The ability of SI1 to control *Salmonella* on sprouting alfalfa seeds was assessed. SI1, in particular, was selected for this study as it caused complete inhibition of *S.* Enteritidis in TSB at all tested MOIs and possessed the greatest burst size (approximately 83 phages) and possessed one of the shortest latent periods (25 min) (**Figure 2**). Moreover, *S.* Enteritidis is a serotype previously linked to North American sprout outbreaks (Centers for Disease Control and Prevention [CDC], 2017) and further, has been implicated in the highest number of salmonellosis outbreaks worldwide (Mattick et al., 2001). On the basis of these factors, they were selected for use in this biocontrol study.

Treatment with SI1 (MOI = 100) resulted in a significant ($p < 0.05$) 2.51 ± 0.24 log CFU/g reduction of *S.* Enteritidis, 2 h after treatment (**Figure 9**), corresponding to a decrease of 38.3 ± 3.0% of the initial viable population. This was accompanied by a 1.02 ± 0.33 log PFU/g increase in phage titer (**Figure 10**). In contrast, previous work by Kocharunchitt et al. (2009) reported a one log CFU/g decrease in *S.* Oranienburg populations following application of phage SSP6 onto alfalfa seeds at the beginning of germination. Similarly, a 1.37 log CFU/g reduction of *Salmonella* populations was observed on mustard seeds at 24 h following phage treatment (Pao et al., 2004). Sprout production standards, as set by Health Canada, recommend

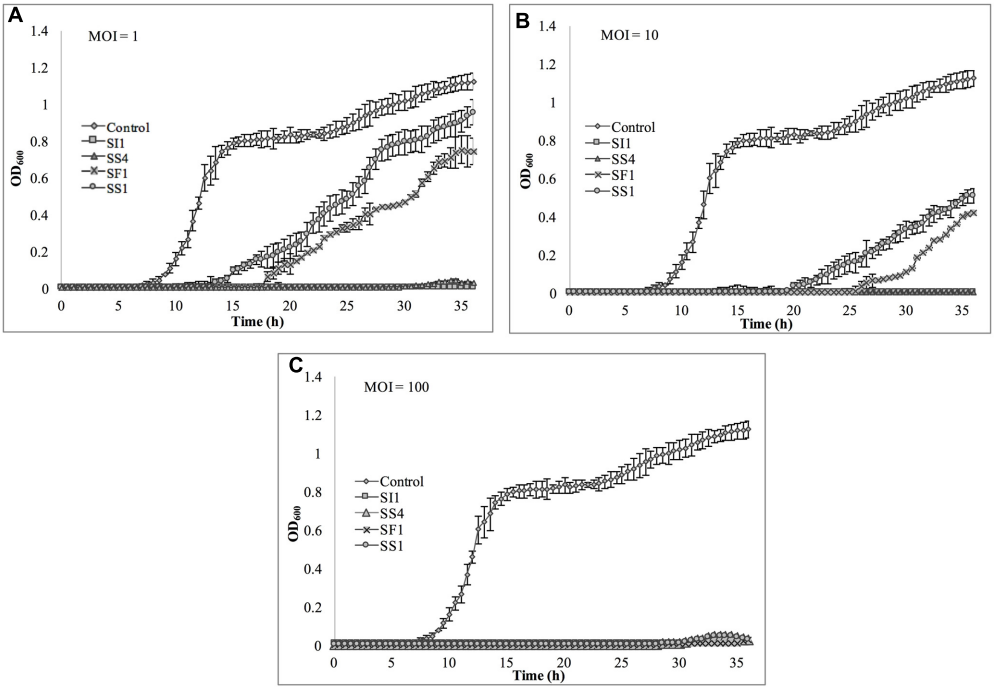


FIGURE 6 | *In vitro* analysis of *S.* Enteritidis inhibition by phages SI1, SS4, SF1, and SS1 at **(A)** MOI = 1, **(B)** MOI = 10, and **(C)** MOI = 100. Data shown are the mean of three replicates ± SD.

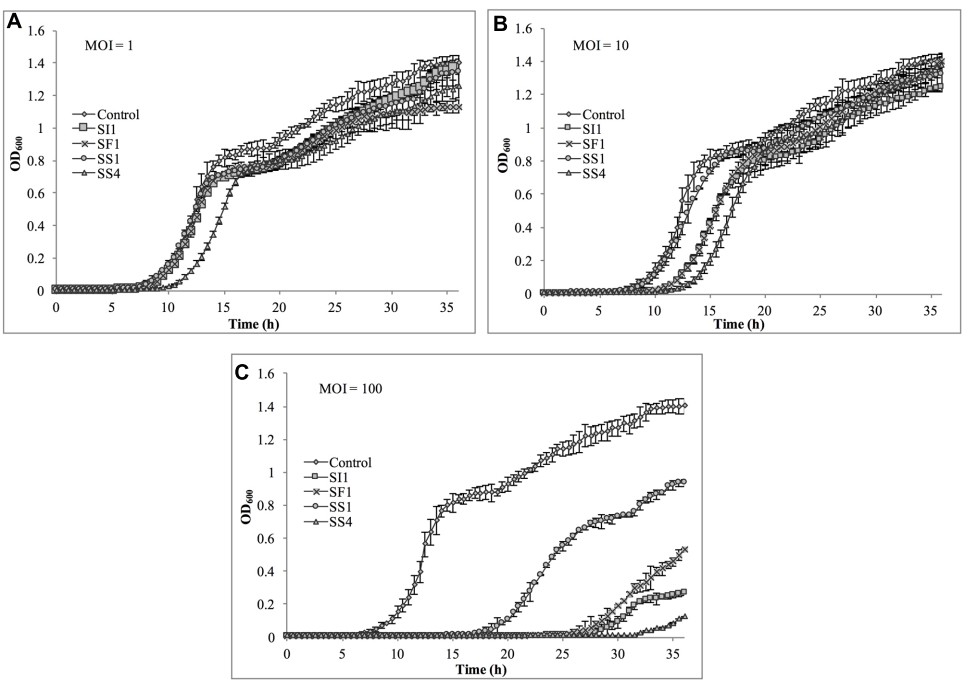


FIGURE 7 | *In vitro* analysis of *S.* Agona inhibition by phages SI1, SS4, SF1, and SS1 at **(A)** MOI = 1, **(B)** MOI = 10, and **(C)** MOI = 100. Data shown are the mean of three replicates ± SD.

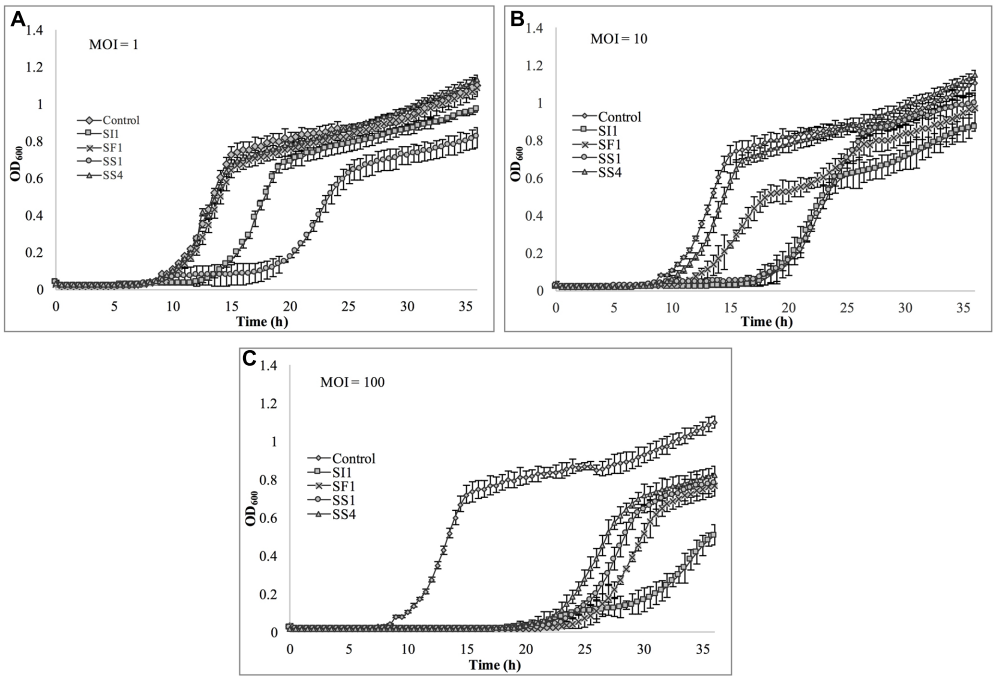


FIGURE 8 | *In vitro* analysis of *S.* Typhimurium inhibition by phages SI1, SS4, SF1, and SS1 at **(A)** MOI = 1, **(B)** MOI = 10, and **(C)** MOI = 100. Data shown are the mean of three replicates ± SD.

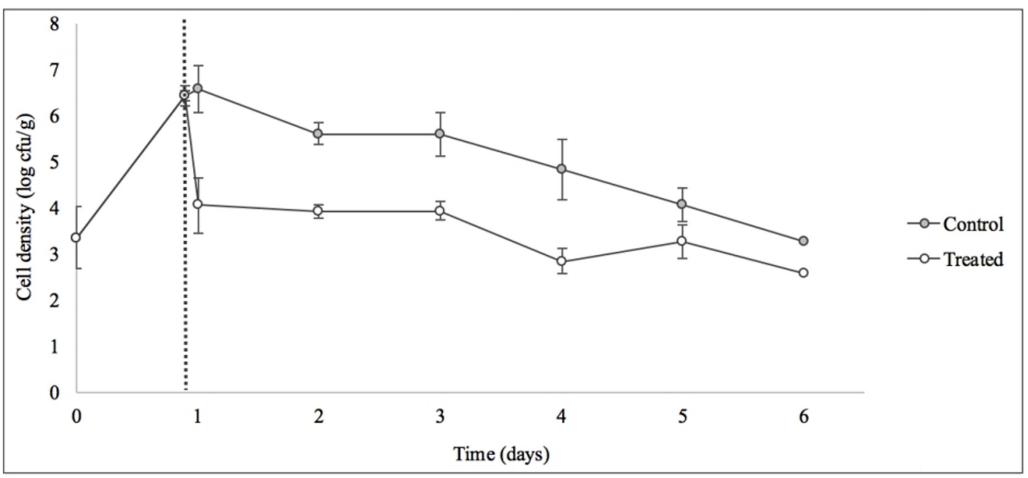


FIGURE 9 | Control of *S.* Enteritidis on sprouting alfalfa seeds with phage SI1. Dotted line indicates the phage treatment at 22 h after artificial contamination of the seeds. Data shown are the mean of three replicates ± SD.

that sprout decontamination methods achieve a minimum three log reduction in pathogen counts (Canadian Food Inspection Agency [CFIA], 2014). Further validation of SI1 infectivity across a range of potential bacterial contaminants and at various stages throughout the sprouting process is therefore required to ensure complete compliance with Health Canada standards, although a >2.5 log CFU/g reduction of *S.* Enteritidis is promising. Additionally, the final weight of the seeds treated with *Salmonella* only (82.40 ± 2.83 CFU/g) was not significantly different ($p > 0.05$) than that of the phage-treated sprouts (80.64 ± 1.41 CFU/g), further demonstrating its potential suitability for use in industry.

On days 2–6 following phage treatment, *Salmonella* cell densities on treated alfalfa seeds were also reduced, but this was not significant ($p > 0.05$) (**Figure 9**). In line with this observation, phage titers increased the day of seed treatment, indicating its replication. However, the initial increase was followed by a stagnation of growth and small decreases in titer

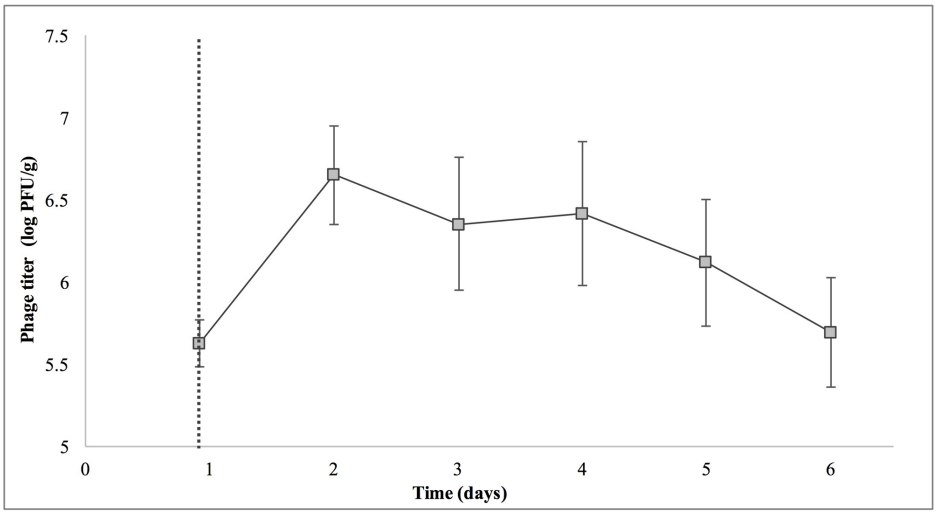


FIGURE 10 | Titer of phage SI1 during control of *S.* Enteritidis on alfalfa sprouts. Dotted line indicates the phage treatment at 22 h after artificial contamination of the seeds. Data shown are the mean of three replicates ± SD.

(**Figure 10**). Notably, it is presumed that the emergence of phage-resistant *Salmonella* may have contributed to the diminished effectiveness in the days following treatment. Indeed, phage-resistant *Salmonella* has been identified in both in *in vitro* systems (Vipra et al., 2013) and foods treated with phage (Kocharunchitt et al., 2009; Guenther et al., 2012). Emergence of bacterial mutants resistant to phage is particularly apparent when MOI values are high, as this enhances the selective pressure to resist infection (Vipra et al., 2013). It has been reported, however, that phage-resistant mutants possess attenuated pathogenicity and diminished fitness (Kong et al., 2011). A possible remedy to control the emergence of such mutants is through the use of a phage cocktail (Spricigo et al., 2013; Pereira et al., 2016a), which may additionally extend the spectrum of lysis to include other *Salmonella* strains (Chan et al., 2013).

Although the present results are not fully consistent with the data obtained *in vitro*, it is hypothesized that the simplicity of an *in vitro* system represents an ideal scenario for phage infection and multiplication. The nature of a food matrix presents with various complicating factors. For instance, possibilities include biofilm production on alfalfa sprouts (Kocharunchitt et al., 2009), which could hinder phage adsorption (Sutherland et al., 2004); growth of endogenous microbiota naturally present on sprout seeds, which may provide alternative adsorption sites (Ye et al., 2010); or internalization of *Salmonella* into the sprouts itself (Erickson, 2012), rendering them unavailable for phage attack. These factors could account for the diminished efficacy of the phage and also its inconsistent increases in phage titer throughout this assay. It is possible that additional phage treatments throughout the sprouting process, or phage treatment in combination with other treatments (e.g., chlorine or organic acid washes), would further reduce the viable *Salmonella* populations on alfalfa seeds. It should also be noted that the high initial load of *Salmonella* used in this assay is unrepresentative of real world situations, yet is important from a technical perspective to determine the log kill. Ye et al. (2010) reported a six log CFU/ml decrease of *Salmonella* on artificially contaminated mung bean sprouts upon treatment with a combination of six *Salmonella* phages and *Enterobacter asburiae*, a naturally competitive microorganism. Interestingly, this combination treatment was significantly more effective than treatment with phage or *E. asburiae* alone.

CONCLUSION

Bacteriophage treatment of produce is an underdeveloped, emerging topic of interest and is currently not extensively used in industry. In this study, four lytic bacteriophages infecting *Salmonella* were assessed to determine their suitability for biocontrol in alfalfa sprout production. The results revealed that all four phages possessed desirable characteristics for use in biocontrol efforts. Among the phages characterized, SI1 proved to be particularly effective for control of *Salmonella* both *in vitro* and upon application onto sprouting alfalfa seeds. Although promising, future work should also aim to optimize this treatment, such as by incorporating hurdled treatments (i.e., with conventional sanitizers) or designing a multi-phage cocktail. Additionally, phage treatment of other sprouts varieties should be investigated to confirm the potential for use in related produce items.

AUTHOR CONTRIBUTIONS

PD, LG, RL, and SW were responsible for the study conception. KF and SW conceived the experimental design. AS designed the phage isolation protocol. KF and BL were responsible for data acquisition. KF isolated the bacteriophages in this study.

LG and MD provided *Salmonella* strains used in this study. BL performed the pH and temperature stability analyses and participated in determination of the host range. KF carried out all other characterizations and performed the *in vitro* analysis and the biocontrol assay with alfalfa sprouts. KF, BL, and SW analyzed and interpreted the data. KF drafted the manuscript. All authors provided critical revisions and approved the manuscript.

FUNDING

This work was supported by grants from Genome Canada (grant number 8505) and the National Sciences and Engineering Research Council of Canada (NSERC Discovery Grant RGPIN-2015-04871).

ACKNOWLEDGMENTS

We thank Ms. Donna Lau and Mr. Justin Falardeau for sample collection and technical assistance with bacteriophage isolation. Some of the *Salmonella* isolates used in this study were kindly provided by Drs. Alexander Gill and Sandeep Tambar at Health Canada and Dr. Martin Wiedmann at Cornell University. Felix-O1 was provided by Dr. Sylvain Moineau at Laval University.

REFERENCES

Adams, M. H. (1959). *Bacteriophages*. New York, NY: Interscience Publishers.

Ahiwale, S., Tagunde, S., Khopkar, S., Karni, M., Gajbhiye, M., and Kapadnis, B. (2013). Liquid based formulations of bacteriophages for the management of waterborne bacterial pathogens in water microcosms. *Indian J. Exp. Biol.* 51, 1038–1045.

Bari, M. L., Enomoto, K., Nei, D., and Kawamoto, S. (2010). Practical evaluation of Mung bean seed pasteurization method in Japan. *J. Food Prot.* 73, 752–757. doi: 10.4315/0362-028X-73.4.752

Bari, M. L., Nei, D., Enomoto, K., Todoriki, S., and Kawamoto, S. (2009). Combination treatments for killing *Escherichia coli* O157:H7 on alfalfa, radish, broccoli, and mung bean seeds. *J. Food Prot.* 72, 631–636. doi: 10.4315/0362-028X-72.3.631

Canadian Food Inspection Agency [CFIA] (2014). *Code of Practice for the Hygienic Production of Sprouted Seeds*. Available at: http://www.inspection.gc.ca/food/fresh-fruits-and-vegetables/food-safety/sprouted-seeds/eng/1413825271044/1413825272091 [accessed April 26, 2017].

Carey-Smith, G. V., Billington, C., Cornelius, A. J., Hudson, J. A., and Heinemann, J. (2006). Isolation and characterization of bacteriophages infecting *Salmonella* spp. *FEMS Microbiol. Lett.* 258, 182–186. doi: 10.1111/j.1574-6968.2006.00217.x

Centers for Disease Control and Prevention [CDC] (2017). *Reports of Selected Salmonella Outbreak Investigations*. Available at: https://www.cdc.gov/salmonella/outbreaks.html [accessed May 1, 2017].

Chan, B. K., Abedon, S. T., and Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiol.* 8, 769–783. doi: 10.2217/fmb.13.47

Charkoswski, A. O., Barak, J. D., Sarreal, C. Z., and Mandrell, R. E. (2002). Differences in growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on alfalfa sprouts. *Appl. Environ. Microbiol.* 68, 3114–3120. doi: 10.1128/AEM.68.6.3114-3120.2002

Cornelissen, A., Ceyssens, P. J., Krylov, V. N., Noben, J. P., Volckaert, G., and Lavigne, R. (2012). Identification of EPS-degrading activity within the tail spikes of the novel *Pseudomonas putida* phage AF. *Virology* 434, 251–256. doi: 10.1016/j.virol.2012.09.030

Deveau, J., Labrie, S. J., Chopin, M. C., and Moineau, S. (2006). Biodiversity and classification of lactococcal phages. *Appl. Environ. Microbiol.* 72, 4338–4346. doi: 10.1128/AEM.02517-05

Ding, J., Fu, T., and Smith, M. (2013). Microbial contamination in sprouts: How effective is seed disinfection treatment? *J. Food Sci.* 78, R495–R501. doi: 10.1111/1750-3841.12064

Ellis, A., Preston, M., Borczyk, A., Miller, B., Stone, P., Hatton, B., et al. (1998). A community outbreak of *Salmonella* Berta associated with a soft cheese product. *Epidemiol. Infect.* 120, 29–35. doi: 10.1017/S0950268897008376

Erickson, M. C. (2012). Internalization of fresh produce by foodborne pathogens. *Annu. Rev. Food Sci. Technol.* 3, 283–310. doi: 10.1146/annurev-food-022811-101211

Fahey, J. W., Ourisson, P. K., and Degnan, F. H. (2006). Pathogen detection, testing, and control in fresh broccoli sprouts. *Nutr. J.* 5:13. doi: 10.1186/1475-2891-5-13

Fong, K., and Wang, S. (2016). Strain-specific survival of *Salmonella enterica* in peanut oil, peanut shell and chia seeds. *J. Food Prot.* 79, 361–368. doi: 10.4315/0362-028X.JFP-15-419

Food and Drug Administration [FDA] (2015). *Microbiological Safety Evaluations and Recommendations on Sprouted Seed*. Available at: https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ProducePlantProducts/ucm078789.htm [accessed April 26, 2017].

Goode, D., Allen, V. M., and Barrow, P. A. (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Appl. Environ. Microbiol.* 69, 5032–5036. doi: 10.1128/AEM.69.8.5032-5036.2003

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., and Loessner, M. J. (2012). Biocontrol of *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2. *Int. J. Food Microbiol.* 154, 66–72. doi: 10.1016/j.ijfoodmicro.2011.12.023

Hagens, S., and Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Appl. Microbiol. Biotechnol.* 76, 513–519. doi: 10.1007/s00253-007-1031-8

Hamdi, S., Rousseau, G., Labrie, S. J., Tremblay, D. M., Kourda, R. S., Slama, K. B., et al. (2017). Characterization of two polyvalent phages infecting *Enterobacteriaceae*. *Sci. Rep.* 7:40349. doi: 10.1038/srep40349

Hoiseth, S. K., and Stocker, B. A. (1981). Aromatic-dependent *Salmonella* Typhimurium are non-virulent and effective as live vaccines. *Nature* 291, 238–239. doi: 10.1038/291238a0

Jepson, C. D., and March, J. B. (2004). Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. *Vaccine* 22, 2413–2419. doi: 10.1016/j.vaccine.2003.11.065

Kalatzis, P. G., Bastias, R., Kokkari, C., and Katharios, P. (2016). Isolation and characterization of two lytic bacteriophages, (St2 and (Grn1: phage therapy application for biological control of *Vibrio alginolyticus* in aquaculture live feeds. *PLOS ONE* 11:e0151101. doi: 10.1371/journal.pone.0151101

Khan Mirzaei, M., and Nilsson, A. S. (2015). Isolation of phages for phage therapy: a comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. *PLOS ONE* 10:e0118557. doi: 10.1371/journal.pone.0118557

Kim, C., Hung, Y. C., Brackett, R. E., and Lin, C. S. (2003). Efficacy of electrolyzed oxidizing water in inactivating *Salmonella* on alfalfa seeds and sprouts. *J. Food Prot.* 66, 208–214. doi: 10.4315/0362-028X-66.2.208

Kocharunchitt, C., Ross, T., and McNeil, D. L. (2009). Use of bacteriophages as biocontrol agents to control *Salmonella* associated with seed sprouts. *Int. J. Food Microbiol.* 128, 453–459. doi: 10.1016/j.ijfoodmicro.2008.10.014

Kong, Q., Yang, J., Liu, Q., Alamuri, P., Roland, K. L., and Curtiss, R. III (2011). Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 79, 4227–4239. doi: 10.1128/IAI.05398-11

Kramer, M. F., and Lim, D. V. (2004). A rapid and automated fiber optic-based biosensor assay for the detection of *Salmonella* in spent irrigation used in the sprouting of sprout seeds. *J. Food Prot.* 67, 46–52. doi: 10.4315/0362-028X-67.1.46

Kumar, M., Hora, R., Kostrzynska, M., Waites, W. M., and Warriner, K. (2006). Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on mung beans, alfalfa,

and other seed types destined for sprout production by using an oxychloro-based sanitizer. *J. Food Prot.* 69, 1571–1578. doi: 10.4315/0362-028X-69.7.1571

Kutter, E. (2009). "Phage host range and efficiency of plating," in *Bacteriophage*, eds M. R. J. Clokie and A. M. Kropinski (New York, NY: Humana Press).

Lang, M. M., Ingham, B. H., and Ingham, S. C. (2000). Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. *Int. J. Food Microbiol.* 58, 73–82. doi: 10.1016/S0168-1605(00)00297-X

Langlet, J., Gaboriaud, F., and Gantzer, C. (2007). Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. *J. Appl. Microbiol.* 103, 1632–1638. doi: 10.1111/j.1365-2672.2007.03396.x

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., et al. (2001). Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *J. Food Prot.* 64, 1116–1121. doi: 10.4315/0362-028X-64.8.1116

Levin, B. R., and Bull, J. J. (2004). Population and evolutionary dynamics of phage therapy. *Nat. Rev. Microbiol.* 2, 166–173. doi: 10.1038/nrmicro822

Li, H., Bhaskara, A., Megalis, C., and Tortorello, M. L. (2012). Transcriptomic analysis of *Salmonella* desiccation resistance. *Foodborne Pathog. Dis.* 9, 1143–1151. doi: 10.1089/fpd.2012.1254

Lilleengen, K. (1950). Typing of *Salmonella* Dublin and *Salmonella* Enteritidis by means of bacteriophage. *Acta Pathol. Microbiol. Scand.* 27, 625–640. doi: 10.1111/j.1699-0463.1950.tb04934.x

Majowicz, S., Musto, J., Scallan, E., Angulo, F., Kirk, M., O'Brien, S., et al. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* 50, 882–889. doi: 10.1086/650733

Mattick, K. L., Jørgensen, F., Wang, P., Pound, J., Vandeven, M. H., Ward, L. R., et al. (2001). Effect of challenge temperature and solute type on heat tolerance of *Salmonella* serovars at low water activity. *Appl. Environ. Microbiol.* 67, 4128–4136. doi: 10.1128/AEM.67.9.4128-4136.2001

Merabishvili, M., Vandenheuvel, D., Kropinski, A. M., Mast, J., De Vos, D., Verbeken, G., et al. (2014). Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*. *PLOS ONE* 9:e104853. doi: 10.1371/journal.pone.0104853

Modi, R., Hirvi, Y., Hill, A., and Griffiths, M. W. (2001). Effect of phage on survival of *Salmonella* Enteritidis during manufacture and storage of Cheddar cheese made from raw and pasteurized milk. *J. Food Prot.* 64, 927–933. doi: 10.4315/0362-028X-64.7.927

Montville, R., and Schaffner, D. W. (2004). Analysis of published sprout seed sanitization studies shows treatments are highly variable. *J. Food Prot.* 67, 758–765. doi: 10.4315/0362-028X-67.4.758

National Advisory Committee on Microbiological Criteria for Foods [NACMCF] (1999). Microbiological safety evaluations and recommendations on sprouted seeds. *Int. J. Food Microbiol.* 52, 123–153.

Neetoo, J., and Chen, H. (2010). Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on artificially contaminated alfalfa seeds using high hydrostatic pressure. *Food Microbiol.* 52, 123–153. doi: 10.1016/j.fm.2009.11.003

Pao, S., Randolph, S., Westbrook, E., and Shen, H. (2004). Use of bacteriophages to control *Salmonella* in experimentally contaminated sprout seeds. *J. Food Sci.* 69, 127–130. doi: 10.1111/j.1365-2621.2004.tb10720.x

Park, M., Lee, J. H., Shin, H., Kim, M., Choi, J., Kang, D. H., et al. (2012). Characterization and comparative genomic analysis of a novel bacteriophage, SFP10, simultaneously inhibiting both *Salmonella enterica* and *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 78, 58–69. doi: 10.1128/AEM.06231-11

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Cunha, A., et al. (2016a). Bacteriophages with potential to inactivate *Salmonella* Typhimurium: Use of single phage suspensions and phage cocktails. *Virus Res.* 220, 179–192. doi: 10.1016/j.virusres.2016.04.020

Pereira, C., Moreirinha, C., Lewicka, M., Almeida, P., Clemente, C., Romalde, J. L., et al. (2016b). Characterization and *in vitro* evaluation of new bacteriophages for the biocontrol of *Escherichia coli*. *Virus Res.* 227, 171–182. doi: 10.1016/j.virusres.2016.09.019

Pérez-Balibrea, S., Moreno, D. A., and García-Viguera, C. (2008). Influence of light on health-promoting phytochemicals of broccoli sprouts. *J. Sci. Food Agric.* 88, 904–910. doi: 10.1002/jsfa.3169

Petty, N. K., Foulds, I. J., Pradel, E., Ewbank, J. J., and Salmond, G. P. C. (2006). A generalized transducing phage (phiIF3) for the genomically sequenced *Serratia marcescens* strain Db11: a tool for functional genomics of an opportunistic human pathogen. *Microbiology* 152, 1701–1708. doi: 10.1099/mic.0.28712-0

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., and Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *J. Clin. Microbiol.* 39, 3461–3465. doi: 10.1128/JCM.39.10.3461-3465.2001

Rakhuba, D. V., Kolomiets, E. I., Szwajcer Dey, E., and Novik, G. I. (2010). Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol. J. Microbiol.* 59, 145–155.

Rohwer, F., and Edwards, R. (2002). The phage proteomic tree: a genome-based taxonomy for phage. *J. Bacteriol.* 184, 4529–4535. doi: 10.1128/JB.184.16.4529-4535.2002

Rombouts, S., Volckaert, A., Venneman, S., Declercq, B., Vandenjeuvel, D., Allonsius, C. N., et al. (2016). Characterization of novel bacteriophages for biocontrol of bacterial blight in leek caused by *Pseudomonas syringae* pv. *porri*. *Front. Microbiol.* 7:279. doi: 10.3389/fmicb.2016.00279

Santander, J., Vasquez, J. I., Segovia, C., Santos, L., Turra, G., Huber, K., et al. (2017). Complete genome sequence of the *Salmonella enterica* serovar Enteritidis bacteriophages fSE1C and fSE4C isolated from food matrices. *Stand. Genome Sci.* 12:1. doi: 10.1186/s40793-016-0218-y

Silva, Y. J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., et al. (2014). Phage therapy as an approach to prevent Vibrio anguillarum infections in fish larvae production. *PLOS ONE* 9:e114197. doi: 10.1371/journal.pone.0114197

Spricigo, D. A., Bardina, C., Cortés, P., and Llagostera, M. (2013). Use of a bacteriophage cocktail to control *Salmonella* in food and the food industry. *Int. J. Food Microbiol.* 165, 169–174. doi: 10.1016/j.ijfoodmicro.2013.05.009

Sutherland, I. W., Hughes, K. A., Skillman, L. C., and Tait, K. (2004). The interaction of phage and biofilms. *FEMS Microbiol. Lett.* 232, 1–6. doi: 10.1016/S0378-1097(04)00041-2

Thorne, C. B., and Holt, S. C. (1974). Cold lability of *Bacillus cereus* bacteriophage CP-51. *J. Virol.* 14, 1006–1012.

Thung, T. Y., Premarathne, J. M. K. J. K., Chang, W. S., Loo, Y. Y., Chin, Y. Z., Tan, C. W., et al. (2017). Use of a lytic bacteriophage to control *Salmonella* Enteritidis in retail food. *LWT Food Sci. Technol.* 78, 222–225. doi: 10.1016/j.lwt.2016.12.044

Vipra, A., Desai, S. N., Junjappa, R. P., Roy, P., Poonacha, N., Ravinder, P., et al. (2013). Determining the minimum inhibitory concentration of bacteriophages: potential advantages. *Adv. Microbiol.* 3, 181–190. doi: 10.4236/aim.2013.32028

Wang, S., Deng, K., Zaremba, S., Deng, X., Lin, C., Wang, Q., et al. (2009). Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. *Appl. Environ. Microbiol.* 75, 6110–6123. doi: 10.1128/AEM.00914-09

Ye, J., Kostrzynska, M., Dunfield, K., and Warriner, K. (2010). Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *J. Food Prot.* 73, 9–17. doi: 10.4315/0362-028X-73.1.9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Fong, LaBossiere, Switt, Delaquis, Goodridge, Levesque, Danyluk and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 November 2017
doi: 10.3389/fmicb.2017.02328

Food Grade Pimenta Leaf Essential Oil Reduces the Attachment of *Salmonella enterica* Heidelberg (2011 Ground Turkey Outbreak Isolate) on to Turkey Skin

*Divek V. T. Nair and Anup Kollanoor Johny**

Department of Animal Science, University of Minnesota, Saint Paul, MN, United States

Salmonella attached to the poultry skin is a major source of carcass contamination during processing. Once attached to the poultry skin, it is difficult to detach and inactivate *Salmonella* by commonly used antimicrobial agents since the pathogen is entrapped deeply in the feather follicles and the crevices on the skin. Essential oils could be natural, safe, and effective alternatives to synthetic antimicrobial agents during commercial and organic processing setup. The present study evaluated the efficacy of pimenta (*Pimenta officinalis* Lindl.) leaf essential oil (PEO), and its nanoemulsion in reducing *Salmonella* Heidelberg attachment on to turkey (*Meleagris gallopavo*) skin during simulated scalding (65°C) and chilling (4°C) steps in poultry processing. A multidrug resistant *S.* Heidelberg isolate from the 2011 ground turkey outbreak in the United States was used in the study. Results showed that PEO and the nanoemulsion resulted in significant reduction of *S.* Heidelberg attachment on turkey skin. Turkey skin samples treated with 1.0% PEO for 5 min resulted in >2 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg at 65 and 4°C, respectively ($n = 6$; $P < 0.05$). Similarly, skin samples treated with 1.0% pimenta nanoemulsion (PNE) for 5 min resulted in 1.5- and 1.8- $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg at 65 and 4°C, respectively ($n = 6$; $P < 0.05$). In addition, PEO and PNE were effective in reducing *S.* Heidelberg on skin during short-term storage at 4 and 10°C (temperature abuse) ($n = 6$; $P < 0.05$). No *Salmonella* was detected in the dipping solution containing 0.5 or 1.0% PEO or PNE, whereas a substantial population of the pathogen survived in the control dipping solution. The results were validated using scanning electron -, and confocal - microscopy techniques. PEO or PNE could be utilized as an effective antimicrobial agent to reduce *S.* Heidelberg attachment to turkey skin during poultry processing.

Keywords: pimenta, essential oil, *Salmonella* Heidelberg, turkey skin, microscopy

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Zhao Chen,
University of California, Davis, United States
Francisco Diez-Gonzalez,
University of Georgia, United States
Francesca Patrignani,
Università di Bologna, Italy

***Correspondence:**
Anup Kollanoor Johny
anupjohn@umn.edu

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *24 May 2017*
Accepted: *13 November 2017*
Published: *28 November 2017*

Citation:
Nair DVT and Kollanoor Johny A (2017) Food Grade Pimenta Leaf Essential Oil Reduces the Attachment of Salmonella enterica Heidelberg (2011 Ground Turkey Outbreak Isolate) on to Turkey Skin. Front. Microbiol. 8:2328. doi: 10.3389/fmicb.2017.02328

INTRODUCTION

Historically, *Salmonella enterica* serovar Heidelberg (*S.* Heidelberg) has been one of the common *Salmonella* associated with poultry and is frequently isolated from turkeys (Foley et al., 2008; Jackson et al., 2013). Being one of the most invasive of *Salmonella* serotypes in humans, the pathogen has surfaced to importance causing significant economic loss to the poultry industry

and public health. In 2011, the consumption of contaminated ground turkey meat resulted in 136 human infections in 34 United States (CDC, 2011). In 2013, multidrug resistant (MDR) *S.* Heidelberg infections were linked to contaminated poultry products from a commercial processor in California (CDC, 2014a). Another outbreak caused by *S.* Heidelberg, including its MDR clones, was reported from a Tennessee correctional facility linked to consumption of contaminated poultry meat (CDC, 2014b). More recently, *S.* Heidelberg was ranked third among the most common etiological agents of human salmonellosis in the United States (CDC, 2013) and is currently an urgent threat to the United States food supply due to their high antibiotic resistance (AR) potential (Folster et al., 2012a,b; Shah et al., 2017)

Turkeys can harbor *S.* Heidelberg in their cecum without showing clinical disease (Poppe et al., 2005; Nair et al., 2016). The excretion of the pathogen through droppings can contaminate farm, and faulty evisceration step during processing could result in the contamination of turkey carcasses with *S.* Heidelberg (WHO, 2009). *Salmonella* attached to the carcass skin is a major source of poultry meat contamination during processing (Kim et al., 1996; Carrasco et al., 2012). During the scalding step, the turkey carcasses are immersed in water at 59 to 63°C for 50 to 125 s (FSIS, 2010) to up to a reported 65°C (Logue et al., 2003) to loosen the hair follicles (Carrasco et al., 2012). This step is considered as the first and critical step where a high likelihood of cross-contamination with pathogenic bacteria, including *Salmonella*, could occur (Russell, 2008; Carrasco et al., 2012). During chilling, the carcasses are immersed in cold water (4.4°C) where the chances of cross-contamination of carcasses with *Salmonella* is high (Nagel et al., 2013). The water uptake and swelling of the poultry skin during immersion chilling also expose deep channels and crevices on the skin, making the conditions favorable for bacterial attachment (Kim et al., 1996). The oily nature of poultry skin, penetration of *Salmonella* into the feather follicles, entrapment of *Salmonella* in the skin crevices, and the presence of high organic load on skin surface during scalding and chilling may reduce the effectiveness of chlorine and other commercially used synthetic disinfectants (Lillard, 1990; Kim et al., 1996; Yang et al., 2001; Nchez et al., 2002; Nagel et al., 2013). Along with these factors, various genetic mechanisms enable *Salmonella* to attach tightly to the skin of poultry carcasses (Salehi et al., 2017).

Recently, there is a tremendous interest in using natural antimicrobials as an alternative to synthetic chemicals against pathogens during food production (Burt, 2004; Lanciotti et al., 2004; Amalaradjou et al., 2010; Kollanoor Johny et al., 2010; Nair et al., 2015; Surendran Nair et al., 2016; Surendran-Nair et al., 2016). Among the emerging and widely researched alternatives, essential oils (EO) or their components are reported effective against foodborne pathogens such as *Salmonella, Campylobacter,* and *Escherichia coli* O157 *in vitro* and *in vivo* (Kollanoor Johny et al., 2008; Amalaradjou et al., 2010; Kollanoor-Johny et al., 2012a,b; Nair et al., 2014, 2015). The United States Food and Drug Administration (USFDA) has approved the use of selected EOs in food matrices (FDA, 2016) and therefore, their efficacy could be evaluated as safe and natural alternatives in commercial and organic poultry production systems (GPO, 2017). The use of EOs is also advantageous since they possess multiple active chemical sites to counteract pathogens using different mechanisms reducing the potential for the development of antimicrobial resistance against EOs (Venkitanarayanan et al., 2013; Surendran Nair et al., 2017).

Pimenta essential oil (PEO), commonly known as allspice oil, is extracted from the leaves of *Pimenta officinalis Lindl.* and is approved as Generally Recognized As Safe (GRAS; CFR-Title 21: Part 182, Sec. 182.20) compound by the Food and Drug Administration (FDA, 2016). The major component of PEO is eugenol (>80%) (Suzuki et al., 2014). The PEO possesses antimicrobial activity against several microorganisms such as *Staphylococcus epidermidis*, *Proteus hauseri*, *Micrococcus yunnanensis,* and *Corynebacterium xerosis* (Suzuki et al., 2014). PEO also possesses *in vitro* antimicrobial activity against *Listeria monocytogenes* (Aureli et al., 1992). However, no studies have been conducted to explore the efficacy of PEO against *Salmonella* in poultry or poultry products. Therefore, the current study evaluated the efficacy of PEO and its nanoemulsion (PNE) on *S.* Heidelberg attachment on turkey skin. The specific objectives of the study were (1) to investigate the effect of PEO or PNE on reducing *S.* Heidelberg attachment (high inoculum and low inoculum) to turkey skin at 4 or 65°C, (2) to determine the effect of PEO or PNE on reducing *S.* Heidelberg (low inoculum) survival on skin during storage for 2 days at 4 or 10°C, and (3) to illustrate the effect of PEO on *S.* Heidelberg (low inoculum) attachment to the turkey skin using confocal microscopy (CM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

All biosafety procedures in Dr. Kollanoor Johny's laboratory are approved by the Institutional Biosafety Committee at the University of Minnesota.

Bacterial Strain and Growth Conditions

An MDR *S.* Heidelberg isolate from the 2011 ground turkey outbreak in the United States (kindly donated by Dr. Irene Hanning, College of Genome Sciences and Technology, University of Tennessee and Dr. Venkitanarayanan, University of Connecticut), was used for the study. Working cultures of *S.* Heidelberg was prepared from the glycerol stock cultures stored at −80°C. *S.* Heidelberg was made resistant to 50 μg/ml nalidixic acid sodium salt (NA; CAS. no. 3374-05-8, Alfa Aesar, Haverhill, MA, United States) for selective enumeration. The growth of *S.* Heidelberg (overnight culture) in tryptic soy broth (TSB; catalog no.C7141, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) was determined on xylose lysine desoxycholate agar plates (XLD; catalog no. C 7322, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) containing, 50 μg/ml NA and incubating at 37°C for 24 h. Then, final inoculum levels of 4 and 7 $\log_{10}$ CFU/ml were prepared from overnight broth culture (∼9 $\log_{10}$ CFU/ml) after centrifugation (3,600 × *g*, 15 min, 4°C) (Allegra X-14 R centrifuge, Beckman Coulter; 5350 Lakeview Parkway S Drive,

Indianapolis, IN, United States) and suspending the pellets in sterile phosphate-buffered saline (PBS; pH 7.2) (Kollanoor-Johny et al., 2012a).

Turkey Skin Preparation

Turkey drumsticks purchased from a local retail store were used for the study. The skin was separated from underlying muscles using a sterile scalpel, and 1 inch × 1 inch skin portions were exposed to UV light for 5 min to kill the background microbial flora before the application of *S.* Heidelberg on the skin surface (Kim et al., 1996).

Inoculation of *S.* Heidelberg on Turkey Skin Surface

The skin samples were dipped in 100 ml PBS containing 4 and 7 $\log_{10}$ CFU/ml *S.* Heidelberg for 20 min. Skin samples were stored at room temperature in a biosafety cabinet for 1 h for *Salmonella* attachment. Unattached and loose *Salmonella* were removed by immersing the skin samples in sterile PBS for 5 min. Non-inoculated skin samples dipped in sterile PBS were used as negative controls (Kim et al., 1996; Tamblyn and Conner, 1997; Tamblyn et al., 1997).

PEO, PNE, and Determination of Particle Size of PNE

PEO (≥99%; Natural, Food Grade; PCcode: 1002115007; Product: W290106-100G-K; Lot# MKBS7421V) was purchased from Sigma–Aldrich (St. Louis, MO, United States). PEO was mixed (by vortexing) in DI water for 30 s to prepare desired concentrations (0.5 and 1.0% v/v) for treating the turkey skin in all the experiments. PNE was prepared using high energy sonication technique as described previously (Ghosh et al., 2013; Bhargava et al., 2015). Briefly, PEO was emulsified in DI water adding tween 80 (2:1) and homogenized under high energy sonication (750 W) using a sonicator (Soniprep 450). The procedure was continued for 20 min with short intervals in an ice containing chamber to reduce the heat generation during sonication and to avoid the evaporative loss of the oil. The stability and particle size of PNE were determined using dynamic light scattering method after storing PNE at room temperature for 7 days (Bhargava et al., 2015).

Dip Treatment of PEO or PNE on *S.* Heidelberg Attached to Turkey Skin at 65°C

PEO or PNE at concentrations of 0.5 and 1.0% (v/v) were freshly prepared in sterile DI water by vortexing for 30 s, and used in the study by maintaining the temperature at 65°C using a hot water bath (Model: 89032-226, VWR international, 1310 Goshen parkway, PA, United States). The temperature of the treatment water was set at 65°C to simulate the scalding step in poultry processing, and the temperature was monitored using a thermocouple for constancy. Each skin sample was inoculated with *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch or 7 $\log_{10}$ CFU/sq. inch) and separately dipped in 0, 0.5 or 1% (v/v) of PEO or PNE for the 30 s, 3 min, or 5 min. The skin samples were homogenized immediately after the dip treatment in fresh 10 ml PBS, and *S.* Heidelberg survival rate was determined. An industry control (chlorine, 50 ppm) and a solvent control (tween 80, 1%) were also tested with the lower inoculum level of *S.* Heidelberg at 65°C following the same protocol as detailed above to determine if these treatments had any effect on the pathogen populations.

Dip Treatment of PEO or PNE on Skin Attached *S.* Heidelberg at Chilling Temperature

Concentrations of PEO or PNE at 0.5 and 1.0% (v/v) were freshly prepared in DI water by vortexing for 30 s, and used in the study by maintaining the temperature at 4°C. The temperature of the treatment water was maintained at 4°C to simulate chilling in poultry processing. Each skin sample was inoculated with *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch or 7 $\log_{10}$ CFU/sq. inch) and separately dipped in 0, 0.5, or 1% (v/v) of PEO or PNE for the 30 s, 3 min, or 5 min. Then skin samples were homogenized immediately after the dip treatment in fresh 10 ml PBS, and *S.* Heidelberg attachment was determined. An industry control (chlorine, 50 ppm) and a solvent control (tween 80, 1%) were also tested with the lower inoculum level of *S.* Heidelberg at 4°C following the same protocol as detailed above to determine if these treatments had any effect on the pathogen populations.

Dip Treatment of PEO or PNE on *S.* Heidelberg Survival on Skin Surface during Storage at Chilling and Abuse Temperature

The PEO or PNE treatments at 1% (v/v) was prepared in sterile DI water by vortexing for 30 s, at 4°C and used for dip treating skin samples; *S.* Heidelberg inoculated (4 $\log_{10}$ CFU/sq. inch) and non-inoculated (control) skin samples for 5 min under chilling temperature were kept. Then the (non-dripping) samples were packaged under aerobic conditions and stored at 4 or 10°C for 48 h (FSIS, 2002; Ingham et al., 2007). *S.* Heidelberg attachment was determined by sampling at 0, 2, 24, and 48 h of storage after PEO and PNE treatments.

Microbiological Analysis

The surviving *S.* Heidelberg attached on the skin after PEO or PNE treatments were determined by enumeration. The skin samples were homogenized with 10 ml sterile PBS in a stomacher (100/125V, 50/60Hz; Neutec Group Inc., 200 Central Ave, Farmingdale, NY, United States) for 2 min at 200 rpm. Then the sample homogenates were serially diluted 10-folds, and a volume of 100 μl from appropriate dilutions was surface plated on XLD containing 50 μg/ml NA. The surviving *S.* Heidelberg were enumerated after 24 h incubation at 37°C (Kollanoor-Johny et al., 2012a). A 100 μl of the dipping solution after all the treatments were directly surface plated on XLD + NA plates to determine any surviving pathogen populations in the dipping solution. Further, 1 ml of the dipping solution was enriched in 10 ml of selenite cysteine broth (Criterion, Hardy Diagnostics, Santa Maria, CA, United States), incubated for 12 h

and streaked on XLD + NA plates for detection of surviving bacteria if any.

CM of Turkey Skin Treated with PEO

Turkey skin (1 inch × 1 inch) portions were inoculated with 4 $\log_{10}$ CFU/sq. inch *S.* Heidelberg. Skin samples were dip treated in 1% PEO for 5 min at 4°C. Immediately after treatments, the skin samples were stained with L7012 LIVE/DEAD® BacLight Bacterial Viability Kit (Catalog number: L7012, Thermo Fisher Scientific, Waltham, MA, United States) as described previously (Seo and Frank, 1999). Briefly, the inoculated and non-inoculated skin samples with or without treatment with PEO were immersed in a solution containing equal volume of SYTO® 9 green-fluorescent nucleic acid stain (Catalog number: L7012, Thermo Fisher Scientific, Waltham, MA, United States) and propidium iodide Catalog number: L7012, Thermo Fisher Scientific, Waltham, MA, United States) red-fluorescent nucleic acid stain. The SYTO® 9 dye stains both live and dead cells. However, propidium iodide penetrates only through damaged cell membranes (dead bacteria) and masks the intensity of SYTO® 9 dye when both dyes are present. Therefore, live and dead bacteria appear as green and red, respectively. The skin samples were examined for live and dead bacteria under a confocal microscope (Nikon A1 spectral confocal microscope, University of Minnesota Imaging Center) after incubating at room temperature for 15 min. Non-inoculated skin samples dipped in sterile DI water or PEO served as negative controls. Similarly, *S.* Heidelberg inoculated skin samples dipped in sterile DI water for 5 min were included as positive controls.

SEM of Turkey Skin Treated with PEO

Turkey skin samples (1 inch × 1 inch) inoculated with *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) was dipped in 1% PEO for 5 min at 4°C. Non-inoculated skin samples dipped in sterile DI water or PEOs were used as negative controls. *S.* Heidelberg dipped in sterile DI water for 5 min served as *S.* Heidelberg controls. Sample preparation for electron microscopy was conducted as previously described with some modifications (Lee et al., 2014). Briefly, immediately after each treatment, the skin samples were stored in primary fixative [3% paraformaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 0.1 M sodium cacodylate buffer with 5mM calcium chloride and 5mM magnesium chloride (pH 7.4)] for 12 h. Then the samples were fixed in 2% osmium tetroxide and 0.1 M sodium cacodylate buffer for 12 h. Then the samples were washed in ultrapure water (NANOpure Infinity®; Barnstead/Thermo Fisher Scientific; Waltham, MD, United States) and dehydrated by ascending grades of ethanol series. Samples were processed in a critical point dryer (Autosamdri-814; Tousimis; Rockville, MD, United States) and mounted on aluminum stubs, sputter-coated with gold-palladium, and observed using a scanning electron microscope (S3500N; Hitachi High Technologies America, Inc.; Schaumberg, Illinois; University of Minnesota Imaging Center) at an accelerating voltage of 5.00 kV.

Statistical Analysis

A completely randomized design was used to analyze the effect of PEO and PNE treatments on *S.* Heidelberg in all experiments. Each skin sample served as the experimental unit. The number of *S.* Heidelberg colonies were logarithmically transformed before analysis to achieve homogeneity of variance (Byrd et al., 2003). The detection limit of *S.* Heidelberg was set at 1.0 $\log_{10}$ CFU/ml on XLD plates. The samples from which no bacteria were recovered after spread plating, but positive after enrichment, were assumed a value of 0.95 for analysis (9 CFU/ml) (Seo et al., 2000; Young et al., 2007). The data were analyzed using the PROC MIXED procedure of SAS (version 9.3) (SAS Institute, 2004). Differences among the least square means were detected using

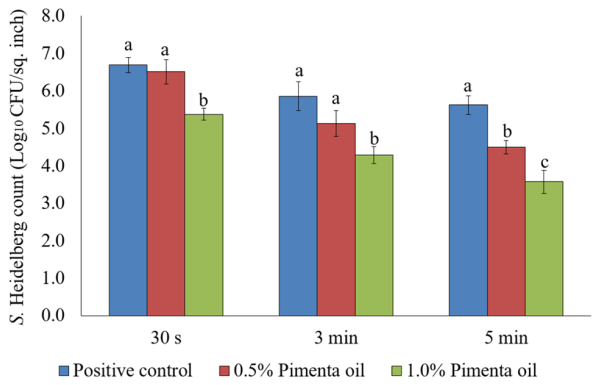


FIGURE 1 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at scalding temperature (65°C) at higher initial inoculum. *S.* Heidelberg (7 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PEO for 30 s, 3 min, or 5 min at 65°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

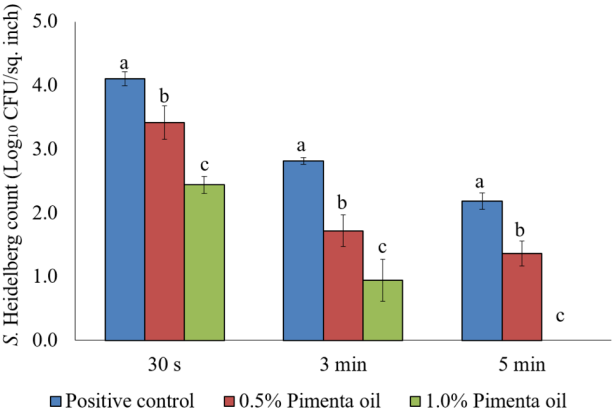


FIGURE 2 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at scalding temperature (65°C) at lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PEO for 30 s, 3 min, or 5 min at 65°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

Fisher's least significant difference test. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of PEO or PNE against *S.* Heidelberg Attachment on Turkey Skin at Scalding Temperature (65°C)

Scalding is the step where the feather follicle is loosened for feather removal (picking) (Carrasco et al., 2012; FSIS, 2015). Scalding tanks are a potential area for carcass cross-contamination with pathogens such as *Salmonella* and *Campylobacter* (FSIS, 2015). Cross-contamination can lead to the attachment of *Salmonella* to the skin surface eventually making it hard to detach the pathogens with commonly used antimicrobial agents (Lillard, 1990; Kim et al., 1996; Yang et al., 2001; Nchez et al., 2002; Nagel et al., 2013).

In the current study, two levels of initial inocula were tested: a higher inoculum level of 7 $\log_{10}$ CFU/sq. inch, and a lower inoculation level of 4 $\log_{10}$ CFU/sq. inch. The application of PEO resulted in a significant reduction of *S.* Heidelberg attachment on turkey skin at scalding temperature when a high-level inoculum of *S.* Heidelberg was used (**Figure 1**). The PEO treatment at a concentration of 0.5% resulted in 1.13 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg after 5 min dip treatment ($P < 0.05$) at 65°C, compared to the *Salmonella* control. Whereas, 1% PEO dip treatment resulted in 1.37, 1.57 and 2.05 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg in 30 s, 3 min, and 5 min, respectively at 65°C ($P < 0.05$) (**Figure 1**). The PEO treatment was also effective when a low initial inoculum of *S.* Heidelberg was tested and was concentration dependent (**Figure 2**). The PEO treatment at the lower concentration of 0.5% resulted in 0.72, 1.09, and 1.87 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg in 30 s, 3 min, and 5 min dip treatment, respectively at 65°C ($P < 0.05$) whereas, 1% PEO dip treatment resulted in 1.66, 1.87, and 2.19 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg in 30 s, 3 min, and 5 min (enrichment negative), respectively 65°C ($P < 0.05$) (**Figure 2**).

Chlorine is a common antimicrobial agent used in poultry processing (Sohaib et al., 2015). However, chlorine may lose its efficacy in scalding tanks due to the high organic load associated with the carcass, and the evaporative loss of the chlorine over time in the scalding tanks (FSIS, 2015). Organic acids can be used in scalding tanks. However, organic acids such as acetic, citric, lactic, malic, mandelic, propionic, or tartaric acid require a concentration of 2.0–6.0% to obtain 2 $\log_{10}$ CFU or more reduction of *Salmonella* attached to the skin of broiler carcasses (Tamblyn and Conner, 1997). In addition, previous studies reported the reduced activity of commonly used synthetic antimicrobial agents such as sodium hypochlorite, acetic acid, trisodium phosphate, and sodium metabisulfite against *Salmonella* attached to poultry skin, and revealed the potential of increased resistance of *Salmonella* to these antimicrobial treatments (Tamblyn et al., 1997). In the current study, *S.* Heidelberg reduction obtained with the use of 1% PEO after 5 min at the lower inoculum level tested was comparable to that of organic acids, and other USDA approved synthetic antimicrobial agents (Tamblyn et al., 1997; Tamblyn and Conner, 1997).

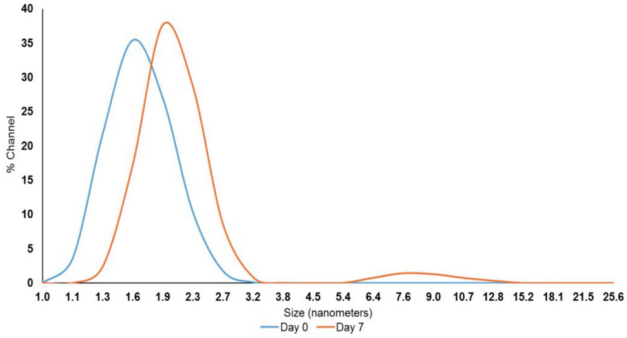


FIGURE 3 | Pimenta nanoemulsion (PNE) was prepared by emulsifying PEO in DI water using tween 80 (2:1) and homogenized under high energy sonication (750 W) using a sonicator. The procedure was continued for 20 min with short intervals. The stability and particle size of PNE were determined using dynamic light scattering method after storing PNE at room temperature for 7 days.

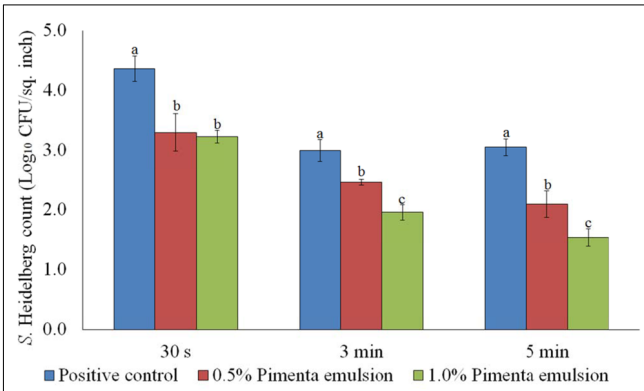


FIGURE 4 | Effect of PNE against *S.* Heidelberg attachment on turkey skin at scalding temperature (65°C) for lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PNE for 30 s, 3 min, or 5 min at 65°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

After exploring the efficacy of PEO at both inoculum levels, we focused on the lower inoculum (4 $\log_{10}$ CFU/sq. inch) to test the effect of PNE. Although the reported contamination level of *Salmonella* on poultry carcasses exiting the chillers is $\sim$ 2.0 $\log_{10}$ CFU/carcass (Waldroup, 1996), we increased the inoculum to 4 $\log_{10}$ CFU/sq. inch to avoid any potential bias in determining the efficacy of PNE treatment. The nanoemulsion of PEO (PNE) could increase the solubility and stability of PEO in water, and the fine droplet size could increase the availability of PEO (Landry et al., 2014). The peak droplet size of the prepared PNE remained in the range of 1.6–2.3 nm during a storage period of 7 days at room

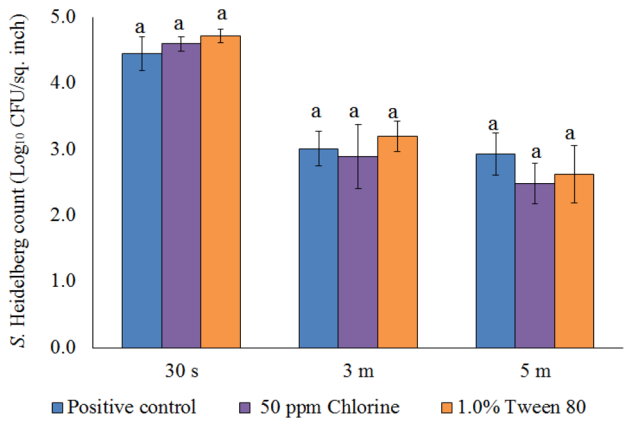


FIGURE 5 | Effect of chlorine (50 ppm) and tween 80 (1.0%) against *S.* Heidelberg attachment on turkey skin at scalding temperature (65°C) for lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 50 ppm chlorine or 1.0% tween 80 for 30 s, 3 min, or 5 min at 65°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined.

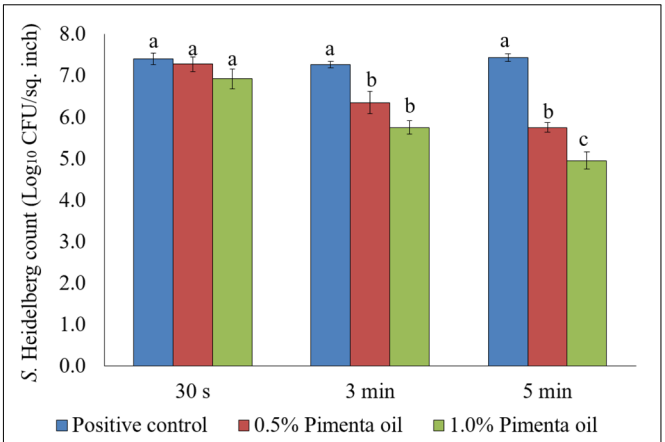


FIGURE 6 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) for higher initial inoculum. *S.* Heidelberg (7 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PEO for 30 s, 3 min, or 5 min at 4°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

temperature which could indicate its increased stability in the water (**Figure 3**).

The PNE treatment resulted in significant reduction of *S.* Heidelberg on the skin which was comparable to the respective concentrations of PEO at 65°C. The PNE treatment at 0.5% resulted in 1.07, 0.53 and 0.95 $\log_{10}$ CFU/sq. inch reduction of skin-attached *S.* Heidelberg after 30 s, 3 min, and 5 min dip treatment, respectively at 65°C ($P < 0.05$). Similarly, 1.0% PNE resulted in 1.14, 1.03, and 1.51 $\log_{10}$ CFU/sq. inch reductions of *S.* Heidelberg after 30 s, 3 min, and 5 min dip treatment, respectively at 65°C ($P < 0.05$; **Figure 4**). In the current experiment, both PEO and PNE showed similar efficacy since PEO also had small droplet size and uniform distribution on the skin surface due to the vigorous homogenization in DI water before application on the skin surface.

After the treatments, no *S.* Heidelberg was detected in the dipping solution containing PNE and PEO treatments (enrichment negative). However, a substantial population of the pathogen survived in the control dipping solution. The positive controls had 2.2-, 1.9-, and 2.3- $\log_{10}$ CFU/ml of *S.* Heidelberg after 30 s, 3 min, and 5 min dip treatments at 65°C. In addition, we conducted experiments using chlorine (50 ppm; industry control) and tween 80 (1.0%; solvent control) and found that these controls had no effect on the skin attached *S.* Heidelberg at 65°C compared to the *Salmonella* control ($P > 0.05$; **Figure 5**).

Effect of PEO or PNE against *S.* Heidelberg Attachment on Turkey Skin at Chilling Temperature (4°C)

Chilling is a critical step in poultry processing where the internal temperature of the poultry carcass is reduced to 40°F (4.4°C) or below within 4–8 h of slaughtering to prevent the growth of pathogenic bacteria including *Salmonella* (FSIS, 2014). The cross-contamination of carcasses with *Salmonella* is high in

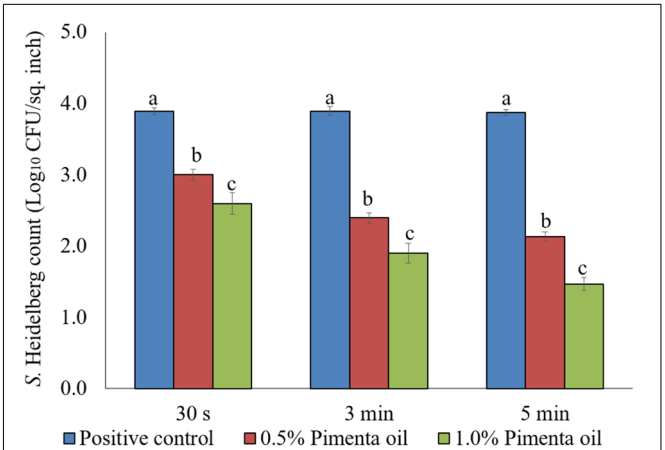


FIGURE 7 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) for lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PEO for 30 s, 3 min, or 5 min at 4°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

chiller where the carcasses are immersed in chilling water, and the carcass exiting the chiller tank often carry a significant amount of the pathogen (Lillard, 1990; Nchez et al., 2002). In the current study, the PEO application as dip treatment at 4°C significantly reduced the attachment of *S.* Heidelberg on turkey skin and the reduction was concentration and contact time dependent after 5 min of exposure for higher inoculation (**Figure 6**). Dip treatment of PEO at 0.5 and 1% did not result in significant reduction of skin attached *S.* Heidelberg for a contact period of 30 s at 4°C when higher initial inoculum of *S.* Heidelberg was used ($P > 0.05$; **Figure 6**). However, 3- and 5- min dip treatment with PEO

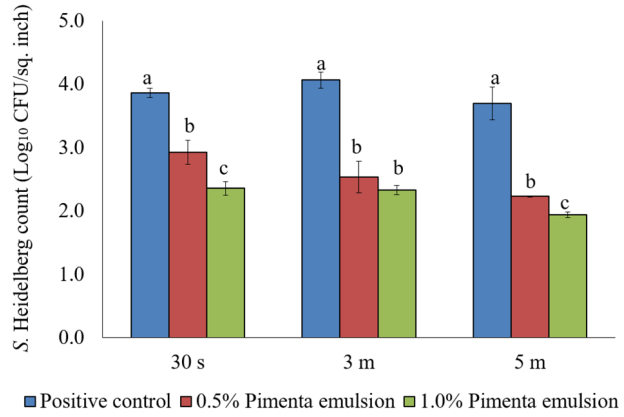


FIGURE 8 | Effect of PNE against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) for lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0, 0.5, or 1.0% PNE for 30 s, 3 min, or 5 min at 4°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined. The treatments were significantly different at $P < 0.05$.

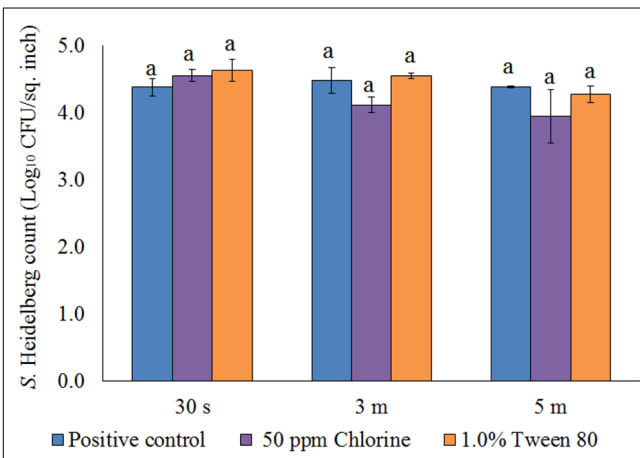


FIGURE 9 | Effect of chlorine (50 ppm) and tween 80 (1.0%) against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) for lower initial inoculum. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 50 ppm chlorine or 1.0% tween 80 for 30 s, 3 min, or 5 min at 65°C. The skin samples were homogenized immediately after the dip treatment and the pathogen populations were determined.

resulted in a significant reduction of *S.* Heidelberg compared to the control at both concentrations tested ($P < 0.05$; **Figure 6**).

The PEO treatment showed higher efficacy against *S.* Heidelberg at 4°C with the low initial inoculum of *Salmonella* (4 $\log_{10}$ CFU/sq. inch skin) compared to the higher inoculation (7 $\log_{10}$ CFU/sq. inch skin). In addition, both PEO concentrations resulted in rapid reduction of *S.* Heidelberg for a contact time as low as 30 s ($P < 0.05$; **Figure 7**). For the 0.5% PEO dip treatment, reduction of 0.89, 1.50, and 1.74 $\log_{10}$ CFU/sq. inch was obtained with 30 s, 3 min, and 5 min contact time, respectively at 4°C, compared to the controls ($P < 0.05$), whereas, a reduction of 1.3, 1.99, and 2.40 $\log_{10}$ CFU/sq. inch was obtained with 1.0% PEO after 30 s, 3 min, and 5 min dip treatment, compared to the control at 4°C ($P < 0.05$; **Figure 7**).

In the current study, PEO was rapidly effective against *S.* Heidelberg attachment on turkey skin (3 or 5 min for higher initial inoculum, and 30 s, 3 min, or 5 min for lower initial inoculum). As a usual practice, poultry carcass is immersed in the chilling tank at 4.4°C, and the antimicrobial agents get sufficient contact time to reduce *Salmonella* (Nagel et al., 2013). However, common antimicrobial agents including chlorine were found to be less effective against skin-attached *Salmonella*. Chlorine reduced less than 1.0 $\log_{10}$ CFU/ml of *S.* Typhimurium attachment on poultry skin at a concentration of 50 ppm for 50 min contact time (Yang et al., 2001). In our study, the PEO dip treatment showed similar or better/higher efficacy compared to other synthetic antimicrobial agents approved for the chilling process. For example, a common antimicrobial, sodium metabisulfite, was ineffective against firmly attached *Salmonella* even after 60 min immersion treatment at 0°C. In addition, acetic acid (5%) and sodium metabisulfite (1%) were ineffective against *S.* Typhimurium attachment on poultry skin under aforementioned chilling conditions (Tamblyn et al., 1997). Additionally, organic acids such as acetic, citric, lactic, malic, mandelic, propionic, or tartaric acid required a concentration of 4.0% or above to get at least 2 $\log_{10}$ reduction of *S.* Typhimurium attachment on poultry skin at 0°C for 60 min contact time (Tamblyn and Conner, 1997). Another antimicrobial, sodium hypochlorite, was effective against skin-attached *S.* Typhimurium only when used at higher concentrations such as 400 and 800 ppm for a contact time of 60 min at 0°C to result in <2 $\log_{10}$ CFU/ml reductions of firmly attached *Salmonella* on the skin surface.

In addition to the potential use of PEO in chilling tanks, it could be used for post-chill dip treatment during poultry processing since PEO dip treatment was effective rapidly against skin-attached *S.* Heidelberg. Post-chill dip application of antimicrobial agents is currently practiced as a part of the multiple hurdle technology along with antimicrobial interventions in the chilling tanks (Nagel et al., 2013; Nair et al., 2015). The advantage of post-chill dip application is that the carcass is in contact with the antimicrobial agent for a shorter duration (30 s) and a higher concentration of antimicrobial agent can be used without deteriorating the carcass quality. The antimicrobial agent would be more efficient since there is less organic load compared to the chilling tank (Nagel et al., 2013). In the current study, 30 s dip treatment of PEO resulted in 0.83 and 1.3 $\log_{10}$ CFU/sq. inch reductions of *S.* Heidelberg with 0.5 and 1.0% PEO, respectively at 4°C. It is comparable to the reduction achieved during post chill dip treatment using other synthetic antimicrobial agents or other EOs (Nagel et al., 2013; Nair et al., 2014, 2015). For example, 40 or 400 ppm chlorine and 1000 or 5000 ppm lysozyme resulted in less than 1 $\log_{10}$ CFU/ml reduction of *S.* Typhimurium on broiler carcass when used as post-chill dip treatment for 20 s (Nagel et al., 2013). Similarly, post chill application of carvacrol at 0.5, 1.0, or 2% for 30 s resulted in less than a $\log_{10}$ CFU/ml

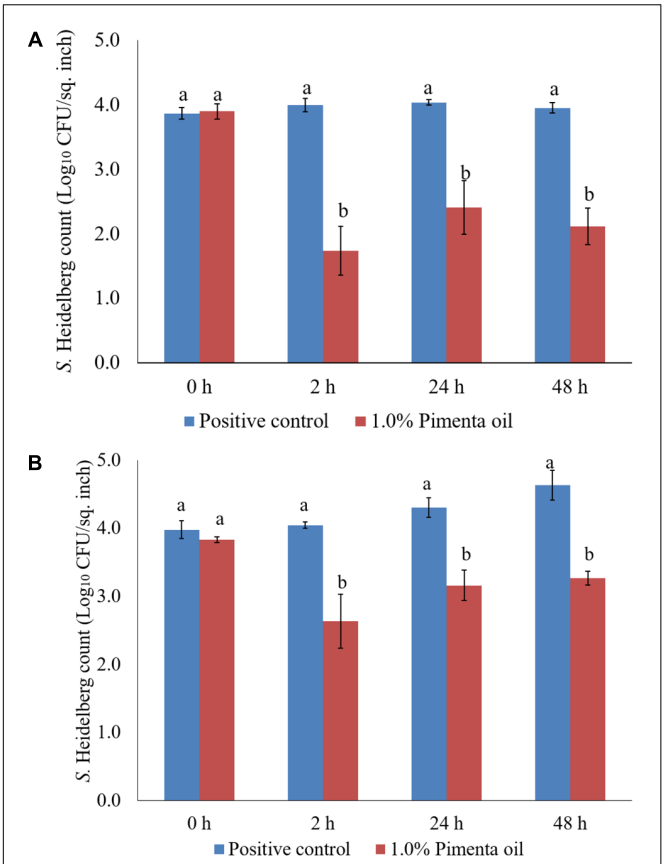


FIGURE 10 | (A) Effect of PEO against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) and storage at 4°C for 48 h. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0 or 1.0% PEO for 5 min at 4°C. The samples were packaged under aerobic conditions and stored at 4°C. *S.* Heidelberg attachment was determined by sampling at 0, 2, 24, and 48 h of storage after PEO treatments. The treatments were significantly different at $P < 0.05$. **(B)** Effect of PEO against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) and storage at 10°C for 48 h. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0 or 1.0% PEO for 5 min at 4°C. The samples were packaged under aerobic conditions and stored at 10°C. *S.* Heidelberg attachment was determined by sampling at 0, 2, 24, and 48 h of storage after PEO treatments. The treatments were significantly different at $P < 0.05$.

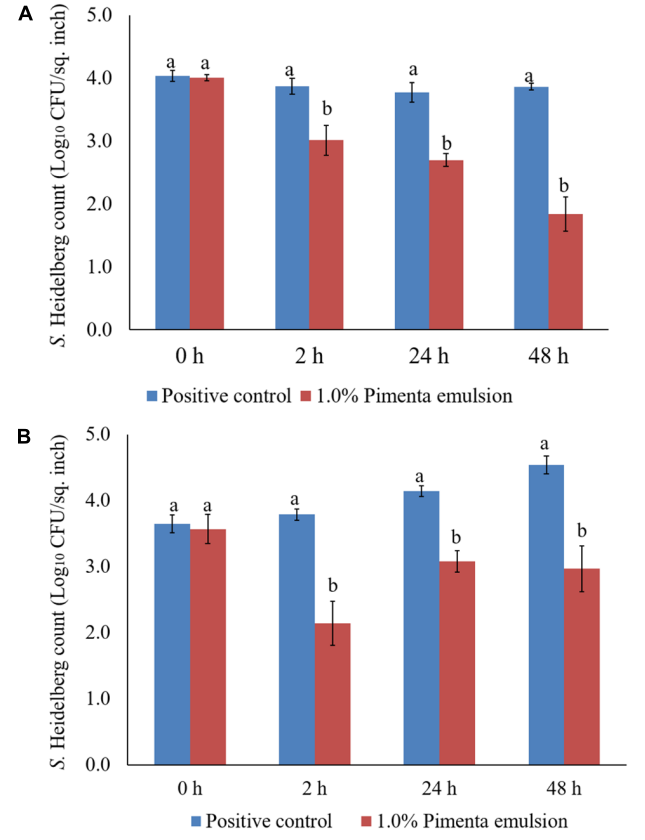


FIGURE 11 | (A) Effect of PNE against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) and storage at 4°C for 48 h. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0 or 1.0% PNE for 5 min at 4°C. The samples were packaged under aerobic conditions and stored at 4°C. *S.* Heidelberg attachment was determined by sampling at 0, 2, 24, and 48 h of storage after PEO treatments. The treatments were significantly different at $P < 0.05$. **(B)** Effect of PNE against *S.* Heidelberg attachment on turkey skin at chilling temperature (4°C) and storage at 10°C for 48 h. *S.* Heidelberg (4 $\log_{10}$ CFU/sq. inch) inoculated skin samples were treated with 0 or 1.0% PEO for 5 min at 4°C. The samples were packaged under aerobic conditions and stored at 10°C. *S.* Heidelberg attachment was determined by sampling at 0, 2, 24, and 48 h of storage after PNE treatments. The treatments were significantly different at $P < 0.05$.

reduction of *Salmonella* spp. (*S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium) on skinless, boneless turkey breast cutlets (Nair et al., 2014).

The PNE treatment also showed significant reduction of skin-attached *S.* Heidelberg at 4°C (**Figure 8**). Lower initial inoculum (4.0 $\log_{10}$ CFU/sq. inch) of *S.* Heidelberg was used to study the effect of PNE on skin-attached *S.* Heidelberg since PEO treatment showed similar or higher efficacy when used against the lower initial inoculum of *S.* Heidelberg. At chilling temperature, PNE at 0.5% resulted in 0.94, 1.53 and 1.47 $\log_{10}$ CFU/sq. inch reductions of *S.* Heidelberg in 30 s, 3 min, and 5 min dip treatment, respectively ($P < 0.05$), compared to the *S.* Heidelberg control. Likewise, PNE at 1% resulted in 1.51, 1.74, and 1.76 $\log_{10}$ CFU/sq. inch reductions of *S.* Heidelberg after 30 s, 3 min, and 5 min dip treatment, respectively at 4°C compared to the control ($P < 0.05$) (**Figure 8**).

After the treatments, no *S.* Heidelberg was detected in the dipping solution containing PNE and PEO treatments (enrichment negative). However, a substantial population of the pathogen survived in the control dipping solution. The positive controls had 2.5-, 2.4-, and 2.6- $\log_{10}$ CFU/ml of *S.* Heidelberg after 30 s, 3 min and 5 min dip treatments at 4°C. In addition, we conducted experiments using chlorine (50 ppm; industry control) and tween 80 (1.0%; solvent control) and found that these controls had no effect on the skin attached *S.* Heidelberg at 4°C compared to the *Salmonella* control. Chlorine treatment resulted in only 0.37 and 0.44 $\log_{10}$ CFU/sq. inch reductions of *S.* Heidelberg ($P > 0.05$; **Figure 9**) for 3 and 5 min dip treatments, respectively whereas tween 80 treatment resulted in same *S.*

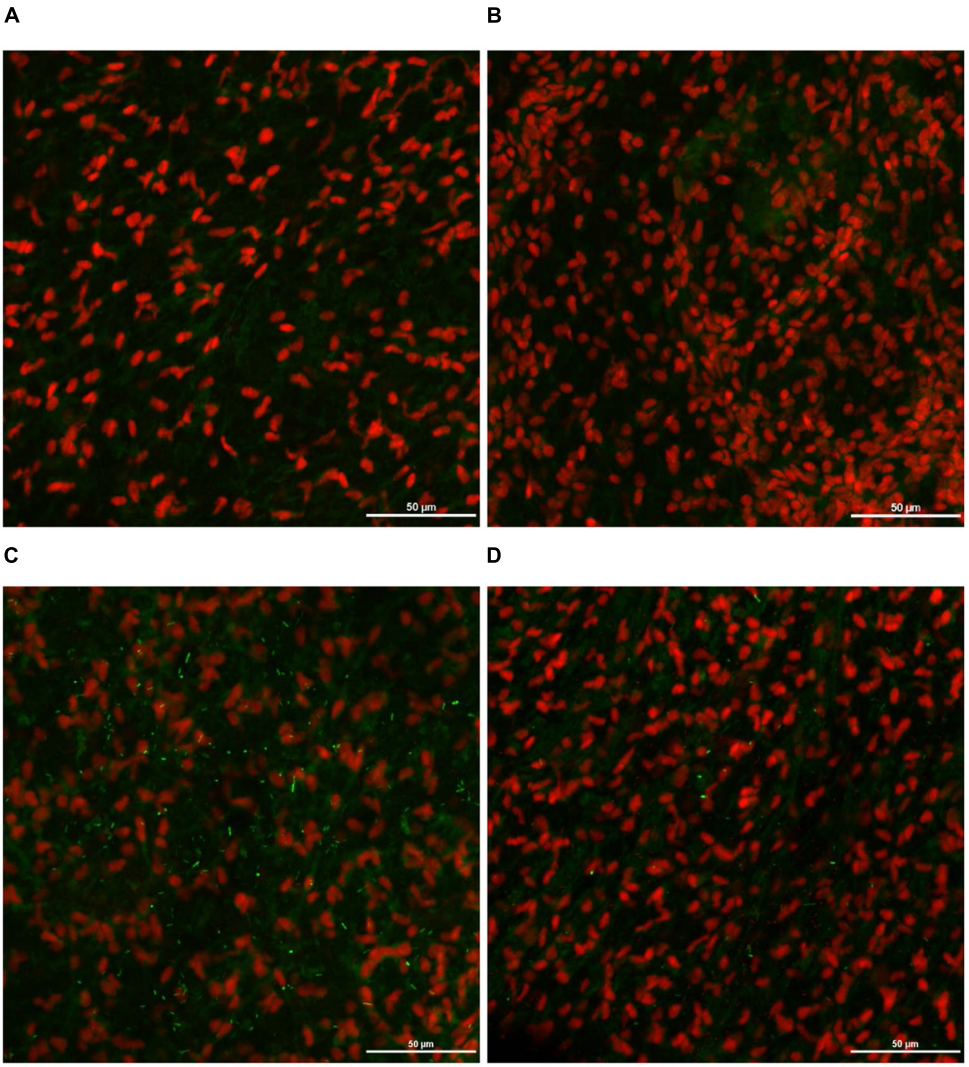


FIGURE 12 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at simulated chilling temperature (4°C) – Confocal microscopy of the outer surface of skin: **(A)** Negative control (Turkey skin + DI water), **(B)** Pimenta control (Turkey skin + DI water + PEO), **(C)** *Salmonella* control (Turkey skin + DI water + *S.* Heidelberg) and **(D)** Treatment (Turkey skin + DI water + *S.* Heidelberg + PEO).

Heidelberg count as that of *Salmonella* control in all the three dip treatments at 4°C ($P > 0.05$; **Figure 9**).

Effect of PEO or PNE against *S.* Heidelberg Attachment on Turkey Skin at 4°C and Stored for 48 h at 4°C and 10°C

The PEO treatment at 1% applied for 5 min at 4°C showed a higher reduction of *S.* Heidelberg attachment on turkey skin (**Figure 7**). Therefore, the same combination was used for determining the efficacy of PEO or PNE treatments against *S.* Heidelberg attachment on turkey skin for 48 h storage at 4°C (**Figure 10A**) and 10°C (**Figure 10B**; abuse temperature). The PEO treatment (1.0%) resulted in 2.27, 1.63, and 1.83 $\log_{10}$ CFU/sq. inch reduction of *S.* Heidelberg after 2, 24 and 48 h of storage at 4°C ($P < 0.05$) (**Figure 10A**). The skin samples were also stored at 10°C to determine *S.* Heidelberg attachment at abuse temperature during refrigerated storage (FSIS, 2002). *S.* Heidelberg multiplied during 10°C storage in the *S.* Heidelberg controls. However, 1% PEO dip treatment for 5 min significantly reduced *S.* Heidelberg growth and multiplication on the skin after 2, 24, and 48 h of storage at 10°C ($P < 0.05$; **Figure 10B**).

The PNE (1.0%) treatment was also effective in reducing *S.* Heidelberg attachment on turkey skin at 4 and 10°C; the efficacy was comparable to the 1.0% PEO treatment for 5 min under similar storage conditions. The PNE treatment resulted in significant reduction in *S.* Heidelberg attachment after 2, 24 and 48 h of storage at 4°C ($P < 0.05$; **Figure 11A**). Likewise, PNE resulted in a similar reduction in *S.* Heidelberg populations after storage at 10°C ($P < 0.05$; **Figure 11B**). Therefore, the results of the present study indicate that PEO and PNE treatments are

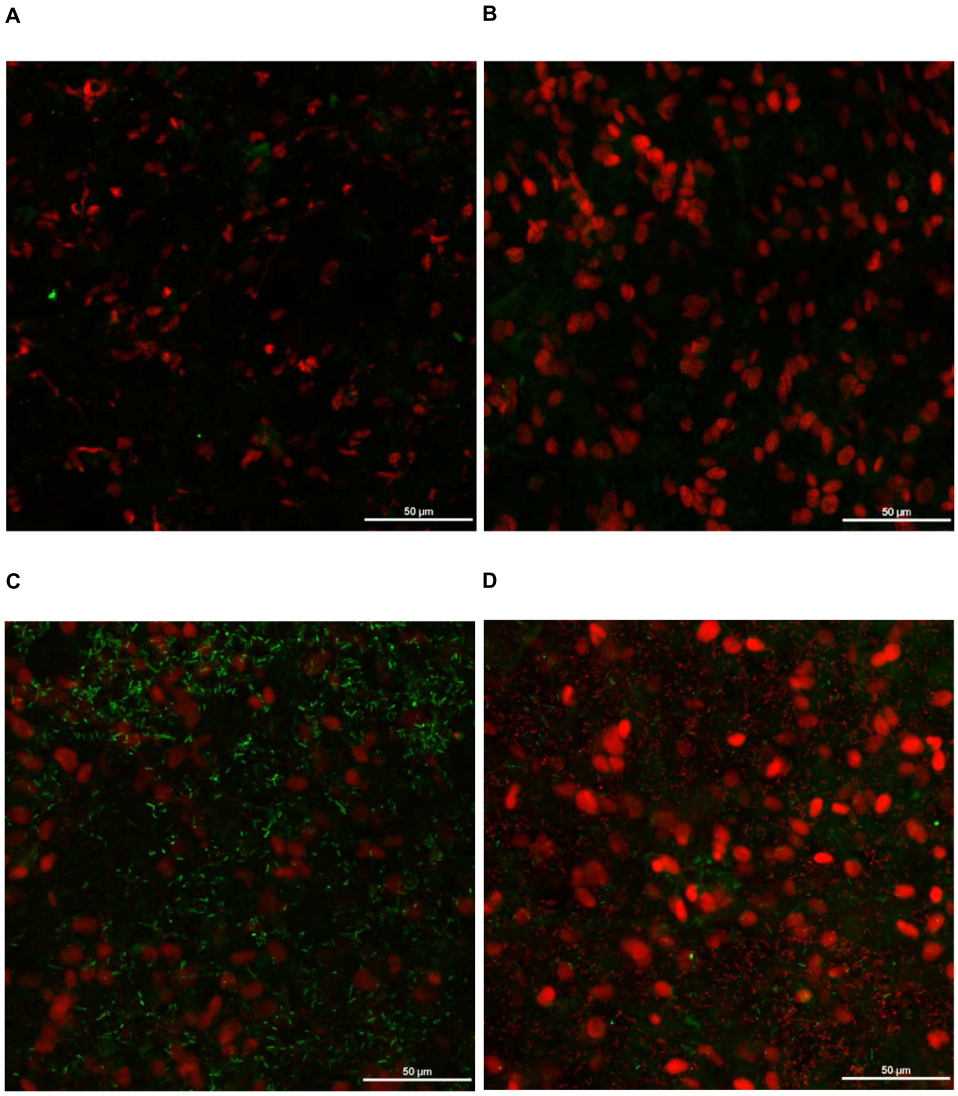


FIGURE 13 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at simulated chilling temperature (4°C) – Confocal microscopy of the inner surface of skin: **(A)** Negative control (Turkey skin + DI water), **(B)** Pimenta control (Turkey skin + DI water + PEO), **(C)** *Salmonella* control (Turkey skin + DI water + *S.* Heidelberg) and **(D)** Treatment (Turkey skin + DI water + *S.* Heidelberg + PEO).

effective in reducing *S.* Heidelberg attachment on turkey skin when stored at 4°C for 48 h. Also, PEO and PNE treatments were effective in reducing *S.* Heidelberg attachment on turkey skin during abuse temperature (10°C) condition for 48 h.

Effect of PEO on *S.* Heidelberg Attachment to Turkey Skin Illustrated Using CM and SEM

As expected, *S.* Heidelberg was not present in the negative controls (**Figures 12A**, **13A**, **14A**, **15A**). The CM and SEM revealed that the PEO treatment had no deleterious effect on turkey skin. The skin cells maintained normal shape (**Figures 12B**, **13B**, **14B**, **15B**). Inoculation of skin samples with *S.* Heidelberg resulted in the attachment of the pathogen to both sides. The physical structure of the skin surface contributed to the firm attachment of *S.* Heidelberg onto the skin surface. The pathogen could penetrate deep into the crevices and feather follicles on the outer skin surface. In addition, the swelling of skin cells during the immersion process could have exposed deep channels and crevices on the skin resulting in enhanced *S.* Heidelberg attachment to the skin surface (Kim et al., 1996). The presence of flagella in *Salmonella* is also a major contributing factor for firm attachment of the pathogen to the poultry skin. Flagellar structural subunits (*flgK*, *fliC*, and *fljB*) and motor units (*motA*, *motB*) play a significant role in the *Salmonella* attachment to the poultry skin (Salehi et al., 2017).

The CM of the *S.* Heidelberg treated skin revealed many live *S.* Heidelberg cells (green rod-shaped cells) on the outer (**Figure 12C**) and inner (**Figure 13C**) surfaces of the skin.

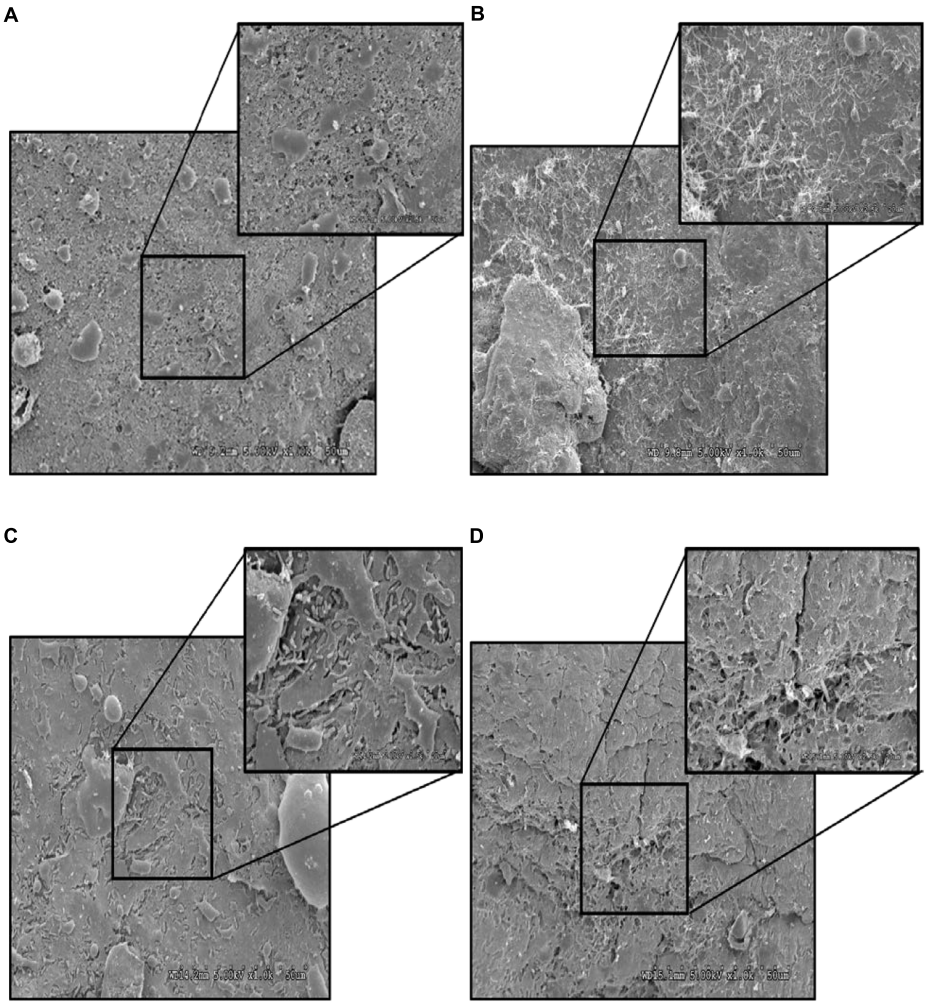


FIGURE 14 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at simulated chilling temperature (4°C) – Scanning electron microscopy of the outer surface of skin: **(A)** Negative control (Turkey skin + DI water), **(B)** Pimenta control (Turkey skin + DI water + PEO), **(C)** *Salmonella* control (Turkey skin + DI water + *S.* Heidelberg) and **(D)** Treatment (Turkey skin + DI water + *S.* Heidelberg + PEO).

However, on the PEO treated skin, *S.* Heidelberg attachment was significantly less (**Figures 12D**, **13D**) as indicated by several dead cells (red rod-shaped cells) (**Figure 13D**). Similarly, SEM also revealed a significant reduction of *S.* Heidelberg on the inner and outer surfaces of the skin in the PEO treated skin samples (**Figures 14D**, **15D**) compared to the *S.* Heidelberg controls (**Figures 14C**, **15C**). The attachment of *S.* Heidelberg was more on the inner surface of the skin relative to the outer surface which could be due to the strong adherence of *Salmonella* in the adipose tissue underneath the skin (Tan et al., 2014).

Potential Mechanisms of Action of EOs against *S.* Heidelberg

The active components present in the EOs elicit antimicrobial property against pathogenic microorganisms (Kollanoor Johny et al., 2008; Amalaradjou et al., 2010; Kollanoor-Johny et al., 2012a,b; Nair et al., 2014, 2015). These components include eugenol, carvacrol, thymol, cinnamaldehyde, *p*-cymene and a multitude of others that kill microorganisms by different modes of actions such as disruption of the cell wall, degradation of cytoplasmic membranes, alteration of membrane potential, disruption of proton motive force, and leakage of cellular contents through multiple targets in the microbial cell (Burt, 2004). However, the mode of action of essential oils could vary between Gram-negative and Gram-positive bacteria. The features of Gram negative bacterial cell such as less lipophilic nature, the presence of outer membrane and the lipopolysaccharide could make Gram negative bacteria more resistant to the effect of essential oil components (Nazzaro et al., 2013). On the other side, we have previously reported high antibacterial activity for essential oil ingredients such as *trans*-cinnamaldehyde and eugenol against *S.* Enteritidis in broiler chickens (Kollanoor-Johny et al., 2012a,b).

In the present study, the PEO treatment caused significant reduction of *S.* Heidelberg attached to turkey skin. The exact

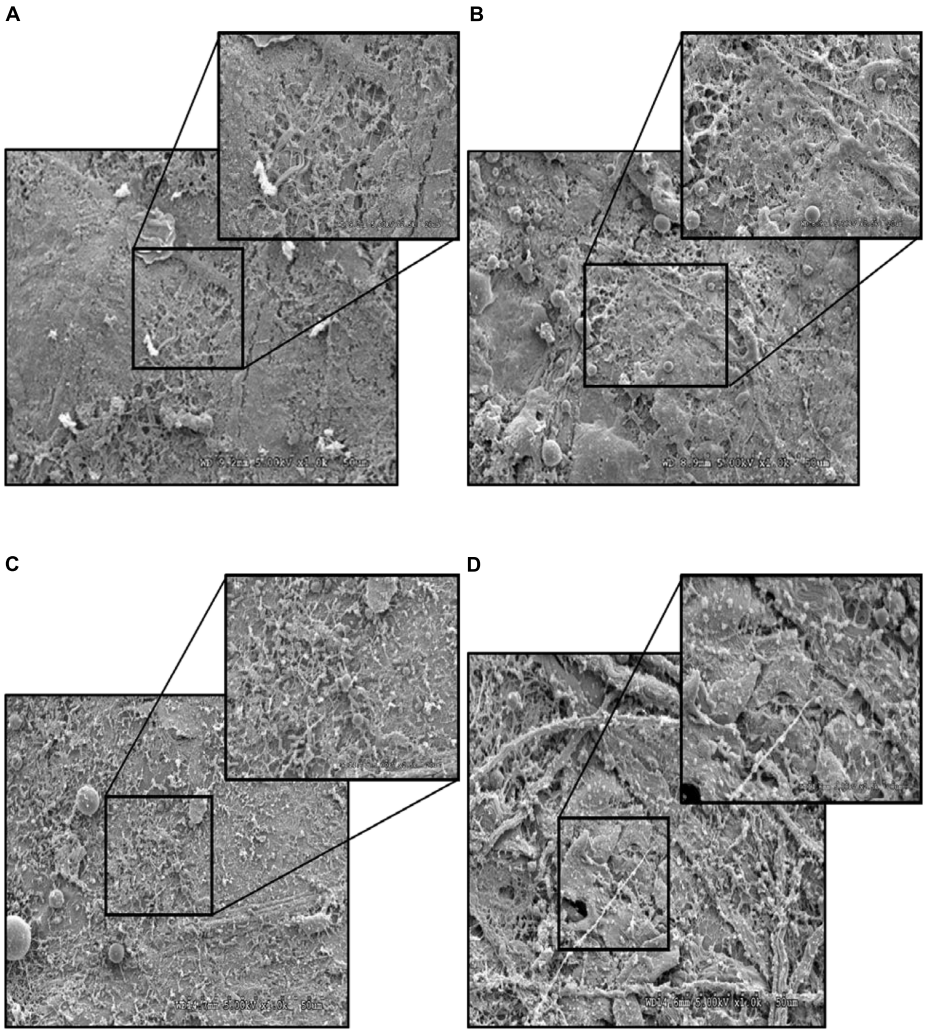


FIGURE 15 | Effect of PEO against *S.* Heidelberg attachment on turkey skin at simulated chilling temperature (4°C) – Scanning electron microscopy of the inner surface of skin: **(A)** Negative control (Turkey skin + DI water), **(B)** Pimenta control (Turkey skin + DI water + PEO), **(C)** *Salmonella* control (Turkey skin + DI water + *S.* Heidelberg) and **(D)** Treatment (Turkey skin + DI water + *S.* Heidelberg + PEO).

mechanism of action of PEO against *S.* Heidelberg has not been understood. However, PEO contains eugenol as a major active component having strong inhibitory activity against both Gram-negative and Gram-positive bacteria (Hyldgaard et al., 2012). The presence of eugenol might have resulted in morphological alterations of the microbial cell, including the disruption of the cell membrane, and formation cleft, and pore leading to the leakage of contents and subsequent death of microorganisms (Suzuki et al., 2014). Moreover, eugenol can down-regulate several critical genes in *S.* Enteritidis responsible to adhesion and invasion of cultured avian epithelial cells *in vitro* (Kollanoor-Johny et al., 2012a; Upadhyaya et al., 2013). More investigations are required to understand the exact mechanism of action of PEO on *S.* Heidelberg attached to turkey skin.

CONCLUSION

In the current study, we investigated the potential of PEO and PNE on *S.* Heidelberg attached to turkey skin in simulated scalding and chilling conditions. The highest reduction of skin attached *S.* Heidelberg was observed when 1.0% PEO or PNE was used for 5 min dip treatment at scalding (65°C) or chilling temperature (4°C). Although comparable *S.* Heidelberg reductions were obtained at both temperatures, a slightly better effect was noticed with 1% PEO at the tested scalding temperature. An antimicrobial treatment that results in 2 log or more reduction of *Salmonella* when applied during chilling is considered effective since *Salmonella* may be present on the poultry carcass in the range of <100 cells after processing (Tamblyn et al., 1997). Interestingly, no *S.* Heidelberg was

detected in the dipping solution containing PNE and PEO treatments, although the pathogens were present in the control dipping solution. This result indicates that PEO and PNE could prevent cross-contamination or recontamination of the pathogens in case the same water is used for dipping/washing carcasses. Moreover, PEO and PNE were effective in reducing *S.* Heidelberg on skin during a storage period of 2 days at chilling and abuse temperatures. CM and SEM revealed a deep infiltration and attachment of *S.* Heidelberg in the inner and outer surfaces of the skin. Treatment with PEO reduced pathogen attachment on either side as evidenced from the CM and SEM images.

Overall, the results indicate that PEO or PNE treatments could be potential alternatives to synthetic antimicrobial agents to reduce *S.* Heidelberg attachment to turkey skin during poultry processing. Although the PEO was solubilized by the nanoemulsion method, the potency did not differ significantly between the PEO and PNE treatments. We are investigating the possibility of scale-up investigations with PEO by incorporating other parameters such as different *Salmonella enterica* serotypes of importance, exploring techniques to lower the levels of PNE and PEO for application on turkey carcasses, varying levels of organic content in the water, the age of scalding and chill water, and the effect of PEO during long-term storage. Furthermore, sensory evaluation of carcasses treated with PEO and PNE treatments must be carried out.

AUTHOR CONTRIBUTIONS

AKJ conceived the idea and designed the experiments. DN performed all experiments and participated in the statistical analysis with AKJ. DN and AKJ jointly wrote and corrected the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank the MN Agricultural Experimentation Station project funds (state special) awarded to the PI, AKJ, for research support. They are thankful to Grant Bartel and Dr. Gail Celio at the University of Minnesota Imaging Center for their technical help with CM and SEM, respectively. They would also like to thank Dr. James Marti at the Nanotechnology Center, the University of Minnesota, for his directions on analyzing the particle size of the PNE treatments, and thank Mr. Jijo Vazhakkattu Thomas, MS student, for his help in surface plating.

REFERENCES

Amalaradjou, M. A. R., Baskaran, S. A., Ramanathan, R., Kollanoor Johny, A., Charles, A. S., Valipe, S. R., et al. (2010). Enhancing the thermal destruction of *Escherichia coli* O157: H7 in ground beef patties by trans-cinnamaldehyde. *Food Microbiol.* 27, 841–844. doi: 10.1016/j.fm.2010.05.006

Aureli, P., Costantini, A., and Zolea, S. (1992). Antibacterial activity of some plant essential oils against *Listeria monocytogenes*. *J. Food Prot.* 55, 344–348. doi: 10.4315/0362-028X-55.5.344

Bhargava, K., Conti, D. S., da Rocha, S. R. P., and Zhang, Y. (2015). Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. *Food Microbiol.* 47, 69–73. doi: 10.1016/j.fm.2014.11.007

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Byrd, J. A., Anderson, R. C., Callaway, T. R., Moore, R. W., Knape, K. D., Kubena, L. F., et al. (2003). Effect of experimental chlorate product administration in the drinking water on *Salmonella* Typhimurium contamination of broilers. *Poult. Sci.* 82, 1403–1406. doi: 10.1093/ps/82.9.1403

Carrasco, E., Morales-Rueda, A., and García-Gimeno, R. M. (2012). Cross-contamination and recontamination by *Salmonella* in foods: a review. *Food Res. Int.* 45, 545–556. doi: 10.1016/j.foodres.2011.11.004

CDC (2011). *Salmonella Heidelberg Infections Linked to Ground Turkey*. Available at: https://www.cdc.gov/salmonella/2011/ground-turkey-11-10-2011.html [accessed January 15, 2017].

CDC (2013). *Morbidity and Mortality Weekly Report: Surveillance for Foodborne Disease Outbreaks — United States, 1998–2008*. Available at: https://www.cdc.gov/mmwr/preview/mmwrhtml/ss6202a1.htm [accessed May 22, 2017].

CDC (2014a). *Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Foster Farms Brand Chicken*. Available at: https://www.cdc.gov/salmonella/heidelberg-10-13/ [accessed January 15, 2017].

CDC (2014b). *Salmonella Outbreak of Salmonella Heidelberg Infections Linked to Tyson Brand Chicken*. Available at: https://www.cdc.gov/salmonella/heidelberg-01-14/ [accessed January 15, 2017].

FDA (2016). *CFR - Code of Federal Regulations Title 21: Part 182 – Substances Generally Recognized as Safe: Sec. 182.20. Essential Oils, Oleoresins (Solvent-Free), and Natural Extractives (Including Distillates)*. Silver Spring, MD: FDA.

Foley, S. L., Lynne, A. M., and Nayak, R. (2008). *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J. Anim. Sci.* 86(Suppl. 14), E149–E162. doi: 10.2527/jas.2007-0464

Folster, J. P., Pecic, G., Rickert, R., Taylor, J., Zhao, S., Fedorka-Cray, P. J., et al. (2012a). Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg from a ground turkey-associated outbreak in the United States in 2011. *Antimicrob. Agents Chemother.* 56, 3465–3466. doi: 10.1128/AAC.00201-12

Folster, J. P., Pecic, G., Singh, A., Duval, B., Rickert, R., Ayers, S., et al. (2012b). Characterization of extended-spectrum cephalosporin–resistant *Salmonella enterica* serovar Heidelberg isolated from food animals, retail meat, and humans in the United States 2009. *Foodborne Pathog. Dis.* 9, 638–645. doi: 10.1089/fpd.2012.1130

FSIS (2002). *Potential Scientific Parameters that must be Considered to Establish Global Dates for Refrigerated Ready-to-Eat Foods*. Available at: https://www.fsis.usda.gov/OPHS/nacmcf/2002/rep_shelflife1.htm [accessed March 11, 2017].

FSIS (2010). *Compliance Guideline for Controlling Salmonella and Campylobacter in Poultry*. Available at: http://www.fsis.usda.gov/PDF/Compliance_Guide_Controling_Salmonella_Campylobacter_Poultry_0510.pdf

FSIS (2014). *FSIS Compliance Guide: Modernization of Poultry Slaughter Inspection: Chilling Requirements*. Washington, DC: Food Safety and Inspection Service.

FSIS (2015). *DRAFT FSIS Compliance Guideline for Controlling Salmonella and Campylobacter in Raw Poultry*. Washington, DC: Food Safety and Inspection Service, 1–109.

Ghosh, V., Mukherjee, A., and Chandrasekaran, N. (2013). Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity. *Ultrason. Sonochem.* 20, 338–344. doi: 10.1016/j.ultsonch.2012.08.010

GPO (2017). *eCFR — Code of Federal Regulations: Subpart C—Organic Production and Handling Requirements and Subpart D—Labels, Labeling, and Market Information*. Available at: http://www.ecfr.gov/cgi-bin/text-idx?SID=0a4045faa17f9676c258733eb6f1b9e7&mc=true&node=pt7.3.205&rgn=div5#sp7.3.205.c [accessed January 15, 2017].

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* 3:12. doi: 10.3389/fmicb.2012.00012

Ingham, S. C., Fanslau, M. A., Burnham, G. M., Ingham, B. H., Norback, J. P., and Schaffner, D. W. (2007). Predicting pathogen growth during short-term temperature abuse of raw pork, beef, and poultry products: use of an isothermal-based predictive tool. *J. Food Prot.* 70, 1446–1456. doi: 10.4315/0362-028X-70.6.1446

Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., and Chai, S. J. (2013). *Salmonella enterica* serotypes and food commodities. *Emerg. Infect. Dis.* 19, 1239–1244. doi: 10.3201/eid1908.121511

Kim, K. Y., Frank, J. F., and Craven, S. E. (1996). Three-dimensional visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy. *Lett. Appl. Microbiol.* 22, 280–282. doi: 10.1111/j.1472-765X.1996.tb01161.x

Kollanoor Johny, A., Darre, M. J., Donoghue, A. M., Donoghue, D. J., and Venkitanarayanan, K. (2010). Antibacterial effect of *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken cecal contents *in vitro*. *J. Appl. Poult. Res.* 19, 237–244. doi: 10.3382/japr.2010-00181

Kollanoor Johny, A., Darre, M. J., Hoagland, T. A., Schreiber, D. T., Donoghue, A. M., Donoghue, D. J., et al. (2008). Antibacterial effect of trans-cinnamaldehyde on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken drinking water. *J. Appl. Poult. Res.* 17, 490–497. doi: 10.3382/japr.2008-00051

Kollanoor-Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A., Babapoor, S., March, B., et al. (2012a). Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds *trans*-cinnamaldehyde and eugenol. *Appl. Environ. Microbiol.* 78, 2981–2987. doi: 10.1128/AEM.07643-11

Kollanoor-Johny, A., Upadhyay, A., Baskaran, S. A., Upadhyaya, I., Mooyottu, S., Mishra, N., et al. (2012b). Effect of therapeutic supplementation of the plant compounds *trans*-cinnamaldehyde and eugenol on *Salmonella enterica* serovar Enteritidis colonization in market-age broiler chickens. *J. Appl. Poult. Res.* 21, 816–822. doi: 10.3382/japr.2012-00540

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M., and Gardini, F. (2004). Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. *Trends Food Sci. Technol.* 15, 201–208. doi: 10.1016/j.tifs.2003.10.004

Landry, K. S., Chang, Y., McClements, D. J., and McLandsborough, L. (2014). Effectiveness of a novel spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and *Escherichia coli* O157: H7 on contaminated mung bean and alfalfa seeds. *Int. J. Food Microbiol.* 187, 15–21. doi: 10.1016/j.ijfoodmicro.2014.06.030

Lee, N. Y., Park, S. Y., Kang, I. S., and Ha, S. D. (2014). The evaluation of combined chemical and physical treatments on the reduction of resident microorganisms and *Salmonella* Typhimurium attached to chicken skin. *Poult. Sci.* 93, 208–215. doi: 10.3382/ps.2013-03536

Lillard, H. S. (1990). The impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. *J. Food Prot.* 53, 202–207. doi: 10.4315/0362-028X-53.3.202

Logue, C. M., Sherwood, J. S., Elijah, L. M., Olah, P. A., and Dockter, M. R. (2003). The incidence of *Campylobacter* spp. on processed turkey from processing plants in the midwestern United States. *J. Appl. Microbiol.* 95, 234–241. doi: 10.1046/j.1365-2672.2003.01969.x

Nagel, G. M., Bauermeister, L. J., Bratcher, C. L., Singh, M., and McKee, S. R. (2013). *Salmonella* and *Campylobacter* reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. *Int. J. Food Microbiol.* 165, 281–286. doi: 10.1016/j.ijfoodmicro.2013.05.016

Nair, D., Thomas, J. V., and Kollanoor-Johny, A. (2016). "*Propionibacterium freudenreichii* reduces cecal colonization of multidrug-resistant *Salmonella* Heidelberg in turkey poults," in *Proceedings of the Poultry Science Association 105th Annual Meeting Abstracts (Schwean-Lardner)*, New Orleans, 21–22.

Nair, D. V. T., Kiess, A., Nannapaneni, R., Schilling, W., and Sharma, C. S. (2015). The combined efficacy of carvacrol and modified atmosphere packaging on the survival of *Salmonella*, *Campylobacter jejuni* and lactic acid bacteria on turkey breast cutlets. *Food Microbiol.* 49, 134–141. doi: 10.1016/j.fm.2015.01.010

Nair, D. V. T., Nannapaneni, R., Kiess, A., Schilling, W., and Sharma, C. S. (2014). Reduction of *Salmonella* on turkey breast cutlets by plant-derived compounds. *Foodborne Pathog. Dis.* 11, 981–987. doi: 10.1089/fpd.2014.1803

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., and De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* 6, 1451–1474. doi: 10.3390/ph6121451

Nchez, M. X. S., Fluckey, W. M., Brashears, M. M., and Mckee, S. R. (2002). Antibiotic susceptibility of *Campylobacter* spp. and *Salmonella* spp. in broilers processed in air-chilled and immersion-chilled environments. *J. Food Prot.* 65, 948–956. doi: 10.4315/0362-028X-65.6.948

Poppe, C., Martin, L. C., Gyles, C. L., Reid-Smith, R., Boerlin, P., McEwen, S. A., et al. (2005). Acquisition of resistance to extended-spectrum cephalosporins by *Salmonella enterica* subsp. *enterica* serovar Newport and *Escherichia coli* in the turkey poult intestinal tract. *Appl. Environ. Microbiol.* 71, 1184–1192. doi: 10.1128/AEM.71.3.1184-1192.2005

Russell, S. M. (2008). Chemical residuals in the environment and on chicken carcasses associated with scalding chickens in an acidic, copper sulfate-based commercial sanitizer during poultry processing. *J. Food Prot.* 71, 226–230. doi: 10.4315/0362-028X-71.1.226

Salehi, S., Howe, K., Lawrence, M. L., Brooks, J. P., Bailey, R. H., and Karsi, A. (2017). *Salmonella enterica* Serovar Kentucky flagella are required for broiler skin adhesion and Caco-2 cell invasion. *Appl. Environ. Microbiol.* 83:e02115-16. doi: 10.1128/AEM.02115-16

SAS Institute (2004). *SAS/STAT 9.1 User's Guide*. Available at: http://books.google.com/books?id=2oEQyOIyCbUC&pgis=1

Seo, K., Holt, P., and Gast, R. (2000). Combined effect of antibiotic and competitive exclusion treatment on *Salmonella* Enteritidis fecal shedding in molted laying hens. *J. Food Prot.* 63, 545–548. doi: 10.4315/0362-028X-63.4.545

Seo, K. H., and Frank, J. F. (1999). Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62, 3–9. doi: 10.4315/0362-028X-62.1.3

Shah, D. H., Paul, N. C., Sischo, W. C., Crespo, R., and Guard, J. (2017). Population dynamics and antimicrobial resistance of the most prevalent poultry-associated *Salmonella* serotypes. *Poult. Sci.* 96, 687–702. doi: 10.3382/ps/pew342

Sohaib, M., Anjum, F. M., Arshad, M. S., and Rahman, U. U. (2015). Postharvest intervention technologies for safety enhancement of meat and meat based products; a critical review. *J. Food Sci. Technol.* 53, 19–30. doi: 10.1007/s13197-015-1985-y

Surendran Nair, M., Lau, P., Belskie, K., Fancher, S., Chen, C.-H., Karumathil, D. P., et al. (2016). Potentiating the heat inactivation of *Escherichia coli* O157:H7 in ground beef patties by natural antimicrobials. *Front. Microbiol.* 7:15. doi: 10.3389/fmicb.2016.00015

Surendran-Nair, M., Kollanoor-Johny, A., Ananda-Baskaran, S., Norris, C., Lee, J.-Y., and Venkitanarayanan, K. (2016). Selenium reduces enterohemorrhagic *Escherichia coli* O157: H7 verotoxin production and globotriaosylceramide receptor expression on host cells. *Future Microbiol.* 11, 745–756. doi: 10.2217/fmb.16.16

Surendran Nair, M., Upadhyaya, I., Amalaradjou, M. A. R., and Venkitanarayanan, K. (2017). "Antimicrobial food additives and disinfectants," in *Foodborne Pathogens and Antibiotic Resistance*, ed. O. V. Singh (Hoboken, NJ: John Wiley & Sons, Inc.), 275–301. doi: 10.1002/9781119139188.ch12

Suzuki, É. Y., Baptista, E. B., Resende Do Carmo, A. M., Miranda Chaves, M. D. G. A., Chicourel, E. L., and Barbosa Raposo, N. R. (2014). Potential of the essential oil from *Pimenta pseudocaryophyllus* as an antimicrobial agent. *Acta Pharm.* 64, 379–385. doi: 10.2478/acph-2014-0024

Tamblyn, K. C., and Conner, D. E. (1997). Bactericidal activity of organic acids against *Salmonella* Typhimurium attached to broiler chicken skin. *J. Food Prot.* 60, 629–633. doi: 10.4315/0362-028X-60.6.629

Tamblyn, K. C., Conner, D. E., and Bilgili, S. F. (1997). Utilization of the skin attachment model to determine the antibacterial efficacy of

potential carcass treatments. *Poult. Sci.* 76, 1318–1323. doi: 10.1093/ps/76.9.1318

Tan, S. M., Lee, S. M., and Dykes, G. A. (2014). Fat contributes to the buffering capacity of chicken skin and meat but enhances the vulnerability of attached *Salmonella* cells to acetic acid treatment. *Food Res. Int.* 66, 417–423. doi: 10.1016/j.foodres.2014.10.007

Upadhyaya, I., Upadhyay, A., Kollanoor-Johny, A., Darre, M., and Venkitanarayanan, K. (2013). Effect of plant derived antimicrobials on *Salmonella* Enteritidis adhesion to and invasion of primary chicken oviduct epithelial cells *in vitro* and virulence gene expression. *Int. J. Mol. Sci.* 14, 10608–10625. doi: 10.3390/ijms140510608

Venkitanarayanan, K., Kollanoor-Johny, A., Darre, M. J., Donoghue, A. M., and Donoghue, D. J. (2013). Use of plant-derived antimicrobials for improving the safety of poultry products. *Poult. Sci.* 92, 493–501. doi: 10.3382/ps.2012-02764

Waldroup, A. L. (1996). Contamination of raw poultry with pathogens. *Worlds Poult. Sci. J.* 52, 20–25. doi: 10.1079/Wps19960002

WHO (2009). *Microbiological Risk Assessment Series-19: Salmonella and Campylobacter in Chicken Meat*. Available at: http://www.who.int/foodsafety/publications/micro/MRA19.pdf [accessed August 21, 2017].

Yang, H., Li, Y. B., and Johnson, M. G. (2001). Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and on chicken skin during poultry scalding and chilling. *J. Food Prot.* 64, 770–776. doi: 10.4315/0362-028X-64.6.770

Young, S. D., Olusanya, O., Jones, K. H., Liu, T., Liljebjelke, K. A., and Hofacre, C. L. (2007). *Salmonella* incidence in broilers from breeders vaccinated with live and killed *Salmonella*. *J. Appl. Poult. Res.* 16, 521–528. doi: 10.3382/japr.2007-00009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Nair and Kollanoor Johny. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 November 2017
doi: 10.3389/fmicb.2017.02351

An Assessment of Different Genomic Approaches for Inferring Phylogeny of *Listeria monocytogenes*

Clémentine Henri[1], Pimlapas Leekitcharoenphon[2], Heather A. Carleton[3], Nicolas Radomski[1], Rolf S. Kaas[2], Jean-François Mariet[1], Arnaud Felten[1], Frank M. Aarestrup[2], Peter Gerner Smidt[3], Sophie Roussel[1], Laurent Guillier[1], Michel-Yves Mistou[1] and René S. Hendriksen[2]*

[1] Agence Nationale de Sécurité Sanitaire de l'Alimentation, Maisons-Alfort Laboratory for Food Safety, University Paris-Est, Maisons-Alfort, France, [2] European Union Reference Laboratory for Antimicrobial Resistance, National Food Institute, WHO Collaborating Center for Antimicrobial Resistance in Food Borne Pathogens and Genomics, Technical University of Denmark, Kongens Lyngby, Denmark, [3] National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, United States

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università di Teramo, Italy

Reviewed by:
Stephen Forsythe,
Nottingham Trent University,
United Kingdom
Eelco Franz,
Centre for Infectious Disease Control
(RIVM), Netherlands

***Correspondence:**
Michel-Yves Mistou
michel-yves.mistou@anses.fr

Specialty section:
This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 07 September 2017
Accepted: 15 November 2017
Published: 29 November 2017

Citation:
Henri C, Leekitcharoenphon P, Carleton HA, Radomski N, Kaas RS, Mariet J-F, Felten A, Aarestrup FM, Gerner Smidt P, Roussel S, Guillier L, Mistou M-Y and Hendriksen RS (2017) An Assessment of Different Genomic Approaches for Inferring Phylogeny of Listeria monocytogenes. Front. Microbiol. 8:2351. doi: 10.3389/fmicb.2017.02351

Background/objectives: Whole genome sequencing (WGS) has proven to be a powerful subtyping tool for foodborne pathogenic bacteria like *L. monocytogenes.* The interests of genome-scale analysis for national surveillance, outbreak detection or source tracking has been largely documented. The genomic data however can be exploited with many different bioinformatics methods like single nucleotide polymorphism (SNP), core-genome multi locus sequence typing (cgMLST), whole-genome multi locus sequence typing (wgMLST) or multi locus predicted protein sequence typing (MLPPST) on either core-genome (cgMLPPST) or pan-genome (wgMLPPST). Currently, there are little comparisons studies of these different analytical approaches. Our objective was to assess and compare different genomic methods that can be implemented in order to cluster isolates of *L. monocytogenes*.

Methods: The clustering methods were evaluated on a collection of 207 *L. monocytogenes* genomes of food origin representative of the genetic diversity of the Anses collection. The trees were then compared using robust statistical analyses.

Results: The backward comparability between conventional typing methods and genomic methods revealed a near-perfect concordance. The importance of selecting a proper reference when calling SNPs was highlighted, although distances between strains remained identical. The analysis also revealed that the topology of the phylogenetic trees between wgMLST and cgMLST were remarkably similar. The comparison between SNP and cgMLST or SNP and wgMLST approaches showed that the topologies of phylogenic trees were statistically similar with an almost equivalent clustering.

Conclusion: Our study revealed high concordance between wgMLST, cgMLST, and SNP approaches which are all suitable for typing of *L. monocytogenes*. The comparable clustering is an important observation considering that the two approaches have been variously implemented among reference laboratories.

Keywords: *Listeria monocytogenes*, WGS, cgMLST, wgMLST, SNPs, PFGE, conventional MLST, surveillance

INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is one out of 17 species belonging to the genus *Listeria*, a Gram-positive rod-shaped bacterium (Weller et al., 2015). *L. monocytogenes* is classified into four major evolutionary lineages, 13 agglutination serotypes, and five molecular serotypes (Doumith et al., 2004; Orsi et al., 2011). *L. monocytogenes* is responsible for the serious foodborne illness, listeriosis caused by consumption of contaminated food such as unpasteurized milk, cheese, smoked salmon, uncooked meat and ready-to-eat food (Law et al., 2015). *L. monocytogenes* has the ability to grow at low temperatures, form bio-films and persist in food processing plants (Carpentier and Cerf, 2011). Subsequently, it represents a significant challenge for the food-producing industry (Ferreira et al., 2014). *L. monocytogenes* is one of the foodborne pathogens that cause the highest rate of mortality, yet its incidence is low (EFSA, 2014). Between 2008 and 2013, a significant increase of 8.6% in the incidence of listeriosis has been recorded in Europe. In 2015, over than 2200 cases were reported in Europe. This highlights *L. monocytogenes* as a serious re-emerging public health concern and it is therefore intensively monitored in developed countries (de Noordhout et al., 2014; EFSA, 2014).

The European surveillance system of *L. monocytogenes* from humans, foods, animals, and environments is still widely based on pulsed field gel electrophoresis (PFGE) (EFSA, 2014). PFGE was developed in the 1980s and the current PFGE scheme requires restriction by two enzymes using a validated standard protocol (Brosch et al., 1994; Michelon et al., 2015). PFGE has been extremely useful in *Listeria* outbreak investigations but its discriminatory power can be suboptimal for source tracking and source attribution (Ribot et al., 2006). The conventional multilocus sequence typing (MLST), based on the nucleotide sequence of seven house-keeping genes, provides a sequence type (ST) allowing strains to be clustered into clonal complexes (CC) (Ragon et al., 2008). Conventional MLST has been used in population diversity studies to investigate the population structure of *L. monocytogenes* (Chenal-Francisque et al., 2011; Haase et al., 2011; Cantinelli et al., 2013; Henri et al., 2016; Maury et al., 2016).

Recently, Whole genome sequencing (WGS)-based subtyping has proven to be extremely powerful for *L. monocytogenes*. A number of studies have demonstrated the advantages of using WGS analysis for national surveillance, outbreak detection and source tracking of *L. monocytogenes* (Chen et al., 2016a; Jackson et al., 2016). Single Nucleotide Polymorphism (SNP) and gene-by-gene approaches (genomic MLST) have been mainly employed at the genome scale. The gene-by-gene approach is based on inference of categorical data based on allelic variation of a predefined set of genes from either core genome only (called hereafter core genome MLST or cgMLST) or on a set of genes from both core and accessory genome (called hereafter whole genome MLST or wgMLST). The core genome consists of all genes present in all genomes of *L. monocytogenes* while the pan-genome consists of all the genes present in any strain of the species (supra-genome). Different cg or wgMLST schemes have been developed: in Germany (Ruppitsch et al., 2015), Austria (Hyden et al., 2016), and USA (Chen et al., 2016b), as well as by a consortium comprising the CDC (USA), the Pasteur Institute (France), the SSI (Denmark), PHAC Canada and PHE (UK) (Moura et al., 2016). The SNP approach is based on mapping raw sequence reads against a reference genome to call variations in both genes and intergenic regions. The choice of the reference genome is fundamental for SNP calling (Pightling et al., 2014). The SNP approach is currently used in Denmark (Agasan et al., 2013; Wingstrand et al., 2015; Jensen et al., 2016) and UK (Awofisayo-Okuyelu et al., 2016), as well as for regulatory purposes by the US Food & Drug Administration (FDA). An additional approach consists in inference of categorical data based on presence or absence of predicted proteins. Similar to the MLST approaches, the profile of presence and absence of predicted proteins could either be performed with the core genome (called hereafter cgMLPPST) or the pan genome (called hereafter wgMLPPST) (Leekitcharoenphon et al., 2014). Phylogenetic inference based on predicted proteins could be tested in order to cluster strains according to predicted phenotypic trait and adaptation abilities, and would be an original surveillance tool for source tracking (Deng et al., 2010).

The rapid implementation of WGS by different laboratories and laboratory networks using different approaches to analyse their data makes necessary to assess the differences between clustering methods. The main aim of this study was to assess the concordance between cgMLST, wgMLST, SNP, cgMLPPST, and wgMLPPST approaches using a well-defined panel of food strains of *L. monocytogenes* isolated in France during the last 20 years.

MATERIALS AND METHODS

Strain Panel

Previously, we have investigated by PFGE and conventional MLST the genetic diversity of approximately 2000 *L. monocytogenes* of food origin isolated in France during the past 20 years. A panel of 207 *L. monocytogenes* strains from this study was selected to be statistically representative of the diversity of *L. monocytogenes*. It included strains isolated between 1989 and 2013, from various food matrixes and food processing environments. Out of the 207 strains, 127 isolates belonged to molecular serotype IIa, 25 to molecular serotype IIc, 17 to molecular serotype IIb, and 38 to molecular serotype IVb (Supplementary Table 1). The 207 *L. monocytogenes* strains belonged to 46 different STs and 38 distinct CCs (Supplementary Figure 1). The 207 strains represented 50 PFGE pulsotype clusters as depicted in the Supplementary Figure 2. The clusters were defined by the *Apa*1/*Asc*1 pulsotype patterns and clustered based on 80% similarity [unweighted pair-group method with arithmetic mean (UPGMA), with Dice's coefficient, tolerance and optimization set up at 1%; Henri et al., 2016]. The two reference strains, EGDe (accession number: NC_003210, ST35, CC9, serotype 1/2a and molecular serotype IIa) and EGD (accession number: HG421741, ST12, CC7, serotype 1/2a, and molecular serotype IIa) were included in the final set and used as reference for SNP calling. The complete list of the 209 genomes is available in Supplementary Table 1.

DNA Extraction and Sequencing

DNA extraction was performed using Easy-DNA™ gDNA Purification Kit from Invitrogen™ (Life Technologies™ Headquarters, 5791 Van Allen Way, Carlsbad, CA 92008 USA). The DNA concentrations were measured using the Qbit dsDNA BR Assay Kit from Invitrogen™.

Libraries preparation and DNA sequencing were performed at the Welcome Trust Center for Human Genetics (Roosevelt Drive, Oxford OX3 7BN, 173 United Kingdom). Libraries were prepared by using the NEB library prep kits with in-house developed modifications. A sample of pooled libraries was loaded into Illumina HiSeq reagent cartridge with a standard flow cell. The 207 strains were subjected to pair-end sequencing. Insertion size of pair-end sequences ranged from 65 to 473 bp, with an average of 231 bp. The reads coverage ranged from 28 × to 442 ×, with an average of 213 × (Supplementary Table 1).

A biosample project was created as repository to store all raw sequence reads of this study with open access. The raw sequence data have been submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession no: PRJ 948.

Genomic MLST

The wgMLST and cgMLST were performed at the Centers for Disease Control and Prevention, the USA (US-CDC) by the Enteric Diseases Laboratory Branch.The wgMLST scheme was developed from a set of over 200 annotated closed and high-quality draft genomes that represented the diversity of serotypes and lineages in *L. monocytogenes*. A total of 4,804 unique loci were identified to compose the wgMLST scheme, whereas 1,748 loci represent the cgMLST scheme. The cgMLST scheme was developed by the Pasteur Institute (Moura et al., 2016) and is available at PubMLST website (https://pubmlst.org/databases.shtml). The wgMLST with the cgMLST schema is included in the commercial software [BioNumerics v7.5 (Applied Maths NV, Belgium)]. Alleles were called for both the wgMLST and cgMLST schemes using BioNumerics v7.5. Unless raw reads (fastq format) were available, assembly-based allele calling (fasta format) was completed. The contigs were assembled using SPAdes 3.5.0, plug-in of the BioNumerics software v7.5. Alleles were named if genes fulfill the following criteria: a start and stop codon were present, the DNA sequence met the 85% minimum homology cut-off, there were no ambiguous base calls in the allele sequence, and had less than 100 gaps in the sequence alignment. Dendrograms of wgMLST and cgMLST were created using the UPGMA algorithm with the allele calls considered categorical data.

Phylogenetic Tree Based on SNPs

The SNP tree was built with the pipeline CSI phylogeny accessible from the Center for Genomic Epidemiology (www.genomicepidemiology.org) (Leekitcharoenphon et al., 2012a; Kaas et al., 2014). The reference strains, EGD (ST35) and EGD-e (ST12) have been previously subjected to thorough genomic investigation and their differences are well documented (Bécavin et al., 2014). Both reference genomes belong to the same lineage II and serovar 1/2a but with different STs (ST35 and ST12, respectively).

The paired-end reads were mapped to the reference genomes using Burrows–Wheeler Aligner (BWA) (Li and Durbin, 2009). Initially, a SNP analysis was performed using the reference genome: EGD-e (accession number NC_003210, length 2,944,528 bp). Subsequently, a second SNP analysis was performed using the second reference genome: EGD (accession number HG421741, length 2,907,193 bp).

SNPs were determined using mpileup commands from SAMTools version 0.1.18. The SNPs were filtered according to five parameters: (1) a minimum distance of 10 bps between each SNP, (2) a minimum of 10x depth and 10% of the breadth coverage, (3) the mapping quality was above 30, (4) the SNP quality was higher than 20, and (5) all indels were excluded. For each genome, SNPs were concatenated to a single alignment corresponding to the positions of the reference genome.

The concatenated SNPs (with either EGD or EGD-e as reference) were inferred with the multi-core architecture (Aberer et al., 2010) of RAxML 8.2.4 (Stamatakis, 2014) based on a bootstrap analysis and search for best-scoring Maximum Likelihood tree with General Time-Reversible model of substitution and secondary structure 16-state model (Pattengale et al., 2009).

Core and Pan-Genome Plot

The raw reads were assembled using Velvet for *de novo* short reads assembly (Zerbino and Birney, 2008). Prediction of Open Reading Frames (ORFs) and proteins was performed using Prodigal in each *de novo* assembly (Hyatt et al., 2010; Jacobsen et al., 2011). Protein families were constructed by first aligning predicted proteins all-against-all using BLASTP with 50/50 rule (two genes were determined as a set if: the alignment length exceeds 50% of the longest sequence with more than 50% of the aligned sequences reported as identical) (Tettelin et al., 2005; Leekitcharoenphon et al., 2012b). Nonetheless, by this process, predicted proteins can be present in different families. Thus, all families sharing predicted proteins(s) were combined to ensure that each predicted proteins belongs to only one protein family (Tettelin et al., 2005; Friis et al., 2010; Lukjancenko et al., 2010, 2012; Vesth et al., 2010; Jacobsen et al., 2011; Kaas et al., 2012).

To each genome corresponds a set of predicted proteins, some of which are also found in other genomes. The pan-genome is the union of the predicted proteins, while the core genome is the intersection of the predicted proteins for the genomes under consideration (Tettelin et al., 2005; Leekitcharoenphon et al., 2012b). The size of the core- and pan genomes according to the number of genomes analyzed in our dataset is shown in Supplementary Figure 3.

CgMLPPST Tree

Multiple alignment for each core predicted proteins (predicted proteins found in all genomes) was performed with MUSCLE version 3.8.31 (Edgar, 2004). The concatenated aligned ORFs, without deletion of invariable positions, were obtained to reconstruct phylogenetic inference with the multi-core architecture (Aberer et al., 2010) of RAxML 8.2.4 (Stamatakis, 2014) based on a bootstrap analysis and search for best-scoring. Maximum Likelihood tree, with General Time-Reversible model

of substitution and secondary structure 16-state model, was built (Pattengale et al., 2009).

WgMLPPST Trees

BlastP, using 50 percent length and 50 percent similarity rules, was performed for each samples against pan-genomes previously defined (Altschul et al., 1990). A profile of absence (0) or presence (1) of all genes was performed for each sample. The wgMLPPST tree was reconstructed from this matrix consisting of gene families (rows) and genomes (columns).

The analysis of presence/absence of the accessory genes across the 207 isolates showed that the genes could be divided into shell (genes that are frequently found) and cloud genes (genes that are rarely found). The wgMLPPST could be constructed by adding more weight either to cloud or shell genes. The trees were constructed using hierarchical clustering of the relative Manhattan distance according to the distance matrix (Snipen and Ussery, 2010; Leekitcharoenphon et al., 2012b).

Trees Visualization and Annotation

All trees were visualized and annotated using iTOL (Letunic and Bork, 2007) and the R software (R Development Core Team, 2008). For better visualization, the trees were all circulated and the results of the standard typing approaches for each strain were displayed in outer external rings.

Concordance between Standard and Genomic Approaches

When all trees were reconstructed (the phylogenetic SNP, cgMLST and wgMLST, pan-genome, and core gene trees) we assessed the concordance of the genomic clustering with conventional groups: lineages, molecular serotype, PFGE pulsotype and ST's. The results were reported in percentage of concordance.

Trees Comparison and Statistical Analyses

A phylogenic tree can be characterized with two properties: the topology and the branch lengths. The topology is the branching structure of the tree and it indicates patterns of relatedness among strains.The comparison of the tree topology and distance were performed using the R packages "ade4," "ape," "dendextend," "phangorn," and "phytools" (Paradis et al., 2004; Dray et al., 2007; Schliep, 2011; Revell, 2012; Galili, 2015). "ade4" package was used for the graphical representation functions, "ape" package was used to read, plot and manipulate phylogenetic trees, "phangorn" and "dendextend" were used to compute pairwise distance between pairs of strains from phylogenetic network and "phytool" was used to visualize and analyse comparative data from species using colors.

Cophenetic and the Cor_cophenetic

The cophenetic is the distance between two strains and the exact height of the dendrogram where the two branches that contain the two strains join into one single branch. The cophenetic correlation (hereafter termed: cor_cophenetic) calculates the correlation between the cophenetic distance matrices of the two trees. The cor_cophenetic value ranges between −1 (perfect negative correlation) and 1 (perfect positive correlation). A value close to 0 (nil) indicates the absence of correlation for the two trees. The cophenetic and cor_cophenetic functions of dendextend and phangorn package were used to evaluate the clustering (Sokal and James, 1962; Cardona et al., 2013).

The Fowlkes-Mallows Index

The dendextend package calculates the Fowlkes-Mallows (FM) index which assess the similarity between two clusters (Fowlkes and Mallows, 1983). The FM index values are comprised between 0 (nil) and 1. The closer it is to 1, the more the clusters are similar. We calculated the asymptotic values, E_FM (Expected_Flowlkes-Mallows) and V_FM (Variance_Flowlkes-Mallows), expected under the null hypothesis (H0) that assumes that the two trees have the same topology if one tree is a random shuffle of the strains of the other tree (for instance no correlation between the trees). If $E_FM+1{,}65 \cdot V_FM^{0.5}$ is below the observed one we can reject H0 at $\alpha = 0.05$.

RESULTS

Comparaison of the Clustering Efficiency of Core and Whole Genome Genomic MLST

Initially, the cgMLST and wgMLST approaches were tested to infer the phylogeny of 207 food strains. Two major clades were observed for both cgMLST and wgMLST which corresponded mainly to lineage I and lineage II. Lineage II was subdivided in three clades that corresponded mainly to (1) CC13, CC193, CC31; (2) CC7, CC155, CC37, CC26, CC20, CC8, CC21, CC204, CC9, and seven singletons (ST19, ST18, ST177, ST200, ST207, ST534, ST620) (all singletons and CC from lineage II); and (3) to CC121 (lineage II) (**Figures 1A,B**). The inferred cgMLST and wgMLST phylogenies were in perfect accordance with the lineage classification whereas for the molecular serotyping, the concordance was slightly lower with a concordance of 96.6% for cgMLST and 97.6% for wgMLST (**Table 1** and Supplementary Table 2). Importantly, the gene by gene approaches displayed a high concordance with conventional MLST i.e., 99.5% concordance with the cgMLST approach and 97.1% with the wgMLST (**Table 1** and Supplementary Table 2). As expected the PFGE clustering showed a much lower performance with only 67.3 and 68.8% of concordance with cgMLST and wgMLST, respectively.

A visual comparison of cgMLST- and wgMLST-inferred phylogenies showed that strains from lineage II were grouped similarly and correctly with both approaches. To make the comparative analysis of the clustering methods easier, the trees to be compared were plotted facing each other with the same strains being connected (**Figure 2**). This data plot highlights the differences between phylogenies reconstructions. No clustering differences were observed in the shape of the trees (**Figure 2**), and only a few positioning differences were observed between strains within the same CC. The Fowlkes-Mallows Index and cor_cophenetic were calculated to quantify the similarity between the cgMLST and wgMLST inferred trees. In case of unrelated trees, the maximum expected value for FM index (E_FM) is 0.174 by taking into account E_FM and V_FM values. The calculated value of 0.885 is much higher than this critical value and indicates

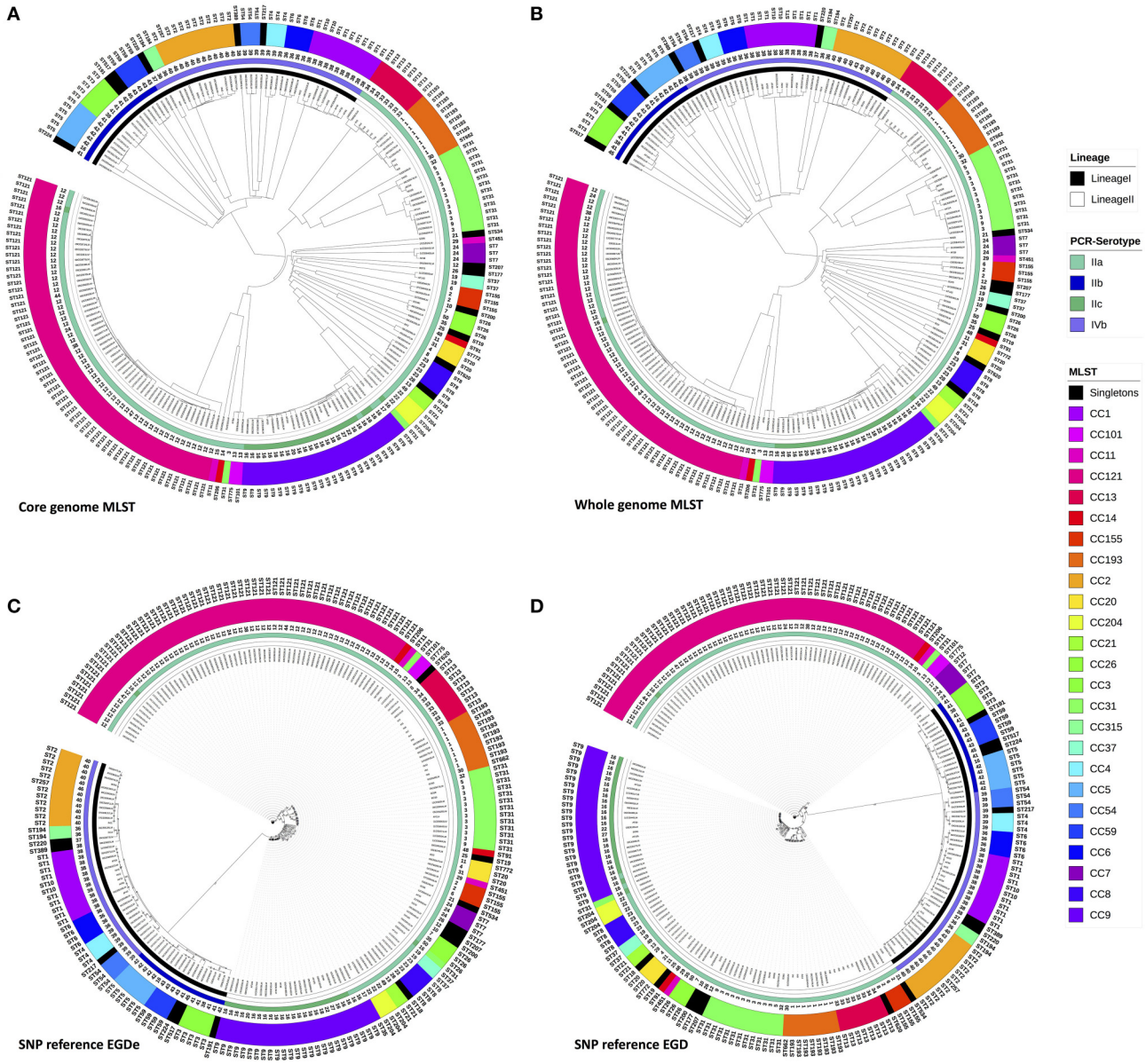


FIGURE 1 | Phylogenic trees with the 208 *L. monocytogenes*, based on genomic MLST scheme define by Bionumerics® (core and pan) and Phylogenic trees based on SNPsvanalysis with EGD-e and EGD as references. Trees were circulated using ItoL. Inner circle represents lineage for each strains, second ring represents PCR-serotype, the third band shows the pulsotype cluster for each strain and the last two rings shows results from conventional seven loci MLST typing for each strains either with CC and ST. Color codes for Lineage, PCR-serotype and conventional seven loci MLST CC are shown aside in the figure legend. **(A)** The Analysis was performed on 1,748 core genes scheme from Bionumerics and dendograms was done using the UPGMA algorithm with the allele calls considered categorical data. **(B)** The Analysis was performed on pan genes scheme from Bionumerics and dendograms was done using the UPGMA algorithm with the allele calls considered categorical data. **(C)** SNP tree was constructed from SNPs that were identified using the pipeline CSI phylogeny accessible from the Center for Genomic Epidemiology (www. genomicepidemiology.org). EGDe was used as the reference genome to called SNPs. The SNP alignments were subjected to maximum-likelihood tree construction using PhyML with 100 bootstrap replicates. **(D)** SNP tree was constructed from SNPs that were identified using the pipeline CSI phylogeny accessible from the Center for Genomic Epidemiology (www.genomicepidemiology.org). EGD was used as the reference genome to called SNPs. The SNP alignments were subjected to maximum-likelihood tree construction using PhyML with 100 bootstrap replicates.

a high similarity between the two trees. In addition the calculated cor_cophenetic value of 0.999 (1 indicating a perfect correlation) statistically supports the conclusion that both methods lead to the same phylogenetic reconstruction.

The SNP Trees

The SNP trees were computed from concatenated SNPs identified from mapping raw reads to the reference genomes, EGD-e or EGD (**Figures 1C,D**). On average, 2.74 Mb (93.9%) of the EGD-e

TABLE 1 | Backward comparison with routine typing methods.

Trees based on genomic methods	Lineage (%)	Serotype (%)	conventional MLST (%)	PFGE (%)
Core genome MLST	100.0	96.6	99.5	67.3
Whole genome MLST	100.0	97.6	97.1	68.8
SNP tree EGD-e	100.0	99.0	94.7	69.2
SNP tree EGD	100.0	97.6	94.7	67.8
CgMLPPST tree based on the study panel	100.0	98.1	97.1	73.1
WgMLPPST tree (Shell)	99.0	96.6	87.50	62.5
WgMLPPST tree (Cloud)	83.2	88.0	87.02	70.2

The performance of genomic methods was measured by concordance with routine methods (Lineage, PCR-Serotype, MLST, PFGE). The 100% means all strains from a particular group for routine method clustered together in corresponding tree. For instance, all strains clustered together according their lineage (I or II) for cgMLST, wgMLST, SNP trees and core genes tree but only 99 and 83.2% of strains for both MLPPST (respectively Shell and Cloud). See detail of count in Supplementary Table 2.

reference genome and 2.73 Mb (93.3%) of EGD reference genome were mapped against the 207 genomes included in the study. The phylogenies were inferred based on the analysis of 38,787 and 38,620 SNPs, using the EGD-e reference and the EGD reference, respectively. The SNP approaches grouped strains into two main clusters that corresponded to lineage I and II with a perfect 100% concordance (**Table 1**, Supplementary Table 2). When molecular serotypes were concerned, the concordance was of 99.0 and 97.6%, for SNP tree based on the EGD-e and the EGD references, respectively (**Table 1**, Supplementary Table 2). The SNP approaches were able to categorized strains according to STs (conventional MLST) with a concordance of 94.7% for both EGD-e and EGD references (**Table 1**, Supplementary Table 2). As expected, thePFGE clustering obtained the poorest concordance with the SNPs clustering with only 69.2 and 67.8%, for EGD-e and EGD references, respectively.

A visual comparison between the SNP analysis based on the EGD-e and EGD references showed that strains from lineage I are arranged in a similar way in the two trees, whereas strains from lineage II showed more variability. To assess the validity of these differences and remove artifacts, we performed a one to one plot with identical strains connected. To optimize matching, branches around nodes were also rotated (**Figure 3**). We noticed that only a few CC's (CC7, CC8 and CC155) and three unique strains (06CEB103LM, 09CEB923LM, and 11CEB445LM) changed positions in the two trees. The statistical analysis revealed that the two trees were similar as the FM index of 0.796 was higher than the E_FM value (0.409). Likewise, the cor_cophenetic equal to 0.999 confirmed the highly similar tree topologies. Finally, the analysis indicated that changing reference for SNP calling produce similar but not identical trees. By comparison, the FM index (0.885) for the wgMLST-cgMLST clustering comparison was 0 closer to 1.

Comparison between the SNP and Genomic MLST

We compared the phylogenic trees based on SNPs with cgMLST and wgMLST approaches, respectively. (**Figure 4**, Supplementary Figure 4). In both comparisons, we observed that six CC's (CC5, CC59, CC8 for lineage I and CC13, CC31, CC193 for lineage II) and three unique strains changed of position in the compared trees (08CEB244LM and IN12 for both comparisons, 10CEB615LM and 05CEB573LM for SNP vs. wgMLST and SNP vs. cgMLST, respectively). The FM Index (0.486) was low but higher than the expected E_FM (0.135) value, providing statistical evidence that the SNP and the wgMLST approaches provide overall similar results. The same conclusion was reached when the SNP and the cgMLST approaches were compared (FM Index of 0.426; with E_FM = 0.146). The cor_cophenetic was not estimated as the two matrices of distance are not based on the same distance scale.

CgMLPPST Tree

The cgMLPPST, as opposed to the allele-based cgMLST tree, was inferred based on the multiple alignment for each core genes found among the genomes included in the study (Supplementary Figure 5). This approach showed 100, 97.1, and 98.1% concordances with lineage, conventional MLST, and molecular serotype, respectively (**Table 1** and Supplementary Table 2). Overall, the cgMLPPST performed better than these conventional methods (**Table 1**, Supplementary Table 2).

The core genes determined in this study might be seen as large due the number of genomes used (129 182 variable positions across 207 genomes). With more genomes, from diverse origin, and if our panel would include strains from lineage III and IV; the number of core genes would probably be lower than 2000 genes and should approach the 1748 genes used in the cgMLST scheme. (Supplementary Figure 3). Indeed, this result indicates that the panel of food strains do not represent the full diversity of *L. monocytogenes*.

WgMLPPST Approaches

We observed five major clades in the wgMLPPST trees (Supplementary Figures 6, 7). The five clades corresponded mainly to (1) lineage I, CC121, (2) CC193, CC31, (3) CC13, (4) CC9, CC204, and (5) CC7, CC8, CC20, CC21, CC21, CC26, CC31, CC37, CC101, and seven unique strains from lineage II (ST19, ST18, ST177, ST200, ST207, ST534, ST620). For the wgMLPPST approaches, we observed that the phylogenetic trees obtained from either the shell or the cloud failed to assign one and three strains to the correct lineage (**Table 1**, Supplementary Table 2). Additionally, strains from different molecular serotypes were interspersed causing a low concordance with molecular serotypes (**Table 1**, Supplementary Table 2). Furthermore, the concordance between conventional MLST and wgMLPPST approaches displayed the lowest scores among genomic methods (**Table 1**, Supplementary Table 2). Like for the previous genomic approaches, the concordance with PFGE clustering were low and decreased to 62.5 and 70.2%, for shell and cloud wgMLPPST, respectively. Those results indicate that wgMLPPST is not relevant for surveillance purpose as strains from different lineage, ST and molecular serotype can be mixed. WgMLPPST approaches failed to group together strains from the same lineage despite their genetic homogeneity (Orsi et al., 2011; Paul et al., 2014). The failure to cluster strains from the same lineage confirmed that wgMLPPST is not suitable for phylogeny

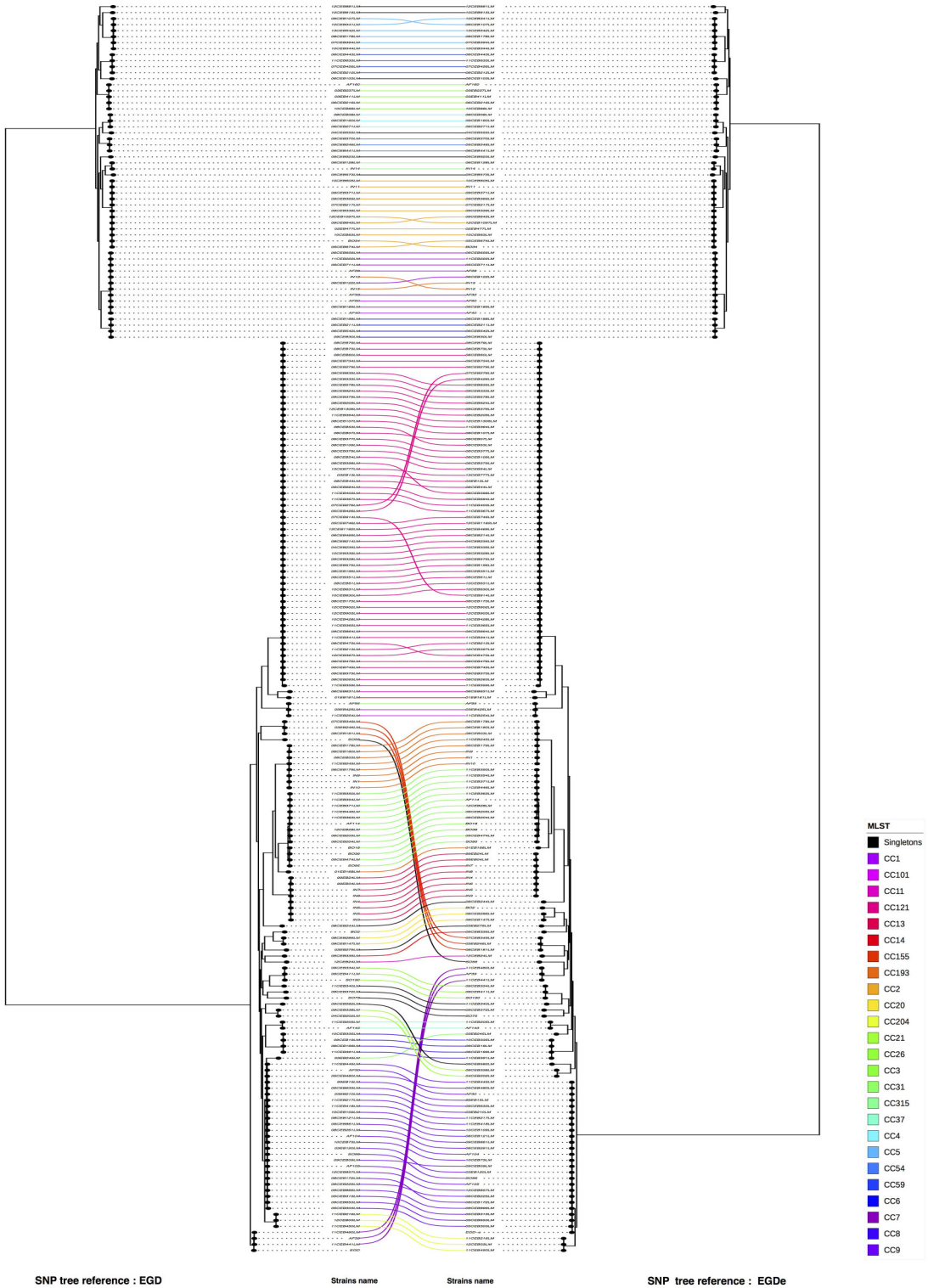


FIGURE 2 | Visual comparison of genome SNP trees using EGD-e or EGD as reference. Using R software, SNP trees performed with the study panel of 208 *L. monocytogenes* were compared. By facing the two trees one in front of the other, corresponding strains were linked (on the left the SNP tree using EGD as reference and on right the SNP tree using EGD-e as reference). The connection between strains was colored according to the CC of the strains (refer to the color code). The two references are indicated in red. Nodes were rotated to optimize matching between corresponding strains in both trees as closely as possible. Similar clusters are connected by straight lines, while curved line connect strains from distinct clusters.

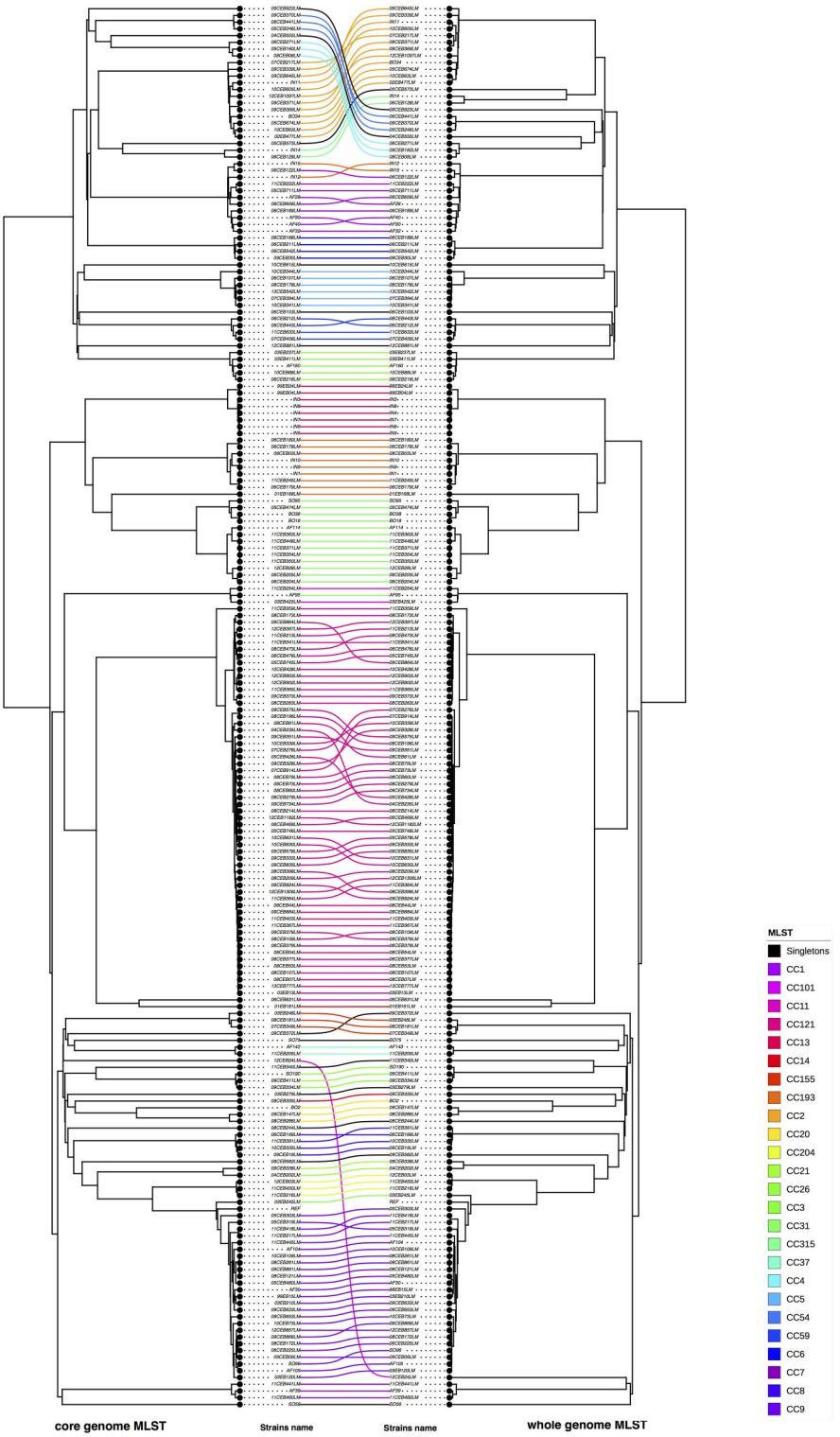


FIGURE 3 | Visual comparison of cgMLST and wgMLST. R software was used to compare core genome and wgMLST on the study panel of 208 *L. monocytogenes*. In this opposite comparison corresponding strains were linked (on the left cgMLST and on right wgMLST). The connection between strains was colored according to the CC of the strains (refer to the color code). Nodes were rotated to optimize matching between corresponding strains in both trees as closely as possible. Similar clusters are connected by straight lines, while curved line connect strains from distinct clusters.

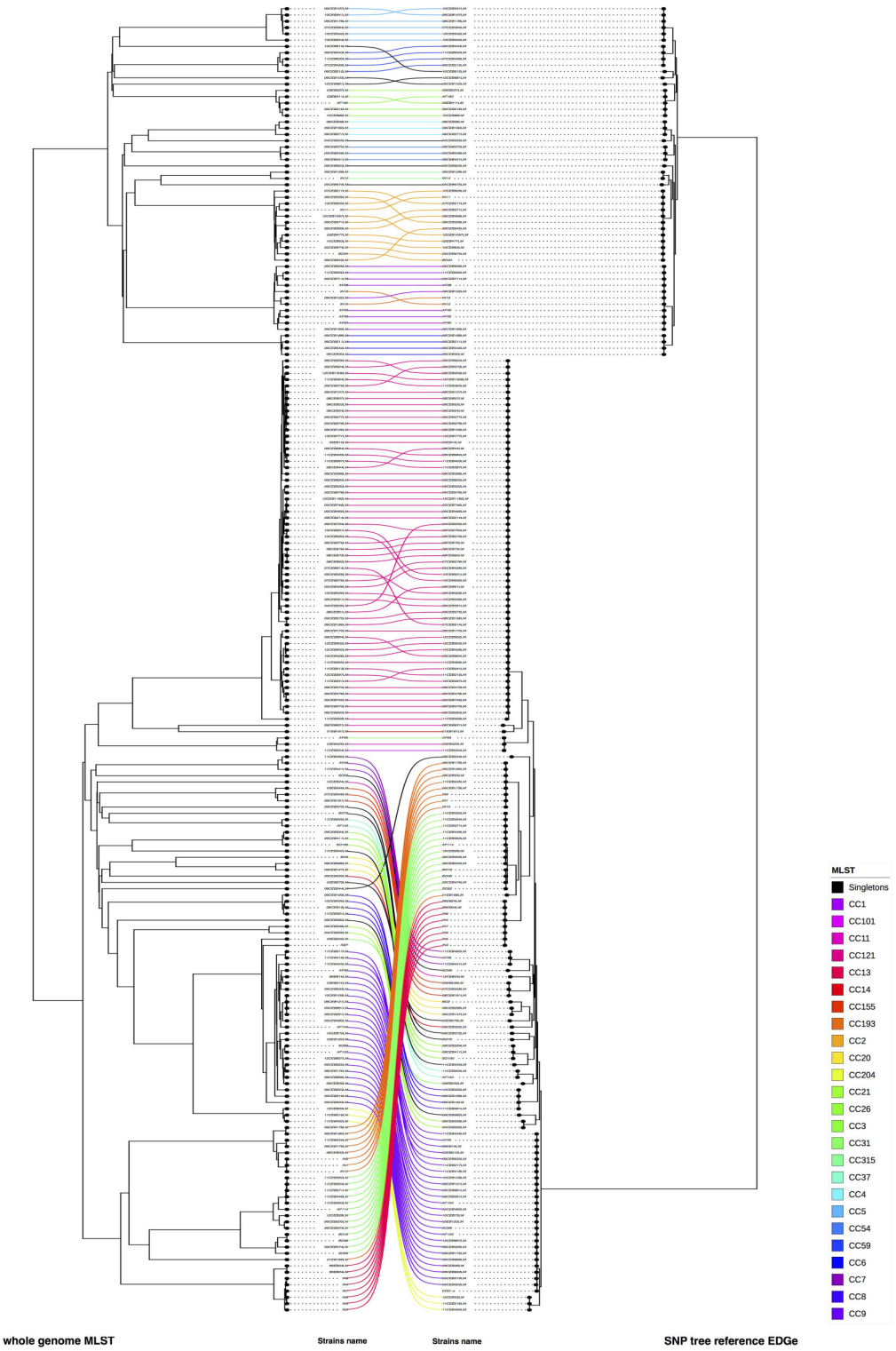


FIGURE 4 | Visual comparison of genome SNP and wgMLST. We compared genome SNP and wgMLST on the study panel using R software (on the left cgMLST and on right wgMLST). Using this face-to-face comparison, we linked corresponding strains. The connection between strains was colored according to the CC of the strains (refer to the color code). Nodes were rotated to optimize matching between corresponding strains in both trees as closely as possible. Similar clusters are connected by straight lines, while curved line connect strains from distinct clusters.

and routine surveillance purposes (Leekitcharoenphon et al., 2014).

DISCUSSION

The speed, cost and efficiency of WGS make it a realistic alternative to most current phenotypic and molecular typing methods for surveillance and outbreak investigation of foodborne pathogens. Currently, WGS is being implemented as a routine diagnostic tool and for surveillance and outbreak detection purposes in a few countries around the world enhancing the public health preparedness. In Europe, EFSA has recognized the strength and power of WGS and already launched pilot projects targeting *L. monocytogenes* and expanding to other foodborne pathogens (Nielsen et al., 2017). There are however, some limitations and obstacles present for the immediate use of WGS for surveillance of foodborne pathogens purpose such as harmonization of the phylogenetic approached, assigning an appropriate nomenclature, and sharing data (EFSA, 2014). For this reason, many European and National projects are currently concentrating their efforts on developing WGS protocols and workflows. The objective of this study was to assess and compare genomic MLST, genomic SNP, predicted protein core- and pan-genomic approaches using a unique and diverse panel of *L. monocytogenes* strain including 36 clonal complexes isolated from food.

The backward comparison to PFGE, lineage and molecular serotype showed that all genomic approaches used in our study: cgMLST, wgMLST, and SNP analyses provide equally reliable results. Our assessment also included the analysis of discrepancies between cgMLST and wgMLST, as well as the influence of the chosen reference genome for SNP investigations. Hence, a strenuous question is the choice of applying SNP analysis vs. genome MLST.

The comparison between the two genome MLST methods, indicate highly similar phylogenetic tree reconstruction regarding both distance and clustering. However, the ease of use of the two methods is not the same. The cgMLST scheme contains a well-defined set of species-wide conserved genes. A precise and calibrated cgMLST is particularly stable, hence suits especially routine epidemiology. This stability may not be provided by wgMLST because of the pan-genome variability and potential continuous expansion. The pan-genome of *Listeria* was calculated several times and comprised between 3,056 genes and 7,000 genes, indicating that it will be necessary to reach a global consensus to define the accessory genes that are part of the whole genome MLST scheme (Deng et al., 2010; Maury et al., 2016). However, the development of methods combining the stability of the core scheme with the accessory genes could certainly be helpful in situation where it is necessary to increase discriminatory power beyond the cgMLST (Maiden et al., 2013).

In a study on a single strain and in a prospective surveillance study of *L. monocytogenes*, it was reported that the choice of the reference genome affects the results of SNP analysis (Pightling et al., 2014). We have extended the investigation to 207 genomes to measure the impact that this reference choice can have on phylogenetic tree reconstruction. Our results showed that the distances between two sets of strains are statistically identical whatever the chosen reference genome, however it impacts the positioning of small groups of strains (**Figure 2**) probably because of unstable transient variants which are retained is this analysis and/or intergenic variants which provide additional discrimination power. One solution to avoid these differences into the tree topologies, would be to remove transient variants from the SNP dataset. *L. monocytogenes* is a clonal species and conventional MLST has proved it robustness for population structure (Ragon et al., 2008; Maury et al., 2016). We believe that the use of SNP analysis for global epidemiological purpose would require a global consensus on a set of CC-specific genomes that could be used as references to perform SNP-calling within ST- or CC-groups. The use of multiple reference genomes would increase the discriminatory power of the method for each CC. Furthermore, using SNP-based phylogeny specific SNP markers, could be proposed to discriminate ST or CC. This SNP-based barcode could cover all main lineages, ST and could classify strains in sub type within ST (Coll et al., 2014). For greater accuracy and efficiency at an international level this should be accompany with the use of a common SNP calling pipeline (Bertels et al., 2014), determining if the variants induced by recombination events must be removed, or not, from the variant dataset before phylogenetic reconstruction (Hedge and Wilson, 2014).

Our results demonstrate with a strong statistical support that the SNP and genomic MLST approaches led to similar phylogenetic reconstruction. This provides microbiologists and epidemiologists working on cluster analysis of *L. monocytogenes* two alternative methods with almost the same discriminatory power and precision. Remarkably, most of the discrepancies observed in the topology concerned full CC or ST. This result shows the noticeable clonality of *L. monocytogenes* and also the robustness of the conventional MLST for population structure since strains of the same CC or ST cluster together irrespective of the genomic methodology used. This study did not find any difference in the discriminatory power of the SNP and the genomic MLST approaches. Despite that the two approaches give similar results, the SNP and genome MLST entail different advantages and disadvantages which should be taken into account in a global epidemiological perspective. None of the approaches require a substantial amount of time and substantial bioinformatics expertise, indeed wgMLST is commercially available from Bionumerics® (and cgMLST in public domain) and numerous open-source SNP calling pipelines are available.

The main difference between the two approaches is that a database of loci and associated alleles is used to identify alleles for cg/wgMLST whereas one reference strain is used for SNP calling. An important benefit of the classification of isolates with cg/wgMLST is that it would be stable over time as new isolates are added, on the other hand it requires a careful curation of new alleles. An additional significant advantage is that the cg/wgMLST can provide a genome sequence type which could lead to a common nomenclature, provided that timely update of alleles databases between servers are adopted. A common nomenclature and a stable scheme should ease data

portability and sharing making communication more effective. Allelic database management requires extensive curation (Jolley et al., 2010) which for the most part can be automated with little manual interference. For these reasons, the genomic MLST approaches appear to be better suited for the use in laboratory surveillance of listeriosis where direct comparability of analytical results by different laboratories is critical, e.g. for global outbreak detection and investigation.

Concerning the SNP-based approaches, a higher discrimination would necessitate the use of different reference genomes for routine surveillance. However, the SNP approach can be fully automated while a question mark remains concerning the automation of the curation process of cg/wgMLST alleles database (Leekitcharoenphon et al., 2012a; Moura et al., 2016). Theoretically, SNP is also more discriminative by taking into account intergenic sequences but it is also more sensitive to parameters variations (reference, SNP calling filters, coverage) inducing divergence in topology of trees as shown in our study (Pightling et al., 2014). It must also be noticed that the SNP-based approaches give the opportunity to detect recombinaison evens (Croucher et al., 2015; Didelot and Wilson, 2015).

As discussed and highlighted in this work the topology of trees is made of branching and distances between strains. These two parameters provide a precise idea of the relationship between strains. This network is used to set-up groups of more and less related strains. Hence, another point of importance is to define thresholds to guide the identification of clusters of related isolates, in a way similar to what has been defined for ST or CC in MLST. This question should be addressed to implement routine surveillance (number of alleles variations for genomes MLST to define an ST or number of SNPs difference for SNP approaches) and a recent study has proposed some answers (Nielsen et al., 2017). An allelic difference threshold for genomic MLST for point source outbreaks has been proposed by Moura and colleagues (Moura et al., 2016) to defined cgMLST type (CT). However, although firm cluster definition criteria may be defined for contamination event point-source outbreaks, it is not possible to define universal cluster criteria for outbreaks that are caused by persistent contamination of a production environment because of the diversity of the situations that enables outbreak strains to evolve and diversify over time (Chen et al., 2017).

The difficulty to define SNP/allele threshold was recently highlighted by Chen et al. (2017) who investigate an outbreak linked to cheese in the USA. In this thorough study, the authors strongly advise to combine multiple WGS analyses (i.e., SNP and allele calling) with relevant phylogenetically reconstruction procedures to confidently delineate related and unrelated isolates (Chen et al., 2017).

Finally, the development of SOP (Standard operating procedure) for production and analysis of WGS data is of paramount importance in order to reach sound conclusions that will be confidently handled by the risk management authorities. In that perspective, the indexes we used in this study to compare clustering and topology will be valuable tools to set out SOP for WGS analysis in the field of microbiological food safety.

CONCLUSION

The backwards comparability between the standard MLST methodology and the genomic MLST and SNP approaches were essentially perfect. Because genomic MLST or SNP approaches provide better resolution, WGS can replace PFGE as the new gold standard for epidemiological typing of *L. monocytogenes*. Moving into the genomic era, it is vital to keep a focus on enhancing the genomic technology, to produce "plug and play solutions" and to provide the technology to diagnostic laboratories responsible for outbreak detection and surveillance. Our results showed concordance between the phylogenetic clustering of *L. monocytogenes* by the genomic MLST and SNP approaches; they are statistically similar in term of tree topology and could be used in combination when facing complex epidemiological situations.

AUTHOR CONTRIBUTIONS

CH was in charge of the whole project and participated in data production, data interpretation, and drafting the manuscript. PL contributed to the data production, data interpretation. HC contributed to the data production and drafting of the manuscript. NR participated to data production. RK participated to data production J-FM participated to the DNA extraction. AF participated to the genomic data production of SNP. FA participated to the design of the study and drafting the manuscript. SR participated in the design of the study. PG. participated in the drafting of the manuscript. LG participated to the study design and design the statistical analysis and contributed in drafting the manuscript. M-YM and RH participated in the design and coordination of the study and in drafting the manuscript. All authors read and approved the final manuscript.

FUNDING

The study was funded by Anses (Maisons-Alfort Laboratory for Food Safety, Maisons-Alfort, France) and supported by the Center for Genomic Epidemiology at the Technical University of Denmark funded by grant 09-067103/DSF from the Danish Council for Strategic Research and the Institut Français du Danemark.

ACKNOWLEDGMENTS

We thank the High-Throughput Genomics Group at the Welcome Trust Center for Human Genetics (Funded by Wellcome Trust grant reference 090532/Z/09/Z and MRC Hub GRANT G090074791070) for sequencing the isolates.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02351/full#supplementary-material

REFERENCES

Aberer, A. J., Pattengale, N. D., and Stamatakis, A. (2010). Parallel computation of phylogenetic consensus trees. *Proc. Comput. Sci.* 1, 1065–1073. doi: 10.1016/j.procs.2010.04.118

Agasan, A., Kornblum, J., Williams, G., Pratt, C. C., Fleckenstein, P., Wong, M., et al. (2013). *Annual Report on Zoonoses in Denmark 2013*. National Food Institute, Technical University of Denmark, Vol. 12, 1–69.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Awofisayo-Okuyelu, A., Arunachalam, N., Dallman, T., Grant, K. A., Aird, H., McLauchlin, J., et al. (2016). An outbreak of human listeriosis in England between 2010 and 2012 associated with the consumption of pork pies. *J. Food Protect.* 79, 732–740. doi: 10.4315/0362-028X.JFP-15-456

Bécavin, C., Bouchier, C., Lechat, P., Archambaud, C., Creno, S., and Gouin, E. (2014). Comparison of widely used Listeria monocytogenesstrains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. *MBio* 5, e00969–e00914. doi: 10.1128/mBio.00969-14

Bertels, F., Silander, O. K., Pachkov, M., Rainey, P. B., and van Nimwegen, E. (2014). Automated reconstruction of whole-genome phylogenies from short-sequence reads. *Mol Biol. Evol.* 31, 1077–1088. doi: 10.1093/molbev/msu088

Brosch, R., Chen, J., and Luchansky, J. B. (1994). Pulsed-field fingerprinting of listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Appl. Environ. Microbiol.* 60, 2584–2592.

Cantinelli, T., Chenal-Francisque, V., Diancourt, L., Frezal, L., Leclercq, A., Wirth, T., et al. (2013). Epidemic clones of listeria monocytogenes are widespread and ancient clonal groups. *J. Clin. Microbiol.* 51, 3770–3779. doi: 10.1128/JCM.01874-13

Cardona, G., Mir, A., Rosselló, F., Rotger, L., and Sánchez, D. (2013). Cophenetic metrics for phylogenetic trees, after Sokal and Rohlf. *BMC Bioinformatics* 14:3. doi: 10.1186/1471-2105-14-3

Carpentier, B., and Cerf, O. (2011). Review-Persistence of listeria monocytogenes in food industry equipment and premises. *Int. J. Food Microbiol.* 145, 1–8. doi: 10.1016/j.ijfoodmicro.2011.01.005

Chen, Y., Burall, L. S., Luo, Y., Timme, R., Melka, D., Muruvanda, T., et al. (2016a). Isolation, enumeration and whole genome sequencing of *listeria monocytogenes* in stone fruits linked to a multistate outbreak. *Appl. Environ. Microbiol.* 82, 7030–7040. doi: 10.1128/AEM.01486-16

Chen, Y., Gonzalez-Escalona, N., Hammack, T. S., Allard, M. W., Strain, E. A., and Brown, E. W. (2016b). Core genome multilocus sequence typing for identification of globally distributed clonal groups and differentiation of outbreak strains of listeria monocytogenes. *Appl. Environ. Microbiol.* 82, 6258–6272. doi: 10.1128/AEM.01532-16

Chen, Y., Luo, Y., Carleton, H., Timme, R., Melka, D., Muruvanda, T., et al. (2017). Whole Genome and Core Genome Multilocus Sequence Typing and Single Nucleotide Polymorphism Analyses of Listeria monocytogenes Isolates Associated with an Outbreak Linked to Cheese, United States, 2013. *Appl. Environ. Microbiol.* 83:e00633-17. doi: 10.1128/AEM.00633-17

Chen, Y., Luo, Y., Curry, P., Timme, R., Melka, D., Doyle, M., et al. (2017). Assessing the Genome Level Diversity of *Listeria monocytogenes* from contaminated ice cream and environmental samples linked to a Listeriosis outbreak in the United States. *PLoS ONE* 12:e171389. doi: 10.1371/journal.pone.0171389

Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., Tran, C., Leclercq, A., et al. (2011). Worldwide distribution of major clones of *Listeria monocytogenes*. *Emerging Infect. Dis.* 17, 1110–1112. doi: 10.3201/eid/1706.101778

Coll, F., McNerney, R., Guerra-Assunção, J. A., Glynn, J. R., Perdigão, J., Viveiros, M., et al. (2014). A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. *Nat. Commun.* 5:4812. doi: 10.1038/ncomms5812

Croucher, N. J., Page, A. J., Connor, T. R., Delaney, A. J., Keane, J. A., Bentley, S. D., et al. (2015). Rapid Phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using gubbins. *Nucleic Acids Res.* 43:e15. doi: 10.1093/nar/gku1196

Deng, X., Phillippy, A. M., Li, Z., Salzberg, S. L., and Zhang, W. (2010). Probing the pan-genome of *Listeria monocytogenes*: new insights into intraspecific niche expansion and genomic diversification. *BMC Genomics* 11:500. doi: 10.1186/1471-2164-11-500

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., et al. (2014). The global burden of Listeriosis: a systematic review and meta-analysis. *Lancet Infect. Dis.* 14, 1073–1082. doi: 10.1016/S1473-3099(14)70870-9

Didelot, X., and Wilson, D. J. (2015). ClonalFrameML: efficient inference of recombination in whole bacterial genomes. *PLoS Comput. Biol.* 11:e1004041. doi: 10.1371/journal.pcbi.1004041

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., and Martin, P. (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.* 42, 3819–3822. doi: 10.1128/JCM.42.8.3819-3822.2004

Dray, S., Dufour, A. B., and Chessel, D. (2007). The ade4 Package—II : Two-Table and K-Table Methods. R News 7, 47–52. doi: 10.18637/jss.v022.i04

Edgar, R. C. (2004). MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

EFSA (2014). *Technical Specifications for the Pilot on the Collection of Data on Molecular Testing of Food-Borne Pathogens from Food, Feed and Animal Samples*. EFSA.

Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J. Food Prot.* 77, 150–170. doi: 10.4315/0362-028X.JFP-13-150

Fowlkes, E. B., and Mallows., C L (1983). A method for comparing two hierarchical clusterings. *J. Am. Stat. Assoc.* 78:553. doi: 10.1080/01621459.1983.10478008

Friis, C., Wassenaar, T. M., Javed, M. A., Snipen, L., Lagesen, K., Hallin, P. F., et al. (2010). Genomic characterization of *Campylobacter jejuni* strain M1. *PLoS ONE* 5:e12253. doi: 10.1371/journal.pone.0012253

Galili, T. (2015). Dendextend: an r package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* 31, 3718–3720. doi: 10.1093/bioinformatics/btv428

Haase, J. K., Murphy, R. A., Choudhury, K. R., and Achtman, M. (2011). Revival of seeliger's historical special listeria culture collection. *Environ. Microbiol.* 13, 3163–3171. doi: 10.1111/j.1462-2920.2011.02610.x

Hedge, J., and Wilson, D. J. (2014). Bacterial phylogenetic reconstruction from whole genomes is robust to recombination but demographic inference is not. *MBio* 5:e02158-14. doi: 10.1128/mBio.02158-14

Henri, C., Félix, B., Guillier, L., Leekitcharoenphon, P., Michelon, D., Mariet, J. F., et al. (2016). Population genetic structure of *Listeria Monocytogenes* strains determined by pulsed-field gel electrophoresis and multilocus sequence typing. *Appl. Environ. Microbiol.* 82, 5720–5728. doi: 10.1128/AEM.00583-16.

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119. doi: 10.1186/1471-2105-11-119

Hyden, P., Pietzka, A., Lennkh, A., Murer, A., Springer, B., Blaschitz, M., et al. (2016). Whole genome sequence-based serogrouping of *Listeria monocytogenes* isolates. *J Biotechnol.* 235, 181–186. doi: 10.1016/j.jbiotec.2016.06.005

Jackson, B. R., Tarr, C., Strain, E., Jackson, K. A., Conrad, A., Carleton, H., et al. (2016). Implementation of nationwide real-time whole-genome sequencing to enhance listeriosis outbreak detection and investigation. *Clin. Infect. Dis.* 63, 380–386. doi: 10.1093/cid/ciw242

Jacobsen, A., Hendriksen, R. S., Aaresturp, F. M., Ussery, D. W., and Friis, C. (2011). The *Salmonella enterica* pan-genome. *Microb. Ecol.* 62, 487–504. doi: 10.1007/s00248-011-9880-1

Jolley, K. A., Maiden, M. C. J., Pettersson, E., Lundeberg, J., Ahmadian, A., Roumagnac, P., et al. (2010). BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. doi: 10.1186/1471-2105-11-595

Jensen, K. A., Nielsen, E. M., Björkman, J. T., Jensen, T., Müller, L., Persson, S., et al. (2016). Whole-genome sequencing used to investigate a nationwide outbreak of listeriosis caused by ready-to-eat delicatessen meat, Denmark, (2014). *Clin. Infect. Dis.* 63, 64–70. doi: 10.1093/cid/ciw192

Kaas, R. S., Friis, C., Ussery, D. W., and Aarestrup, F. M. (2012). Estimating variation within the genes and inferring the phylogeny of 186 sequenced diverse *Escherichia coli* genomes. *BMC Genomics* 13:577. doi: 10.1186/1471-2164-13-577

Kaas, R. S., Leekitcharoenphon, P., Aarestrup, F. M., and Lund, O. (2014). Solving the problem of comparing whole bacterial genomes across different

sequencing platforms. *PLoS ONE* 9:e104984. doi: 10.1371/journal.pone.0104984

Law, J. W., Ab Mutalib, N. S., Chan, K. G., and Lee, L. H. (2015). An insight into the isolation, enumeration, and molecular detection of *Listeria monocytogenes* in food. *Front. Microbiol.* 6:1227. doi: 10.3389/fmicb.2015.01227

Leekitcharoenphon, P., Kaas, R. S., Thomsen, M. C., Friis, C., Rasmussen, S., and Aarestrup, F. M. (2012a). snpTree–a Web-Server to Identify and Construct SNP Trees from Whole Genome Sequence Data. *BMC Genomics* 13(Suppl. 7), S6. doi: 10.1186/1471-2164-13-S7-S6

Leekitcharoenphon, P., Lukjancenko, O., Friis, C., Aarestrup, F. M., and Ussery, D. W. (2012b). Genomic variation in salmonella enterica core genes for epidemiological typing. *BMC Genomics* 13:88. doi: 10.1186/1471-2164-13-88

Leekitcharoenphon, P., Nielsen, E. M., Kaas, R. S., Lund, O., and Aarestrup, F. M. (2014). Evaluation of whole genome sequencing for outbreak detection of *Salmonella enterica*. *PLoS ONE* 9:e87991. doi: 10.1371/journal.pone.0087991

Letunic, I., and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127–128. doi: 10.1093/bioinformatics/btl529

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with burrows-wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Lukjancenko, O., Ussery, D. W., and Wassenaar, T. M. (2012). Comparative genomics of bifidobacterium, *Lactobacillus* and related probiotic genera. *Microb. Ecol.* 63, 651–673. doi: 10.1007/s00248-011-9948-y

Lukjancenko, O., Wassenaar, T. M., and Ussery, D. W. (2010). Comparison of 61 sequenced *Escherichia coli* genomes. *Microb. Ecol.* 60, 708–720. doi: 10.1007/s00248-010-9717-3

Maiden, M. C., Jansen van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., et al. (2013). MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat. Rev. Microbiol.* 11, 728–736. doi: 10.1038/nrmicro3093

Maury, M. M., Tsai, Y. H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., et al. (2016). Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nat. Genet.* 48, 308–313. doi: 10.1038/ng.3501

Michelon, D., Félix, B., Vingadassalon, N., Mariet, J. F., Larsson, J. T., Møller-Nielsen, E., et al. (2015). PFGE Standard operating procedures for listeria monocytogenes: harmonizing the typing of food and clinical strains in Europe. *Foodborne Pathog. Dis.* 12, 244–252. doi: 10.1089/fpd.2014.1877

Moura, A., Criscuolo, A., Pouseele, H., Maury, M. M., Leclercq, A., Tarr, C., et al. (2016). *Whole genome-based population biology and epidemiological surveillance of Listeria Monocytogenes. Nat. Microbiol.* 2:16185. doi: 10.1038/nmicrobiol.2016.185

Nielsen, E. M., Björkman, J. T., Kiil, K., Grant, K., Dallman, T., Painset, A., Amar, C., et al. (2017). *Closing Gaps for Performing a Risk Assessment on Listeria monocytogenes in Ready-to-eat (RTE) Foods: Activity 3, the Comparison of Isolates from Different Compartments Along the Food Chain, and from Humans using Whole Genome Sequencing (WGS) Analysis*. EFSA Supporting Publications.

Orsi, R. H., den Bakker, H. C., and Wiedmann, M. (2011). *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol.* 301, 79–96. doi: 10.1016/j.ijmm.2010.05.002

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* 20, 289–290. doi: 10.1093/bioinformatics/btg412

Pattengale, N. D., Alipour, M., Bininda-Emonds, O. R., Moret, B. M., and Stamatakis, A. (2009). "How Many Bootstrap Replicates Are Necessary?" in *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*, Vol. 5541 LNBI, 184–200.

Paul, D., Steele, C., Donaldson, J. R., Banes, M. M., Kumar, R., Bridges, S. M., et al. (2014). Genome comparison of *Listeria monocytogenes* serotype 4a strainHCC23 with selected lineage i and lineage II L. *Monocytogenes* strains and other *Listeria* strains. *Genomics Data* 2, 219–225. doi: 10.1016/j.gdata.2014.06.010

Pightling, A. W., Petronella, N., and Pagotto, F. (2014). Choice of reference sequence and assembler for alignment of *Listeria monocytogenes* short-read sequence data greatly influences rates of error in SNP analyses. *PLoS ONE* 9:e104579. doi: 10.1371/journal.pone.0104579

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing. Vienna Austria R Foundation for Statistical Computing*. Vienna: R Foundation For Statistical Computing.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., et al. (2008). A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog.* 4:e1000146. doi: 10.1371/journal.ppat.1000146

Revell, L. J. (2012). Phytools: an r package for phylogenetic comparative biology (and Other Things). *Methods Ecol. Evol.* 3, 217–223. doi: 10.1111/j.2041-210X.2011.00169.x

Ribot, E. M., Swaminathan, B., and Pulsenet Taskforce (2006). PulseNet USA: A Five-Year Update 3. *Foodborne Pathog Dis.* 3, 9–19. doi: 10.1089/fpd.2006.3.9

Ruppitsch, W., Pietzka, A., Prior, K., Bletz, S., Fernandez, H. L., Allerberger, F., et al. (2015). Defining and evaluating a core genome multilocus sequence typing scheme for whole-genome sequence-based typing of *Listeria monocytogenes*. *J. Clin. Microbiol.* 53, 2869–2876. doi: 10.1128/JCM.01193-15

Schliep, K. P. (2011). Phangorn: phylogenetic analysis in R. *Bioinformatics* 27, 592–593. doi: 10.1093/bioinformatics/btq706

Snipen, L., and Ussery, D. W. (2010). Standard operating procedure for computing pangenome trees. *Stand. Genomic Sci.* 2, 135–141. doi: 10.4056/sigs.38923

Sokal, R., and James, R. J. (1962). The Comparison of dendrograms by objective methods. *Taxon* 11, 33–39. doi: 10.2307/1217208

Stamatakis, A. (2014). RAxML Version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Tettelin, H., Masignani, V., Cieslewicz, M. J., Donati, C., Medini, D., Ward, N. L., et al. (2005). Genome analysis of multiple pathogenic isolates of streptococcus agalactiae: implications for the microbial 'pan-Genome'. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13950–13955. doi: 10.1073/pnas.0506758102

Vesth, T., Wassenaar, T. M., Hallin, P. F., Snipen, L., Lagesen, K., and Ussery, D. W. (2010). On the origins of a *Vibrio* species. *Microb. Ecol.* 59, 1–13. doi: 10.1007/s00248-009-9596-7

Weller, D., Andrus, A., Wiedmann, M., and den Bakker, H. C. (2015). *Listeria booriae* Sp. Nov. and *Listeria newyorkensis* Sp. Nov., from food processing environments in the USA. *Int. J. Syst. Evol. Microbiol.* 65, 286–292. doi: 10.1099/ijs.0.070839-0

Wingstrand, A., Sørensen, A. I. V., Helwigh, B., and Müller, L. (eds.). (2015). *Annual Report on Zoonoses in Denmark 2014*. Søborg: DTU Food.

Zerbino, D. R., and Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de bruijn graphs. *Genome Res.* 18, 821–829. doi: 10.1101/gr.074492.107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Henri, Leekitcharoenphon, Carleton, Radomski, Kaas, Mariet, Felten, Aarestrup, Gerner Smidt, Roussel, Guillier, Mistou and Hendriksen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 December 2017
doi: 10.3389/fmicb.2017.02254

Prevalence and Antibiotic Resistance against Tetracycline in *Campylobacter jejuni* and *C. coli* in Cattle and Beef Meat from Selangor, Malaysia

Jayasekara M. K. J. K. Premarathne[1,2], Aimi S. Anuar[1], Tze Young Thung[1], Dilan A. Satharasinghe[3,4], Nuzul Noorahya Jambari[5], Noor-Azira Abdul-Mutalib[5], John Tang Yew Huat[6], Dayang F. Basri[7], Yaya Rukayadi[1], Yoshitsugu Nakaguchi[8], Mitsuaki Nishibuchi[8] and Son Radu[1,9]**

[1] Faculty of Food Science and Technology, Food Safety Research Center, Universiti Putra Malaysia, Seri Kembangan, Malaysia, [2] Department of Livestock and Avian Science, Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Kuliyapitiya, Sri Lanka, [3] Institute of Bio Science, Universiti Putra Malaysia, Seri Kembangan, Malaysia, [4] Department of Basic Veterinary Science, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka, [5] Food Safety Research Center (FOSREC), Faculty of Food Science and Technology, Universiti Putra Malaysia, Seri Kembangan, Malaysia, [6] Faculty of Food Technology, Universiti Sultan Zainal Abidin, Kuala Terengganu, Malaysia, [7] Faculty of Health Sciences, School of Diagnostic and Applied Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [8] Center for Southeast Asian Studies, Kyoto University, Kyoto, Japan, [9] Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Seri Kembangan, Malaysia

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Beatrix Stessl, Veterinärmedizinische Universität Wien, Austria
Heriberto Fernandez, Universidad Austral de Chile, Chile

***Correspondence:**
Jayasekara M. K. J. K. Premarathne
krissjayaruk@yahoo.com
Son Radu
son@upm.edu.my

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *04 July 2017*
Accepted: *31 October 2017*
Published: *04 December 2017*

Citation:
Premarathne JMKJK, Anuar AS, Thung TY, Satharasinghe DA, Jambari NN, Abdul-Mutalib N-A, Huat JTY, Basri DF, Rukayadi Y, Nakaguchi Y, Nishibuchi M and Radu S (2017) Prevalence and Antibiotic Resistance against Tetracycline in Campylobacter jejuni and C. coli in Cattle and Beef Meat from Selangor, Malaysia. Front. Microbiol. 8:2254. doi: 10.3389/fmicb.2017.02254

Campylobacter is a major foodborne pathogen frequently associated with human bacterial gastroenteritis in the world. This study was conducted to determine the prevalence and antibiotic resistance of *Campylobacter* spp. in the beef food system in Malaysia. A total of 340 samples consisting of cattle feces ($n = 100$), beef ($n = 120$) from wet markets and beef ($n = 120$) from hypermarkets were analyzed for *Campylobacter* spp. The overall prevalence of *Campylobacter* was 17.4%, consisting of 33% in cattle fecal samples, 14.2% in raw beef from wet market and 7.5% in raw beef from the hypermarket. The multiplex-polymerase chain reaction (PCR) identified 55% of the strains as *C. jejuni*, 26% as *C. coli*, and 19% as other *Campylobacter* spp. A high percentage of *Campylobacter* spp. were resistant to tetracycline (76.9%) and ampicillin (69.2%), whilst low resistance was exhibited to chloramphenicol (7.6%). The MAR Index of *Campylobacter* isolates from this study ranged from 0.09 to 0.73. The present study indicates the potential public health risk associated with the beef food system, hence stringent surveillance, regulatory measures, and appropriate interventions are required to minimize *Campylobacter* contamination and prudent antibiotic usage that can ensure consumer safety.

Keywords: *Campylobacter*, prevalence, beef, antibiotic suspetibility, MPN-PCR

INTRODUCTION

Thermophilic *Campylobacter* is a major bacterial pathogen that causes foodborne infections around the world (EFSA, 2011; WHO, 2012). *C. jejuni* and *C. coli* have been identified as the most common species that lead to campylobacteriosis (Silva et al., 2011). The high incidence rate of campylobacteriosis can be associated with the low minimal infective dose of thermophilic

Campylobacter which is around 500–800 cells (Nachamkin et al., 2008). *Campylobacter* spp. colonize the enteric tract of birds, sheep, cattle and pigs (Stanley and Jones, 2003; Humphrey et al., 2007). Food originated from animals act as the primary source of *Campylobacter* infection (Doorduyn et al., 2010).

Reduce susceptibility of foodborne pathogens to antimicrobials significantly affect global public health (Chatre et al., 2010; Aarestrup, 2015). Increasing resistance in *Campylobacter* to antimicrobials particularly to tetracycline, erythromycin, and (fluoro)quinolones (Gaudreau and Gilbert, 2003; Zhu et al., 2006) was associated with reduce response to therapy leading to higher morbidity and mortality rates in humans (Zhu et al., 2006).

Campylobacter a major public health concern around the world, especially in developed countries and these countries have *Campylobacter* surveillance systems (Scallan et al., 2011; EFSA and ECDC, 2014). Even in the South-East Asia, *Campylobacter* is becoming a key foodborne pathogen (Premarathne et al., 2017). According to a hospital-based study conducted by Lee and Puthucheary (2002), *Campylobacter* spp. was reported at 5% prevalence level and placed among the first five causative agents associated with child's diarrhea. Furthermore, *Campylobacter* were reported in humans from Indonesia (Tjaniadi et al., 2003), Lao People's Democratic Republic (Yamashiro et al., 1998), Singapore (Chau et al., 2016), and Thailand (Bodhidatta et al., 2013). Singapore is the only South-East Asian country that performs national surveillance of *Campylobacter* infection in humans. Estimation and control of campylobacteriosis in South-East Asian countries, including Malaysia, is hindered due to insufficient regulations for food safety and inadequate data on epidemiology of *Campylobacter* cases (Premarathne et al., 2017). Therefore, prevalence and antimicrobial resistance of *Campylobacter* are important to be assessed in these countries.

In Malaysia, beef is sold in traditional wet markets and modern hypermarkets (Chamhuri and Batt, 2013). Beef available in wet markets were supplied by locally slaughtered and dressed cattle at the municipal abattoir or none-abattoir premises (Marimuthu et al., 2015). The wet markets were operated for a short time around 6 h per day and were able to provide fresh meat every day (Tang et al., 2009). The hypermarkets offered chilled or frozen meat that locally produced or from imported beef (Ariff et al., 2015). According to a consumer survey, Malaysians considered that fresh meat is more succulent, healthier and assure the halalness. Therefore, they preferred purchasing meat from wet markets over hypermarkets (Chamhuri and Batt, 2013).

Naturally *Campylobacter* are present in cattle; therefore, feces can contaminate the beef carcasses during slaughtering (Hakkinen et al., 2007). Further, a study conducted in Malaysisa found that *C. jejuni* can survive in food processing environments through formation of biofilms (Teh et al., 2014). The prevalence of *Campylobacter* species in poultry (Tang et al., 2009; Mansouri-Najand et al., 2012; Rejab et al., 2012), duck (Nor Faiza et al., 2013) and salad vegetables (Chai et al., 2007) have been reported in Malaysia. However, limited data exist on the prevalence of *Campylobacter* in other food commodities, including beef food system. Impelled by scarcity of data, this study was conducted to determine the prevalence of *Campylobacter* in the beef food system together with assessing the antibiogram profiles of *Campylobacter* spp.

MATERIALS AND METHODS

Bacterial Strains

In this study *C. jejuni* ATCC 33221 and *C. coli* ATCC 33559 were used as the positive controls.

Sample Collection

A total of 100 fecal samples were collected from apparently healthy beef cattle in three different farms, while purchased 120 chilled beef samples from hypermarkets ($n = 6$) and 120 fresh beef samples from wet markets ($n = 6$) in Selangor, Malaysia. All collected samples were immediately transported to the laboratory in ice and processed within the same day of sample collection.

Sample Processing

The standardized and published protocol by Chai et al. (2007) and Tang et al. (2009) was used for sample processing. A 10 g portion of the sample was aseptically placed in a stomacher bag containing 90 mL Bolton broth base (Merck KGaA, Darmstadt, Germany) and homogenized using a stomacher lab-blender (Interscience, France) at normal speed for 60 s. The Bolton broth was supplemented with Bolton broth selective supplement contained Vancomycin, Cefoperazone, Trimethoprim Lactate and Amphotericin B (Merck KGaA) and 5% lysed horse blood.

Control samples were prepared only with supplemented Bolton broth and inoculated with *C. jejuni* ATCC 33221 and *C. coli* ATCC 33559. The controls and samples were incubated at 42°C for 48 h under microaerophilic conditions comprised of 8–10% (v/v) CO_2 and 5–6% (v/v) O_2 generated by the Anaerocult C system (Merck KGaA) in an anaerobic jar.

Microbiological Isolation

After incubation, a loop full of the enriched sample was streaked in duplicates onto modified charcoal-cefoperazone-deoxycholate agar (mCCDA; Merck KGaA) with CCDA selective supplement contained Cefoperazone and Amphotericin B (Merck KGaA) and incubated microaerobically (Anaerocult C system; Merck KGaA) for 48 h at 42°C. Presumptive identification of *Campylobacter* colonies was based on ISO method that includes (ISO, 2006a,b); oxidase test, catalase test, gram-staining and typical microscopic *Campylobacter* morphology. The presumptive colonies were confirmed by using PCR (Polymerase Chain Reaction) assay.

MPN-Enrichment

Campylobacter cells were counted using the MPN method with triplicate of three 10-fold dilution series prepared by transferring 100 µL of homogenized sample into 900 µL of Bolton broth base (Merck KGaA) supplemented with Bolton broth selective supplements. A control tube was prepared with supplemented Bolton broth and inoculated with *C. jejuni* ATCC 33221 and *C. coli* ATCC 33559. The MPN tubes were incubated at 42°C for 48 h under microaerophilic conditions generated by the Anaerocult C system (Merck KGaA) in an anaerobic jar.

DNA Extraction

Bacterial DNA was extracted from the enriched sample tubes and presumptive bacterial colonies using boiled-cell method (Tang et al., 2009). Briefly, enriched samples in the tubes were pelleted by centrifuging at 10,000 × g for 10 min. The supernatant was carefully removed, next the pellet was washed once with 500 μL sterile distilled water and harvested bacterial cells were suspended in 500 μL of sterile TE buffer (pH 8.0). Bacterial cells were lysed by subjecting to boiling for 10 min, followed by rapid cooling at −20°C for 10 min. Next the sample was centrifuged at 10,000 × g for 10 min. Finally, the supernatant containing bacterial DNA was stored at −20°C until used in PCR.

PCR Identification

All MPN tubes were subjected to identification of *Campylobacter* spp. by multiplex PCR reaction using primers specific for *Campylobacter* genes, *C. jejuni* and *C. coli* (**Table 1**).

The PCR was carried out in 25 μL reactions, and each reaction contained 5 μL of 5× PCR buffer (Promega, USA); 3 μL of 25 mM $MgCl_2$ (Promega, USA); 1 U *Taq* Polymerase (Promega, USA); 1 μL of 10 mM deoxynucleoside triphosphate (dNTP) mix (Promega, USA); 1 μM of forward and reverse primer (Sigma, UK) and 5 μL of template DNA.

The cycling conditions were set as follows on the VeritiTM 96-Well Thermal Cycler (Applied Biosystems, USA), initial denaturing at 94°C for 4 min followed by 33 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 5 min.

A 5 μL of all PCR amplicons were horizontally electrophoresed through a 1.25% agarose gel stained with ethidium bromide in 1× Tris-acetate-EDTA (TAE) (1 mM EDTA, 40 mM Tris-acetate) buffer at 90 V for 40 min. The agarose gel was visualized under ultraviolet (UV) light transilluminator (SynGene, Frederick, USA) and photographed. In each gel a positive control, non-template control and a DNA-molecular marker (100-bp ladder) (Vivantis Technologies, Malaysia) were included. The DNA extracted from *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560) were used as the positive controls, while PCR mixture without DNA template was used as the non-template control.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility tests were conducted using following antimicrobial impregnated disks (Oxoid, England, UK); ampicillin (AMP; 10 μg), cephalothin (CEF; 30 μg), chloramphenicol (CHL; 30 μg), ciprofloxacin (CIP; 5 μg), enrofloxacin (ENR; 5 μg), erythromycin (ERY; 15 μg), gentamicin (GEN; 10 μg), norfloxacin (NORr; 10 μg), nalidixic acid (NAL; 30 μg), sterptomycin (STR; 25 μg), and tetracycline (TET; 30 μg) according to the standard Kirby- Bauer disk diffusion method (Bauer et al., 1966) and performed according to the recommendations of the Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2012).

All isolates were revived from glycerol stocks using Bolton broth supplemented with 5% lysed horse blood. The suspensions were incubated for 48 h at 42°C under microaerophilic conditions. Revived cultures were grown in Brain heart infusion (BHI: Merck KGaA) broth at 42°C under microaerophilic conditions for 24 h and the turbidity of the suspension was adjusted to 0.5 McFarland standard (Clinical and Laboratory Standards Institute, 2012). Then the culture was swabbed uniformly using sterile cotton swabs onto MH agar plates (Merck KGaA) supplemented with 5% horse blood. The plates were incubated under microaerophilic conditions (Anaerocult C system; Merck KGaA) at 37°C for 48 h. *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as reference strains.

The diameters of the inhibition zones around the antibiotic disk were measured. The breakpoints used to categorize isolates as susceptible (s), intermediate (i) and resistant (r) were based on CLSI recommendations (Clinical and Laboratory Standards Institute, 2012). As there were no CLSI recommendations for *Campylobacter* strains, the standards assigned to the *Enterobacteriaceae* family were used as breakpoints to interpret *Campylobacter* resistance.

Multiple Antimicrobial Resistance (MAR) Indexing

Multi-resistance of *Campylobacter* isolates were quantified using the MAR indexing.

$$\text{MAR index} = a/b$$

where "a" indicate the number of antimicrobials to which the particular isolate was resistant and "b" indicate the total number of antimicrobials to which the particular isolate was tested (Krumperman, 1983).

Statistical Analysis

The difference in prevalence level between the two detection methods (MPN_plating and MPN_PCR) and various sample types obtained from cattle, wet market and hypermarket were analyzed using Pearson chi-square test (X^2 test). The results were

TABLE 1 | PCR primers set used for detection of *Campylobacter* spp., *C. jejuni* and *C. coli*.

Targeted species	Targeted gene	Sequence 5′-3′	Amplicon size (bp)	References
Campylobacter	*Cadf*	F:TTG AAG GTA ATT TAG ATA TG R:CTA ATA CCT AAA GTT GAA AC	400	Konkel et al., 1999
C. jejuni	*Oxidoreductase subunit*	F:ATG AAA AAA TAT TTA GTT TTT GCA R:ATT TTA TTA TTT GTA GCA GCG	160	Winters and Slavik, 1995
C. coli	*Ceu*	F:CAA ATA AAG TTA GAG GTA GAA TGT R:GGA TAA GCA CTA GCT AGC TGA T	894	Gonzalez et al., 1997

considered statistically significant at $P < 0.05$ at 95% confidence level.

RESULTS

Culture-Based Detection and Colony Confirmation Applying PCR Method

Presumptive *Campylobacter* spp. were detected in 8% (8 of 100 samples) of the cattle feces samples and 1.6% (4 of 240) of the beef meat samples and all presumptive isolates were confirmed as *Campylobacter* spp. by PCR.

MPN-Plating Media Based Enumeration

The prevalence of *Campylobacter* spp. in cattle and beef meat samples determined by using the MPN-plating is presented in **Table 2**. The MPN-plating detected *Campylobacter* spp. in 3.5% of the total samples (**Table 2**). *Campylobacter* was detected only in 8 out of 100 fecal samples, including *C. jejuni* in 5% and *C. coli* in 2% of the fecal samples. The prevalence of *C. jejuni* was significantly higher ($P \leq 0.05$) than the *C. coli* in fecal samples.

The majority (66%) of isolates were identified as *C. jejuni* and the remainder as *C. coli* (34%). *Campylobacter* spp. were detected only in 2% of the beef samples from wet and hypermarket by the MPN-plating method. The MPN-plating found *C. jejuni* and *C. coli* in 0.8% of beef samples from wet and hypermarket (**Table 2**). The prevalence of *Campylobacter* spp. was statistically not significant ($P \leq 0.05$) between the wet and hypermarkets. However, the prevalence of *Campylobacter* spp. in fecal samples was significantly higher ($P \leq 0.05$) than that in beef samples.

MPN-PCR-Based Enumeration

The prevalence of *Campylobacter* spp determined by using the MPN-PCR method is presented in **Table 3**. Based on the MPN-PCR results *Campylobacter* spp. was detected in 59 (17.4%) of the total 340 examined samples. Detection of *Campylobacter* spp. by MPN-PCR method was significantly higher ($P \leq 0.05$) comparative to the MPN-plating (**Table 3**).

The MPN-PCR method detected *Campylobacter* in 33 out of 100 cattle fecal samples. According to the MPN-PCR, fecal samples contained 16, 8, and 5% of *C. jejuni*, both *Campylobacter* spp. and *C. coli* respectively. The prevalence of *C. jejuni* was significantly higher ($P \leq 0.05$) than the *C. coli* in fecal samples.

Campylobacter was detected in a total of 17/120 (14.2%) beef from wet markets and in 9/120 (7.5%) beef from hypermarkets (**Table 3**). The predominant species of *Campylobacter* detected in beef samples from wet market (55%) was *C. jejuni* and remainder was *C. coli* (20%) and other *Campylobacter* spp. (25%). Similarly, at the hypermarket, *C. jejuni* was the most prevalent (67%) while *C. coli* and other *Campylobacter* spp. were detected in 22 and 11% of the beef samples respectively. The prevalence of *Campylobacter* spp. was statistically not significant ($P \leq 0.05$) between the wet and hypermarkets.

Campylobacter concentration in cattle and beef was enumerated using the MPN method (**Table 4**). The highest number of *Campylobacter* found in fecal samples from cattle, including 3-460 MPN/g of *C. jejuni* and 3-43 MPN/g of *C. coli* and 3-75 MPN/g of other thermophilic *Campylobacter* spp. Beef from wet markets observed to carry *Campylobacter* spp. in the range of 3-75 MPN/g while the beef samples from hypermarkets harbored low *Campylobacter* spp. concentration ranged from 3 to 15 MPN/g.

Antimicrobial Resistance Testing

Eleven antimicrobials named under the different antimicrobial groups were employed in this study to determine the resistance of *Campylobacter* isolates. All the isolates were resistant at least one or more antimicrobials (**Table 5**). Antimicrobial resistance pattern among the tested *Campylobacter* spp. Indicated that majority of the isolates were resistant to tetracycline (76.9%) and ampicillin (69.2%). Least resistance was observed for chloramphenicol (7.6%), cephalothin (15.4%), ciprofloxacin (15.4%) and gentamicin (15.4%).

The MAR index of *Campylobacter* species that were isolated from the current study indicate in **Table 6**. *Campylobacter* species exhibited 7 different antibiotic resistant patterns with MAR index ranging from 0.09 to 0.73. The highest MAR index was 0.73 and showed S, E, En, Na, Nor, Te, C, Amp resistant pattern. Meanwhile, lowest MAR index of 0.09 was demonstrated in isolates that indicate resistance to Te or Amp (**Table 5**). Moreover, 53.8% of the isolates were resistant to the three or more antimicrobials and demonstrated the Multi Drug Resistance (MDR).

DISCUSSION

Though the prevalence of *Campylobacter* in chicken and broilers was well documented in Malaysia; no or limited information was available for *Campylobacter* in other animals and food commodities, particularly for cattle and beef in the

TABLE 2 | Culture-based detection of *Campylobacter* spp. in cattle and beef.

Source	Sample type	Sample size	*C. jejuni*	*C. coli*	*C. jejuni/ C. coli*	OTC	Total
Beef cattle	Feces	100	16 (16)	5 (5)	8 (8)	4 (4)	33 (33)
Wet market	Beef	120	8 (6.7)	1 (0.8)	3 (2.5)	5 (4.2)	17 (14.2)
Hyper market	Beef	120	6 (5)	2 (1.7)	0 (0)	0 (0)	9 (7.5)
	Total	340	30 (8.8)	8 (6.7)	11 (9.2)	10 (8.2)	59 (17.4)

OTC, Other thermophilic Campylobacter spp.

TABLE 3 | MPN-PCR based detection of *Campylobacter* spp. in cattle and beef.

Source	Sample type	Sample size	*C. jejuni*	*C. coli*	*C. jejuni/ C. coli*	OTC	Total
Beef cattle	Feces	100	16 (16)	5 (5)	8 (8)	4 (4)	33 (33)
Wet market	Beef	120	8 (6.7)	1 (0.8)	3 (2.5)	5 (4.2)	17 (14.2)
Hyper market	Beef	120	6 (5)	2 (1.7)	0 (0)	0 (0)	9 (7.5)
	Total	340	30 (8.8)	8 (6.7)	11 (9.2)	10 (8.2)	59 (17.4)

OTC, Other thermophilic Campylobacter spp.

TABLE 4 | Concentration of *Campylobacter* spp. in cattle and beef.

Source	Sample type	Positive sample no	*C. jejuni*			*C. coli*			OTC		
			Max	Med	Min	Max	Med	Min	Max	Med	Min
Farm	Feces	33	460	6.2	3	43	6.1	3	75	15	3
Wet market	Beef	17	29	3.6	3	3.6	3	3	75	21	3
Hyper market	Beef	9	6.1	3	3	15	9.3	3.6	ND	ND	ND

Max, Maximum; Med, Median; Min, Minimum; OTC, Other thermophilic Campylobacter spp., ND, not detected.

TABLE 5 | The frequency of antibiotic drug resistance in *Campylobacter* spp.

Antibiotic group	Resistance profile	Resistant isolates		
		No %	*C. jejuni*	*C. coli*
Aminoglycosides	Gentamycin	2 (15.4)	1	1
	Streptomycin	3 (23.1)	1	2
Macrolides	Erythromycin	4 (30.8)	1	3
Quinolones and Fluoroquinolones	Ciprofloxacin	2 (15.4)	2	0
	Enrofloaxacin	5 (38.5)	2	3
	Nalidixix acid	7 (53.8)	4	3
	Norfloaxacin	4 (30.8)	2	2
Tetracyclines	Tetracycline	10 (76.9)	5	5
Chloramphenicol	Chloramphenicol	1 (7.6)	0	1
Penicillins	Ampicillin	9 (69.2)	5	4
Cephalexins	Cephalothin	2 (15.4)	0	2

TABLE 6 | Multiple antibiotic resistance (MAR) index of *Campylobacter* spp.

MAR index	*Campylobacter* spp.	Isolate code	Antibiogram
0.09	*C. jejuni*	CF3	TET
0.09	*C. coli*	BH158	TET
0.09	*C. jejuni*	CF58	TET
0.09	*C. jejuni*	CF85	AMP
0.18	*C. jejuni*	CF47	NAL, AMP
0.18	*C. jejuni*	BH70	TET, AMP
0.27	*C. jejuni*	BW188	NAL, TET, AMP
0.36	*C. coli*	CF46	NAL, TET, AMP, CEF
0.55	*C. coli*	BW189	STR, ERY, ENR, TET, AMP, CEF
0.55	*C. jejuni*	CF70	NAL, CIP, ENR, NOR, TET, AMP
0.64	*C. coli*	CF84	GEN, ERY, ENR, NAL, NOR, TET, AMP
0.64	*C. jejuni*	CF84	GEN, STR, ERY, CIP, ENR, NAL, NOR
0.73	*C. coli*	CF69	STR, ERY, ENR, NAL, NOR, TET, CHL, AMP

country. Therefore, the present study aimed to address the prevalence, concentration and antimicrobial resistant profiles of thermophilic *Campylobacter* species present in cattle fecal samples and beef.

The overall prevalence of thermophilic *Campylobacter* spp. in fecal samples of apparently healthy cattle was 33% (**Table 2**). Findings of this study was similar to the previous findings reported on prevalence level of *Campylobacter* spp. in cattle from countries including; Japan (39.6%) (Haruna et al., 2013) and Chile (35.9%) (Fernández and Hitschfeld, 2009). Sanad et al. (2011) found only 19.2% prevalence level in samples collected from feedlot, mature cows and bulls presented for slaughter across USA. Meanwhile, low mean *Campylobacter* prevalence level of 5.6% (Nonga et al., 2010) and 13.2% (Karikari et al., 2017) was reported from Tanzania and Ghana respectively. However, a study conducted in 5 different states in the U.S. reported a high prevalence (72.2%) of *Campylobacter* in feedlot cattle (Tang et al., 2017). While, 75–83.3% *Campylobacter* prevalence level was reported in dairy cattle from Lithuania (Ramonaite et al., 2013). Therefore, this study also contributes to prior discussions that cattle can act as a potential reservoir for transmitting *Campylobacter* spp. into the food system (Karenlampi et al., 2007; Cha et al., 2017). In the present study, *C. jejuni* (58.5%) was the predominantly isolated organism in cattle followed by *C. coli* (31.7%), and other thermophilic *Campylobacter* spp. The prevalence of *C. jejuni* and *C. coli* detected in this study was consistent with previous studies (Haruna et al., 2013). However, Karikari et al. (2017) reported higher prevalence level of *C. coli* (47.8%) comparatively *C. jejuni* was detected in 35.8% samples. Similarly, *C. jejuni* was detected in 35.2% cattle feacal samples with a high prevelance of *C. coli* (72.9%) (Sanad et al., 2011). However, Okunlade et al. (2015) reported low *C. coli* prevalence (20.4%) level in rectal swabs collected from cattle from Ibadan, Oyo State, Nigeria. A study conducted in Michigan, USA reported 69.2% prevalence level for *C. jejuni* (Cha et al., 2017). Other *Campylobacter* spp. (0.09%) detected in this study needs to be further characterized. Differences in the reported prevalence level can be resultant due to geographical location, sample size and method of analysis.

The low contamination frequency of *Campylobacter* species detected in beef in this study was in agreement with recent studies. The *Campylobacter* prevalence of fresh beef samples from Poland was 10.1% in Korsak et al. (2015), 16.2% in retail ground beef from Saskatchewan, Canada (Trokhymchuk et al., 2014) and 17.4% in whole beef cuts from retail shops in USA (Vipham et al., 2012). Meanwhile, some studies conducted previously could only detect very low *Campylobacter* contamination level in beef, including 1.9% in Tanzania (Nonga et al., 2010) and 3.3% in Belgium (Ghafir et al., 2007). Chilling reduce the surface humidity of red meat which can be related to low prevalence level *Campylobacter* in beef compared to chicken as *Campylobacter* is very sensitive to dehydration (Silva

et al., 2011). The current study detected high *Campylobacter* prevalence in beef from wet markets (14.2%) comparative to beef from hypermarkets (7.5%) can be associated with the availability of fresh meat in the wet markets in Malaysia. Fresh meat is sold without chilling in the wet markets as it is considered fresh and succulent since it retains moisture. This may facilitate the survival of *Campylobacter* on raw red meat. Further, the low hygienic conditions in the wet markets (Chamhuri and Batt, 2013) may also contribute to the comparatively higher *Campylobacter* contamination rate and increase the potential for cross contamination. Moreover, no-abattoir slaughtering procedures (Marimuthu et al., 2015) can also result in high contamination of beef in wet markets with *Campylobacter*. We speculate that the main reason for the difference of *Campylobacter* prevalence in cattle and beef from various countries can be the result of differences in sampling methods, storage duration, microbiological and molecular methods employed. The results of this study indicate that not only cattle but also beef can be a potential reservoir for *Campylobacter* infection.

Findings of this study reported that *Campylobacter* spp. in cattle range from 460-3 MPN/g, while a lower concentration was detected in beef (29-3 MPN/g). Further this study was in line with Nielsen (2002) who reported that mean *Campylobacter* concentration in cattle feces was 126 MPN/g. A study conducted by Stanley et al. (1998) observed a maximum of *Campylobacter* concentration at the level of 6.3×10^7 MPN/g in cattle. *Campylobacter* concentration in cattle intestine can be lower than that of broilers (Stanley and Jones, 2003). However, owed to a very low infective dose of *Campylobacter* the reported prevalence and concentration indicate the potential of contaminating beef during slaughter procedure. Therefore, proper sanitary and food safety measures should be implemented to ensure safety of beef. Further, an upward trend was observed among Malaysian consumers toward higher value meat such as beef and mutton (Yeong-Sheng et al., 2008). Therefore, beef can be a potential source of *Campylobacter* infection and need more attention focused on improving safety.

Isolation and identification of *Campylobacter* using conventional culture based methods can be challenging due to the fastidious growth requirements and discrepancies in biochemical tests (Nachamkin et al., 2008). Application of molecular methods for identification of foodborne pathogens has increasingly used for food samples to complement conventional microbiological methods owing to its rapid turnaround time and sensitivity (Inglis and Kalischuk, 2003). In this study, detection of *Campylobacter* spp. using PCR method was higher than plating on mCCDA agar. Previous studies on the prevalence of foodborne pathogens in food indicate a difference between the findings through the molecular biological methods comparative to findings of culturing methods (Inglis and Kalischuk, 2003; Chai et al., 2007; Tang et al., 2009). When growth conditions are not favorable *Campylobacter* cells can transition from vegetative stage into a viable but non-culturable (VBNC) state, in which the bacteria cannot be cultured using conventional culture methods (Oliver, 2010; Ramamurthy et al., 2014). The molecular amplification techniques can overcome the limitation of detecting VBNC cells with providing high specificity and sensitivity (Singh et al., 2011).

The high resistance detected in *Campylobacter* isolates against tetracycline in the current study was consistent with previously reported work. (Hong et al., 2007; Kashoma et al., 2015). Hong et al. (2007) reported that 93.4% *Campylobacter* strains from Korean beef samples were resistant to multiple antimicrobials and detected high resistance to tetracycline (94.6%). *Campylobacter* isolated from raw beef in Iran demonstrated the highest resistance to tetracycline (Rahimi et al., 2013). *Campylobacter* resistance to the tetracycline was mainly mediated through *tet* (O) plasmid (Pratt and Korolik, 2005) and worldwide 60–100% *C. jejuni* and *C. coli* were reported to carry tetracycline-resistant plasmids (Lee et al., 1994; Kim et al., 2010). High resistance to tetracycline in this study may be resultant due to *tet* (O) plasmid; however *Campylobacter* isolates from Malaysia need further investigation on prevalence and resistance profile of *tet* (O) plasmid. Similar to findings from the current study, the majority of the *Campylobacter* strains from food animals in Tanzania were resistant to ampicillin (70.3%) (Kashoma et al., 2015). Inherently resistance can be overserved in *Campylobacter* strains to β-lactams including ampicillin (Engberg, 2006; Li et al., 2007) which may be contributory to high ampicillin resistance observed in this study. The high resistance to tetracycline and ampicillin detected in this study could be associated with frequent usage of those antimicrobials in humans and animal husbandry (Chopra and Roberts, 2001; Hao et al., 2014).

Least resistance was observed for chloramphenicol, cephalothin, ciprofloxacin and gentamicin. Similarly low resistance was detected against gentamicin (1.8%) and chloramphenicol (4.5%) in the *Campylobacter* isolates from Tanzania (Kashoma et al., 2015). Further, *Campylobacter* spp. isolated from raw meat in Iran were resistant to gentamicin and chloramphenicol 3.2 and 6.5%, respectively (Rahimi et al., 2013). Contrary to the current study, *Campylobacter* isolates in beef from Korea showed high resistant to ciprofloxacin (95.9%) and nalidixic acid (94.6%) in Hong et al. (2007). Discrepancies and similarities in antibiotic resistance patterns can be attributed to variation in sample type, sampling procedure, type and frequency of antibiotic usage in animal husbandry practices and human therapy.

In the current study multiple antibiotic resistances was detected only in 53.8% of the isolates. Slightly higher multiple antibiotic resistance was observed in *Campylobacter* isolates from broilers in Malaysia (Saleha, 2002) and beef liver from USA (Noormohamed and Fakhr, 2014). The present study, 46.2% *Campylobacter* isolates reported to have a MAR less than 0.2. A bacterium that has a MAR index less than 0.2 has been identified to be isolated from animals that antimicrobials were seldom used. While, if the strain has a MAR index greater than 0.2 considered to be originated from producing animals that have a high potential for contamination (Marian et al., 2012). According to the National Pharmaceutical Control Bureau (NPCB) of the Ministry of

Health, Malaysia, more than 97 antimicrobials have been registered for use in producing animals in Malaysia. However, comparative to poultry, a lower number of products has been registered to be used in cattle. The MAR index detected in *Campylobacter* isolates from cattle and beef of this study may be associated with low frequency of antibiotic usage in cattle compared to poultry in Malaysia. However, multiple antibiotic resistances associated with isolates from cattle and beef exacerbate the public health concern associated with *Campylobacter* infections.

CONCLUSION

Based on the findings of this study indicate the importance of considering the potential public health risk associated with *Campylobacter* in the beef food system in Malaysia. Therefore, implementation of good hygienic practices at the farm, slaughterhouse, and retail level can minimize the *Campylobacter* contamination. Furthermore, the presence of multiple antibiotic resistant *Campylobacter* spp. urge the prudent use of antimicrobials in animal husbandry, farmer awareness and application of good veterinary practices to minimize the likelihood of emerging superbugs.

AUTHOR CONTRIBUTIONS

JP and AA conducted the experiment. TT and DS did the data analysis. JP prepared the manuscript. NJ, N-AA-M, JH, DB, YR, YN, MN, and SR supervised and assisted in the preparation of the manuscript. NJ and N-AA-M provided consumables.

FUNDING

The authors wish to acknowledge the Malaysian Ministry of Higher Education for the financial support through Fundamental Research Grant Scheme (FRGS-02-01-14-1475FR) under the project FRGS/1/2014/SG05/UPM/01/2 and Fund for Research on international cooperation in medical science, Research on global health issues, Health and Labor Science Research Grants, the Ministry of Health, Labor, Kyoto University Research Coordination Alliance, Japan, and by Kakenhi Grant-in-Aid for Scientific Research and from the Japan Society for the Promotion of Sciences.

REFERENCES

Aarestrup, F. M. (2015). The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. *Philos. Trans. R. Soc. B* 370:20140085. doi: 10.1098/rstb.2014.0085

Ariff, O. M., Sharifah, N. Y., and Hafidz, A. W. (2015). Status of beef industry of Malaysia. *Mal. J. Anim.* 18, 1–21. Available online at: http://www.msap.my/pdf/mjas_18_2/1.Status-Ariff_r4-2.pdf

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Path.* 45, 493.

Bodhidatta, L., Srijan, A., Serichantalergs, O., Bangtrakulnonth, A., Wongstitwilairung, B., McDaniel, P., et al. (2013). Bacterial pathogens isolated from raw meat and poultry compared with pathogens isolated from children in the same area of rural Thailand. *Southeast Asian J. Trop. Med. Public Health* 44, 259–272. Available online at: https://pdfs.semanticscholar.org/b58b/d0c295026cbd8fb674f1a00db4ca80e3b498.pdf

Cha, W., Mosci, R. E., Wengert, S. L., Vargas, C. V., Rust, S. R., Bartlett, P. C., et al. (2017). Comparing the genetic diversity and antimicrobial resistance profiles of *Campylobacter jejuni* recovered from cattle and humans. *Front. Microbiol.* 8:818. doi: 10.3389/fmicb.2017.00818

Chai, L. C., Robin, T., Ragavan, U. M., Gunsalam, J. W., Bakar, F. A., Ghazali, F. M., et al. (2007). Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. *Int. J. Food Microbiol.* 117, 106–111. doi: 10.1016/j.ijfoodmicro.2007.02.014

Chamhuri, N., and Batt, P. J. (2013). Exploring the factors influencing consumers' choice of retail store when purchasing fresh meat in Malaysia. *Int. Food Agribus. Man.* 16, 99–122. Available online at: https://www.ifama.org/resources/Documents/v16i3/Chamhuri-Batt.pdf

Chatre, P., Haenni, M., Meunier, D., Botrel, M. A., Calavas, D., and Madec, J. (2010). Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from cattle between 2002 and 2006 in France. *J. Food Prot.* 73, 825–831. doi: 10.4315/0362-028X-73.5.825

Chau, M. L., Hartantyo, S. H. P., Yap, M., Kang, J. S. L., Aung, K. T., Gutiérrez, R. A., et al. (2016). Diarrheagenic pathogens in adults attending a hospital in Singapore. *BMC Infect. Dis.* 16:1. doi: 10.1186/s12879-016-1354-0

Chopra, I., and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260. doi: 10.1128/MMBR.65.2.232-260.2001

Clinical and Laboratory Standards Institute (2012). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M11- S22*. Wayne, PA: CLSI.

Doorduyn, Y., Van Den Brandhof, W. E., Van Duynhoven, Y. T. H. P., Wannet, W. J. B., and Van Pelt, W. (2010). Risk factors for indigenous *Campylobacter jejuni* and *Campylobacter coli* infections in The Netherlands: a case-control study. *Epidemiol. Infect.* 138, 1391–1404. doi: 10.1017/S09502688100 0052X

Engberg, J. (2006). Contributions to the epidemiology of *Campylobacter* infections. *Dan. Med. Bull.* 53, 361–389. Available online at: http://www.danmedj.dk/dmb_2006/0406/0406-disputatser/DMB3853.pdf

European Food Safety Authority (EFSA) (2011). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and Salmonella on broiler carcasses in the EU. *EFSA J.* 8, 1503 doi: 10.2903/j.efsa.2011.2017

European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (2014). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. *EFSA J.* 12:3547. doi: 10.2903/j.efsa.2014.3547

Fernández, H., and Hitschfeld, M. (2009). Occurrence of *Campylobacter jejuni* and *Campylobacter coli* and their biotypes in beef and dairy cattle from the south of Chile. *Braz. J. Microbiol.* 40, 450–454. doi: 10.1590/S1517-83822009000300005

Gaudreau, C., and Gilbert, H. (2003). Antimicrobial resistance of *Campylobacter jejuni* subsp. *jejuni* strains isolated from humans in 1998 to 2001 in Montreal, Canada. *Antimicrob. Agents Chemother.* 47, 2027–2029. doi: 10.1128/AAC.47.6.2027-2029.2003

Ghafir, Y., China, B., Dierick, K., De Zutter, L., and Daube, G. (2007). Seven-year survey of *Campylobacter* contamination in meat at different production stages in Belgium. *Int. J. Food Microbiol.* 116, 111–120. doi: 10.1016/j.ijfoodmicro.2006.12.012

Gonzalez, I., Grant, K. A., Richardson, P. T., Park, S. F., and Collins, M. D. (1997). Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the ceuE gene encoding a putative virulence determinant. *J. Clin. Microbiol.* 35, 759–763.

Hakkinen, M., Heiska, H., and Hanninen, M. L. (2007). Prevalence of *Campylobacter* spp. in cattle in Finland and antimicrobial susceptibilities of bovine *Campylobacter jejuni* strains. *Appl. Environ. Microbiol.* 73, 3232–3238. doi: 10.1128/AEM.02579-06

Hao, H., Cheng, G., Iqbal, Z., Ai, X., Hussain, H. I., Huang, L., et al. (2014). Benefits and risks of antimicrobial use in food-producing animals. *Front. Microbiol.* 5:288. doi: 10.3389/fmicb.2014.00288

Haruna, M., Sasaki, Y., Murakami, M., Mori, T., Asai, T., Ito, K., et al. (2013). Prevalence and antimicrobial resistance of *Campylobacter* isolates from beef cattle and pigs in Japan. *J. Vet. Med. Sci.* 75, 625–628. doi: 10.1292/jvms.12-0432

Hong, J., Berrang, M. E., Liu, T., Hofacre, C. L., Sanchez, S., Wang, L., et al. (2007). Prevalence and antibiotic resistance of *Campylobacter* spp. isolated from chicken meat, pork, and beef in Korea, from 2001 to 2006. *J. Food Prot.* 70, 860–866. doi: 10.4315/0362-028X-70.4.860

Humphrey, T., O'Brien, S., and Madsen, M. (2007). *Campylobacter* as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol.* 117, 237–257. doi: 10.1016/j.ijfoodmicro.2007.01.006

Inglis, G. D., and Kalischuk, L. D. (2003). Use of PCR for direct detection of *Campylobacter* species in bovine feces. *Appl. Environ. Microbiol.* 69, 3435–3447. doi: 10.1128/AEM.69.6.3435-3447.2003

ISO (2006a). *Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for Detection and Enumeration of Campylobacter* spp. – *Part 1: Detection Method*. Geneva: International organization for standardization.

ISO (2006b). *Microbiology of Food And Animal Feeding Stuffs – Horizontal Method for Detection and Enumeration of Campylobacter* spp. – *part 2: Colony-Count Technique*. Geneva: International organization for standardization.

Karenlampi, R., Rautelin, H., and Hänninen, M. L. (2007). Longitudinal study of Finnish *Campylobacter jejuni* and *C. coli* isolates from humans, using multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle. *Appl. Environ. Microbiol.* 73, 148–155. doi: 10.1128/AEM.01488-06

Karikari, A. B., Obiri-Danso, K., Frimpong, E. H., and Krogfelt, K. A. (2017). Antibiotic resistance of campylobacter recovered from faeces and carcasses of healthy livestock. *BioMed. Res. Int.* 2017:4091856. doi: 10.1155/2017/4091856

Kashoma, I. P., Kassem, I. I., Kumar, A., Kessy, B. M., Gebreyes, W., Kazwala, R. R., et al. (2015). Antimicrobial resistance and genotypic diversity of Campylobacter isolated from pigs, dairy, and beef cattle in Tanzania. *Front. Microbiol.* 6:1240. doi: 10.3389/fmicb.2015.01240

Kim, J. M., Hong, J., Bae, W., Koo, H. C., Kim, S. H., and Park, Y. H. (2010). Prevalence, antibiograms, and transferable tet (O) plasmid of *Campylobacter jejuni* and *Campylobacter coli* isolated from raw chicken, pork, and human clinical cases in Korea. *J. Food Prot.* 73, 1430–1437. doi: 10.4315/0362-028X-73.8.1430

Konkel, M. E., Gray, S. A., Kim, B. J., Garvis, S. G., and Yoon, J. (1999). Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. *J. Clin. Microbiol.* 37, 510–517.

Korsak, D., Maćkiw, E., Rozynek, E., and Zyłowska, M. (2015). Prevalence of *Campylobacter* spp. in retail chicken, turkey, pork, and beef meat in Poland between 2009 and 2013. *J. Food Prot.* 78, 1024–1028. doi: 10.4315/0362-028X.JFP-14-353

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherischia coli* to identify high-risk sources of fecal contamination of food. *Appl. Environ. Microbiol.* 46, 165–170.

Lee, C. Y., Tai, C. L., Lin, S. C., and Chen, Y. T. (1994). Occurrence of plasmids and tetracycline resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from whole market chickens and clinical samples. *Int. J. Food Microbiol.* 24, 161–170. doi: 10.1016/0168-1605(94)90115-5

Lee, W. S., and Puthucheary, S. D. (2002). Bacterial enteropathogens isolated in childhood diarrhoea in Kuala Lumpur–the changing trend. *Med. J. Malaysia,* 57, 24–30. Available online at: http://www.e-mjm.org/2002/v57n1/Childhood_Diarrhoea.pdf

Li, X. Z., Mehrotra, M., Ghimire, S., and Adewoye, L. (2007). β-Lactam resistance and β-lactamases in bacteria of animal origin. *Vet. Microbiol.* 121, 197–214. doi: 10.1016/j.vetmic.2007.01.015

Mansouri-Najand, L., Saleha, A. A., and Wai, S. S. (2012). Prevalence of multidrug resistance *Campylobacter jejuni* and *Campylobacter coli* in chickens slaughtered in selected markets, Malaysia. *Trop. Biomed.* 29, 231–238. Available online at: http://www.msptm.org/files/231_-_238_Saleha_A_A.pdf

Marian, M. N., Aminah, S. S., Zuraini, M. I., Son, R., Maimunah, M., Lee, H. Y., et al. (2012). MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. *Food Control* 28, 309–314. doi: 10.1016/j.foodcont.2012.05.030

Marimuthu, M., Adamu, L., Abdullah, F. F. J., Abubakar, M., and Mohammed, K. (2015). Antimicrobial residues in beef animals slaughtered in abattoir and non-abattoir small holders slaughter houses in negeri sembilan, Malaysia. *Alexandria J. Vet. Sci.* 44, 1–8. doi: 10.5455/ajvs.167605

Nachamkin, I., Szymanski, M. C., and Blaser, J. M. (2008). *Campylobacter, 3rd Edn.* Washington, DC: ASM Press.

Nielsen, E. M. (2002). Occurrence and strain diversity of thermophilic campylobacters in cattle of different age groups in dairy herds. *Lett. Appl. Microbiol.* 35, 85–89. doi: 10.1046/j.1472-765X.2002.01143.x

Nonga, H. E., Sells, P., and Karimuribo, E. D. (2010). Occurrences of thermophilic Campylobacter in cattle slaughtered at Morogoro municipal abattoir, Tanzania. *Trop Anim. Health Prod.* 42, 73. doi: 10.1007/s11250-009-9387-7

Noormohamed, A., and Fakhr, M. (2014). Molecular Typing of *Campylobacter jejuni* and *Campylobacter coli* isolated from various retail meats by MLST and PFGE. *Foods* 3, 82–93. doi: 10.3390/foods3010082

Nor Faiza, S., Saleha, A. A., Jalila, A., and Fauziah, N. (2013). Research note occurrence of *campylobacter* and *salmonella* in ducks and duck eggs in Selangor, Malaysia. *Trop. Biomed.* 30, 155–158. Available online at: http://msptm.org/files/155_-_158_Saleha_A_A.pdf

Okunlade, A. O., Ogunleye, A. O., Jeminlehin, F. O., and Ajuwape, A. T. P. (2015). Occurrence of Campylobacter species in beef cattle and local chickens and their antibiotic profiling in Ibadan, Oyo State, Nigeria. *Afr. J Microbiol. Res.* 9, 1473–1479. doi: 10.5897/AJMR2014.7105

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34, 415–425. doi: 10.1111/j.1574-6976.2009.00200.x

Pratt, A., and Korolik, V. (2005). Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *J. Antimicrob. Chemother* 55, 452–460. doi: 10.1093/jac/dki040

Premarathne, J. M. K. J. K., Satharasinghe, D. A., Huat, J. T. Y., Basri, D. F., Rukayadi, Y., Nakaguchi, Y., et al. (2017). Impact of human Campylobacter infections in South-East Asia: the contribution of the poultry sector. *Crit. Rev. Food Sci. Nutr.* 57, 3971–3986. doi: 10.1080/10408398.2016.1266297

Rahimi, E., Ameri, M., Alimoradi, M., Chakeri, A., and Bahrami, A. R. (2013). Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from raw camel, beef, and water buffalo meat in Iran. *Comp. Clin. Path.* 22, 467–473. doi: 10.1007/s00580-012-1434-5

Ramamurthy, T., Ghosh, A., Pazhani, G. P., and Shinoda, S. (2014). Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Front. Public Health* 2:103. doi: 10.3389/fpubh.2014.00103

Ramonaite, S., Rokaityte, A., Tamulevičiene, E., Malakauskas, A., Alter, T., and Malakauskas, M. (2013). Prevalence, quantitative load and genetic diversity of Campylobacter spp. in dairy cattle herds in Lithuania. *Acta. Vet. Scand.* 55:87. doi: 10.1186/1751-0147-55-87

Rejab, S. B. M., K. H., Zessin, R., and Fries, and, P., Patchanee (2012). *Campylobacter* in chicken carcasses and slaughterhouses in Malaysia. *Southeast Asian J. Trop. Med. Public Health* 43, 96–104. Available online at: http://www.tm.mahidol.ac.th/seameo/2012-43-1/13-5196.pdf

Saleha, A. A. (2002). Isolation and characterization of *Campylobacter jejuni* from broiler chickens in Malaysia. *Int. J. Poult. Sci.* 1, 94–97. doi: 10.3923/ijps.2002.94.97

Sanad, Y. M., Kassem, I. I., Abley, M., Gebreyes, W., LeJeune, J. T., and Rajashekara, G. (2011). Genotypic and phenotypic properties of cattle-associated Campylobacter and their implications to public health in the USA. *PLoS ONE* 6:e25778. doi: 10.1371/journal.pone.0025778

Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V., and Hoekstra, R. M. (2011). Foodborne illness acquired in the United States–unspecified agents. *Emerg. Infect. Infect. Dis.* 17, 16–22. doi: 10.3201/eid1701.P21101

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Singh, H., Rathore, R. S., Singh, S., and Cheema, P. S. (2011). Comparative analysis of cultural isolation and PCR based assay for detection of *Campylobacter jejuni* in food and faecal samples. *Braz. J. Microbiol.* 42, 181–186. doi: 10.1590/S1517-83822011000100022

Stanley, K. N., Wallace, J. S., Currie, J. E., Diggle, P. J., and Jones, K. (1998). Seasonal variation of thermophilic campylobacters in lambs at slaughter. *J. Appl. Microbiol.* 84, 1111–1116.

Stanley, K., and Jones, K. (2003). Cattle and sheep farms as reservoirs of *Campylobacter*. *J. Appl. Microbiol.* 94, 104S–113S. doi: 10.1046/j.1365-2672.94.s1.12.x

Tang, J. Y. H., Mohamad Ghazali, F., Abdul Aziz, S., Nishibuchi, M., and Radu, S. (2009). Comparison of thermophilic *Campylobacter* spp. occurrence in two types of retail chicken samples. *Int. Food Res. J.* 16, 277–288. Available online at: http://www.ifrj.upm.edu.my/16%20(3)%202009/2[1]%20John.pdf

Tang, Y., Meinersmann, R. J., Sahin, O., Wu, Z., Dai, L., Carlson, J., et al. (2017). Wide but variable distribution of a hypervirulent *Campylobacter jejuni* clone in beef and dairy cattle in the United States. *Appl. Environ. Microbiol.* 83:AEM.01425-17. doi: 10.1128/AEM.01425-17

Teh, A. H. T., Lee, S. M., and Dykes, G. A. (2014). Does *Campylobacter jejuni* form biofilms in food-related environments? *Appl. Environ. Microbiol.* 80, 5154–5160. doi: 10.1128/AEM.01493-14

Tjaniadi, P., Lesmana, M., Subekti, D., Machpud, N., Komalarini, S., Santoso, W., et al. (2003). Antimicrobial resistance of bacterial pathogens associated with diarrheal patients in Indonesia. *Am. J. Trop. Med. Hyg.* 68, 666–670. Available online at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.558.4922&rep=rep1&type=pdf

Trokhymchuk, A., Waldner, C., Chaban, B., Gow, S., and Hill, J. E. (2014). Prevalence and diversity of *Campylobacter* species in Saskatchewan retail ground beef. *J. Food Prot.* 77, 2106–2110. doi: 10.4315/0362-028X.JFP-14-247

Vipham, J. L., Brashears, M. M., Loneragan, G. H., Echeverry, A., Brooks, J. C., Chaney, W. E., et al. (2012). Salmonella and *Campylobacter* baseline in retail ground beef and whole-muscle cuts purchased during 2010 in the United States. *J. Food Prot.* 75, 2110–2115. doi: 10.4315/0362-028X.JFP-12-077

Winters, D. K., and Slavik, M. F. (1995). Evaluation of a PCR based assay for specific detection of *Campylobacter jejuni* in chicken washes. *Mol. Cell. Probes* 9, 307–310. doi: 10.1016/S0890-8508(95)91556-7

World Health Organization (WHO) (2012). *The Global View of Campylobacteriosis.* Report of Expert Consultation, World Health Organization. Utrecht; Geneva.

Yamashiro, T., Nakasone, N., Higa, N., Iwanaga, M., Insisiengmay, S., Phounane, T., et al. (1998). Etiological study of diarrheal patients in Vientiane, Lao People's Democratic Republic. *J. Clin. Microbiol.* 36, 2195–2199.

Yeong-Sheng, T., Mohamed Arshad, F., Shamsudin, M. N., Mohamed, Z., and Radam, A. (2008). *Demand for Meat Quantitu and Quality in Malaysia: Implications to Australia.* Munich: University Library of Munich.

Zhu, J., Zhang, Y., Hua, X., Hou, J., and Jiang, Y. (2006). Antibiotic resistance in *Campylobacter*. *Rev. Med. Microbiol.* 17, 107–121. doi: 10.1097/MRM.0b013e3280c4d106

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Premarathne, Anuar, Thung, Satharasinghe, Jambari, Abdul-Mutalib, Huat, Basri, Rukayadi, Nakaguchi, Nishibuchi and Radu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 December 2017
doi: 10.3389/fmicb.2017.02567

Genetic Diversity, Antimicrobial Susceptibility, and Biofilm Formation of *Cronobacter* spp. Recovered from Spices and Cereals

Yuanhong Li[1†], *Huan Yu*[2†], *Hua Jiang*[1], *Yang Jiao*[1], *Yaodong Zhang*[1] *and Jihong Shao*[1*]

[1] School of Public Health, Xuzhou Medical University, Xuzhou, China, [2] Department of Pharmacy, Wuhan No.1 Hospital, Wuhan, China

Cronobacter species are important food-borne opportunistic pathogens which have been implicated in the cause of necrotizing enterocolitis, sepsis, and meningitis in neonates and infants. However, these bacteria are routinely found in foodstuffs, clinical specimens, and environmental samples. This study investigated the genetic diversity, antimicrobial susceptibility, and biofilm formation of *Cronobacter* isolates ($n = 40$) recovered from spices and cereals in China during 2014–2015. Based on the *fus*A sequencing analysis, we found that the majority (23/40, 57.5%) of *Cronobacter* isolates in spices and cereals were *C. sakazakii*, while the remaining strains were *C. dublinensis* (6/40, 15.0%), *C. malonaticus* (5/40, 12.5%), *C. turicensis* (4/40, 10.0%), and *C. universalis* (2/40, 5.0%). Multilocus sequence typing (MLST) analysis produced 30 sequence types (STs) among the 40 *Cronobacter* isolates, with 5 STs (ST4, ST13, ST50, ST129, and ST158) related to neonatal meningitis. The pattern of the overall ST distribution was diverse; in particular, it was revealed that ST148 was the predominant ST, presenting 12.5% within the whole population. MLST assigned 12 isolates to 7 different clonal complexes (CCs), 4, 13, 16, 17, 72, 129, and 143, respectively. The results of O-antigen serotyping indicated that *C. sakazakii* serotype O1 and O2 were the most two prevalent serotypes. The antimicrobial susceptibility testing showed that the 40 *Cronobacter* isolates were susceptible to most of the antibiotics tested except for ceftriaxone, meropenem, and aztreona. Of the 40 *Cronobacter* strains tested, 13 (32.5%) were assessed as weak bioflim producers, one (2.5%) was a moderate biofilm producer, one (2.5%) was strong biofilm producer, and the others (62.5%) were non-biofilm producers. MLST and O-antigen serotyping have indicated that *Cronobacter* strains recovered from spices and cereals were genetically diverse. Isolates of clinical origin, particularly the *C. sakazakii* ST4 neonatal meningitic pathovar, have been identified from spices and cereals. Moreover, antimicrobial resistance of *Cronobacter* strains was observed, which may imply a potential public health risk. Therefore, the surveillance of *Cronobacter* spp. in spices and cereals should be strengthened to improve epidemiological understandings of *Cronobacter* infections.

Keywords: *Cronobacter* spp., Multilocus sequence typing, serotyping, antimicrobial susceptibility, biofilm formation

OPEN ACCESS

Edited by:
Rosanna Tofalo,
Università di Teramo, Italy

Reviewed by:
Beatrix Stessl,
Veterinärmedizinische Universität Wien, Austria
Pasquale Russo,
University of Foggia, Italy

***Correspondence:**
Jihong Shao
sjh2653@xzhmu.edu.cn

[†] *These authors have contributed equally to this work.*

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *18 August 2017*
Accepted: *11 December 2017*
Published: *19 December 2017*

Citation:
Li Y, Yu H, Jiang H, Jiao Y, Zhang Y and Shao J (2017) Genetic Diversity, Antimicrobial Susceptibility, and Biofilm Formation of Cronobacter spp. Recovered from Spices and Cereals. Front. Microbiol. 8:2567. doi: 10.3389/fmicb.2017.02567

INTRODUCTION

The *Cronobacter* genus, belonging to the family Enterobacteriaceae, includes seven species: *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. muytjensii*, *C. turicensis*, *C. universalis*, and *C. condimenti* (Iversen et al., 2008; Joseph et al., 2012). Among them, three species in the genus *Cronobacter*, including *C. sakazakii*, *C. malonaticus*, and *C. turicensis* have been implicated in fatal neonatal infections resulting in sepsis, necrotizing enterocolitis and meningitis, with a high mortality rate and probability of neurological sequelae (Hunter and Bean, 2013; Ogrodzki and Forsythe, 2015). Although neonatal infections caused by *Cronobacter* spp. were highlighted, recent studies indicated that these bacteria can cause illness in both infants and adults, especially for newborns, the elderly, and individuals with weakened immune systems (Patrick et al., 2014; Alsonosi et al., 2015). Outbreaks of *Cronobacter* infections have been reported in many countries in recent years (Friedemann, 2009; Holý et al., 2014; Patrick et al., 2014).

The genus *Cronobacter* includes many ubiquitous species that are found in foodstuffs or raw materials, and clinical specimens as well as environmental samples (Reich et al., 2010; Alsonosi et al., 2015; Song et al., 2016; Brandão et al., 2017), but the exact reservoir and routes of transmission has still not been ascertained (Sani and Odeyemi, 2015). Understanding the transmission routes (e.g., waterborne, foodborne, or environmental) and vehicles (e.g., powdered infant formula, vegetables, meat, spices, or cereals) of a *Cronobacter* outbreak is of great public health importance. Thus, evaluation of a wide variety of foods might be necessary to reveal possible routes for transmission of infections caused by the genus *Cronobacter*.

Molecular typing techniques have become an important tool to study the genetic diversity of *Cronobacter* spp. and to trace individual strains that cause human infections. In recent years, a number of molecular typing techniques such as MLST (Baldwin et al., 2009), PCR-restriction fragment length polymorphism (PCR-RFLP) (Vlach et al., 2017), pulsed field gel electrophoresis (PFGE) (Lou et al., 2014), amplified fragment length polymorphism (AFLP) (Turcovský et al., 2011), and random amplified polymorphic DNA (RAPD) (Drudy et al., 2006), have been established to differentiate these pathogens. Among these typing techniques, MLST is currently considered to be the best tool for epidemiological studies of *Cronobacter* spp. due to its high reproducibility and discriminatory ability. Serotyping is another important diagnosis tool widely used for identifying food-borne pathogens. Recent studies indicated that *Cronobacter* spp. have been differentiated into 17 serotypes by PCR-based O-antigen serotyping assays targeting the *wzx* (O-antigen flippase) and the *wzy* (O-antigen polymerase) genes (Jarvis et al., 2011, 2013; Sun et al., 2011, 2012a,b). The development of these molecular techniques is greatly helpful to distinguish *Cronobacter* species and may further assist in epidemiological investigation of outbreaks of *Cronobacter* infections.

Owing to the improper and abusive usage of antimicrobial agents, the emergence and spread of multidrug-resistant strains have become a serious threat to public health worldwide. Current studies indicated that *Cronobacter* spp. seemed to be less resistance to commonly used antibiotics compared to other foodborne pathogens such as *Listeria monocytogenes*, *Campylobacter jejuni,* and *Salmonella* spp. (Wang et al., 2013; Han et al., 2016; Komora et al., 2017). However, drug resistant strains of *Cronobacter* spp. were found in several studies (Lee et al., 2012; Xu et al., 2015; Fei et al., 2017), some of which were characterized as multidrug-resistant strains (Kilonzo-Nthenge et al., 2012). Therefore, it is necessary to investigate the antibiotic resistance of *Cronobacter* spp. recovered from various food samples in order to classify the patterns of resistance and to formulate an effective strategy to prevent the potential spread of these strains.

In recent years, attachment and biofilm formation of foodborne pathogens has become a matter of increasing concern for food safety research because the high likelihoods of potential cross-contamination may lead to serious food safety problems (Simoes et al., 2010). Recently, some researchers have found that strains of *Cronobacter* spp. were able to form biofilms on many kinds of materials such as stainless steel, polyvinyl chloride, silicone, and polycarbonate (Jo et al., 2010; Park and Kang, 2014). Established biofilms are very difficult to remove due to the tolerance to sanitizing agents, and thereby pose a potential health risk to human health because microorganisms within biofilms might result in a persistent release of bacteria to foods and environment. The aim of the present study was to investigate the genetic diversity, by MLST and serotyping, the antimicrobial susceptibility, and biofilm formation of 40 *Cronobacter* isolates from spices and cereals.

MATERIALS AND METHODS

Strain Collection, Culture Condition, and DNA Extraction

A total of 40 *Cronobacter* isolates recovered from spices and cereal food samples in China between September 2014 and June 2015 were analyzed (**Table 1**). Twenty-one strains were from spices and 19 from cereals. These strains have been confirmed as *Cronobacter* spp. by genus specific PCR confirmation based on the outer membrane protein A (*Omp*A) and internal transcribed spacer (ITS) gene, and 16S rRNA sequencing in our previous work (Li Y. H. et al., 2016; Li et al., 2017). The bacterial strains were routinely grown in Tryptic Soy Broth (TSB; QingDao Hope Bio-technology Co., Ltd, Qingdao, China) at 37°C overnight without shaking. Then genomic DNA was extracted with the EZNA Genomic DNA Isolation Kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's protocols.

Multilocus Sequence Typing and Sequence Analysis

MLST was performed by PCR amplification and sequencing of the fragments of typically 7 housekeeping genes (*atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps*A) (Baldwin et al., 2009). Alleles and STs were assigned in accordance with the *Cronobacter* MLST database website (http://pubmlst.org/cronobacter/). The *fus*A allele sequence analysis was also performed with the aim to

TABLE 1 | Molecular identification and biofilm formation profiles of *Cronobacter* strains used in this study.

Origin	Strain	ID[a]	*fus*A allele	*fus*A sequencing	ST[b]	CC	Serotype[c]	Biofilm formation 595 nm	Biofilm formation category
SPICES									
White pepper	XZCRO001	1705	**148**	*C. dublinensis*	**498**		NF	0.141 ± 0.012	Non-biofilm producer
White pepper	XZCRO002	1706	8	*C. sakazakii*	**495**		Csak O1	0.142 ± 0.004	Non-biofilm producer
White pepper	XZCRO003	1707	18	*C. sakazakii*	136		Csak O2	0.158 ± 0.012	Weak
White pepper	XZCRO004	1708	36	*C. sakazakii*	224		Csak O7	0.141 ± 0.018	Non-biofilm producer
Red pepper powder	XZCRO005	1709	20	*C. dublinensis*	**522**		Cdub O1	0.145 ± 0.014	Non-biofilm producer
Prickly ash powder	XZCRO006	1710	18	*C. sakazakii*	**500**		Csak O7	0.143 ± 0.017	Non-biofilm producer
Prickly ash powder	XZCRO007	1711	**149**	*C. sakazakii*	**501**		NF	0.174 ± 0.020	Weak
Prickly ash powder	XZCRO008	1712	26	*C. turicensis*	**502**		Ctur O3	0.129 ± 0.007	Non-biofilm producer
Dried bay leaves	XZCRO009	1713	22	*C. turicensis*	72	72	Ctur O3	0.132 ± 0.003	Non-biofilm producer
Chinese cinnamon	XZCRO010	1714	22	*C. turicensis*	72	72	Ctur O3	0.134 ± 0.007	Non-biofilm producer
Aniseed powder	XZCRO011	1715	7	*C. malonaticus*	**504**		Cmal O2	0.171 ± 0.020	Weak
Prickly ash powder	XZCRO012	1716	68	*C. sakazakii*	143	143	Csak O3	0.173 ± 0.010	Weak
White pepper	XZCRO013	1717	**144**	*C. dublinensis*	**570**		NF	0.202 ± 0.021	Weak
Fennel	XZCRO014	1718	**146**	*C. universalis*	**512**		Cuni O1	0.734 ± 0.034	Strong
Red pepper powder	XZCRO015	1719	**147**	*C. turicensis*	**506**		NF	0.142 ± 0.017	Non-biofilm producer
Red pepper powder	XZCRO039	1743	67	*C. sakazakii*	148	16	Csak O1	0.139 ± 0.010	Non-biofilm producer
Cumin	XZCRO040	1744	67	*C. sakazakii*	148	16	Csak O1	0.137 ± 0.015	Non-biofilm producer
Black pepper	XZCRO041	1745	13	*C. malonaticus*	**511**		Cmal O1	0.135 ± 0.017	Non-biofilm producer
Prickly ash powder	XZCRO042	1746	17	*C. sakazakii*	158		Csak O1	0.143 ± 0.012	Non-biofilm producer
CEREALS									
Mung bean flour	XZCRO016	1720	40	*C. malonaticus*	371		NF	0.145 ± 0.016	Non-biofilm producer
Red bean flour	XZCRO017	1721	20	*C. dublinensis*	**524**		Cdub O1	0.177 ± 0.030	Weak
Maize flour	XZCRO018	1722	40	*C. malonaticus*	371		NF	0.135 ± 0.003	Non-biofilm producer
Soybean flour	XZCRO019	1723	17	*C. sakazakii*	158		Csak O1	0.127 ± 0.004	Non-biofilm producer
Buckwheat flour	XZCRO020	1724	12	*C. sakazakii*	17	17	Csak O2	0.155 ± 0.006	Weak
Proso millet	XZCRO021	1725	36	*C. sakazakii*	224		Csak O7	0.126 ± 0.015	Non-biofilm producer
Black soya bean	XZCRO022	1726	67	*C. sakazakii*	148	16	Csak O1	0.123 ± 0.011	Non-biofilm producer
Wheat flour	XZCRO023	1727	67	*C. sakazakii*	148	16	Csak O1	0.175 ± 0.018	Weak
Buckwheat flour	XZCRO024	1728	7	*C. malonaticus*	129	129	Cmal O2	0.172 ± 0.009	Weak
Mung bean flour	XZCRO025	1729	20	*C. dublinensis*	175		NF	0.140 ± 0.009	Non-biofilm producer
Mung bean flour	XZCRO026	1730	1	*C. sakazakii*	4	4	Csak O2	0.146 ± 0.005	Non-biofilm producer
Glutinous rice	XZCRO027	1731	1	*C. sakazakii*	**508**		Csak O2	0.147 ± 0.015	Non-biofilm producer
Oatmeal flour	XZCRO028	1732	12	*C. sakazakii*	17	17	Csak O2	0.129 ± 0.009	Non-biofilm producer
Black rice	XZCRO029	1733	20	*C. dublinensis*	176		Cdub O1	0.136 ± 0.010	Non-biofilm producer
Maize flour	XZCRO030	1734	8	*C. sakazakii*	50		Csak O1	0.129 ± 0.012	Non-biofilm producer
Wheat flour	XZCRO031	1735	18	*C. sakazakii*	136		Csak O2	0.154 ± 0.008	Weak
Barley flour	XZCRO032	1736	8	*C. sakazakii*	68		Csak O2	0.159 ± 0.005	Weak
Maize flour	XZCRO033	1737	1	*C. sakazakii*	**509**		Csak O2	0.152 ± 0.003	Weak
Oatmeal flour	XZCRO034	1738	**146**	*C. universalis*	**510**		Cuni O1	0.160 ± 0.005	Weak
Wheat flour	XZCRO035	1739	14	*C. sakazakii*	13	13	Csak O2	0.146 ± 0.017	Non-biofilm producer
Soybean flour	XZCRO043	1747	67	*C. sakazakii*	148	16	Csak O1	0.381 ± 0.012	Moderate
Negative control								0.125 ± 0.008	

[a] *ID, Strain identification code in the Cronobacter PubMLST database.* [b] *Newly determined alleles and STs are in bold type.* [c] *NF, Not found.*

identify and differentiate the isolates into species as previously described (Alsonosi et al., 2015; Brandão et al., 2017).

O-Antigen Serotype Analysis

The serotypes of *Cronobacter* isolates obtained from spices and cereals in the present study were determined using the PCR-based O-antigen serotyping technique as previously described (Sun et al., 2012a,b; Jarvis et al., 2013). Primers and PCR cycling conditions used for serotyping of *Cronobacter* strains are listed in **Table 2**.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of *Cronobacter* strains was investigated by the Kirby-Bauer disk diffusion method

TABLE 2 | Lists of primers and PCR cycling conditions used for serotyping of *Cronobacter* strains.

Serogroup	Target gene	Primer sequence	PCR cycling conditions	Amplicon size (bp)	References	No. of strains	
						spices	cereals
CsakO1	*wzy*	CCCGCTTGTATGGATGTT CTTTGGGAGCGTTAGGTT	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	364	Sun et al., 2012b	4	5
CsakO2	*wzy*	ATTGTTTGCGATGGTGAG AAAACAATCCAGCAGCAA	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	152	Sun et al., 2012b	1	8
CsakO3	*wzy*	CTCTGTTACTCTCCATAGTGTTC GATTAGACCACCATAGCCA	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	704	Sun et al., 2012b	0	1
CsakO4	*wzy*	ACTATGGTTTGGCTATACTCCT ATTCATATCCTGCGTGGC	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	890	Sun et al., 2012b	0	0
CsakO5	*wzy*	GATGATTTTGTAAGCGGTCT ACCTACTGGCATAGAGGATAA	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	235	Sun et al., 2012b	0	0
CsakO6	*wzy*	ATGGTGAAGGGAACGACT ATCCCCGTGCTATGAGAC	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	424	Sun et al., 2012b	0	0
CsakO7	*wzy*	CCCGCTTGTATGGATGTT CTTTGGGAGCGTTAGGTT	95°C, 5 min; (94°C, 30 s; 53°C, 30 s; 72°C, 1 min) x 30; 72°C, 7 min	364	Sun et al., 2012b	2	1
CmalO1	*wzx*	AGGGGCACGGCTTAGTTCTGG CCCGCTTGCCCTTCACCTAAC	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x 25; 72°C, 5 min	323	Jarvis et al., 2011	1	0
CmalO2	*wzx*	TGGCCCTTGTTAGCAAGACGTTTC ATCCACATGCCGTCCTTCATCTGT	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x 25; 72°C, 5 min	394	Jarvis et al., 2011	1	1
CdubO1	*wzx*	TCGTTTTGATGCTCTCGCTGCG ACAAATCGCGTGCTGGCTTGAA	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x 25; 72°C, 5 min	435	Jarvis et al., 2013	1	2
CdubO2	*wzx*	CTCGGTTCATGGATTTGCGGC CAGCGTGAAAACAGCCAGGT	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x 25; 72°C, 5 min	227	Jarvis et al., 2013	0	0
CturO1	*wzx*	AGGGGCACGGCTTAGTTCTGG CCCGCTTGCCCTTCACCTAAC	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x 25; 72°C, 5 min	323	Jarvis et al., 2013	0	0
CturO2	*wzy*	TTTCTTGTTATTGCCTGTGT AACAAAATCAGCGAGACTAA	95°C, 5 min; (94°C, 30 s; 50°C, 30 s; 72°C, 1 min) x 30; 72°C, 5 min	438	Sun et al., 2012a	0	0
CturO3	*wzx*	GCATCCCTTCAGAGTAGCGCA ACCACCTGCCATTGTCCTACTG	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x25, 72°C, 5 min	236	Jarvis et al., 2013	3	0
CuniO1	*wzx*	CATTCTCGCTTCCGCAGTTGC CCCAACCATCATTAGGGCCGAG	95°C, 2 min; (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) x25, 72°C, 5 min	145	Jarvis et al., 2013	1	1
Uncertain	–	–		–	–	4	3
Total						21	19

using Mueller-Hinton agar (Hangzhou Microbial Reagent Co., Ltd, Hangzhou, China) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). Thirteen antibiotics were tested: ampicillin (10 μg), ticarcillin-clavulanic acid (75:10 μg), cefixime (5 μg), amikacin (30 μg), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin

(5 μg), nitrofurantoin (300 μg), chloramphenicol (30 μg), meropenem (10 μg), aztreonam (30 μg), ceftriaxone (30 μg), trimethoprim (5 μg). All *Cronobacter* isolates and the two quality control strains (*Escherichia coli* ATCC 29522 and *Staphylococcus aureus* ATCC 29213) were grown in nutrient agar plates (Hangzhou Microbial Reagent Co., Ltd, Hangzhou, China) at 37°C overnight during antimicrobial susceptibility testing.

Biofilm Formation Assay

Microtiter plate assays (MPA) were performed to investigate the biofilm-forming ability of *Cronobacter* strains with minor modification, as previously described (Lee et al., 2012). Briefly, overnight cultures (1 ml) of *Cronobacter* strains ($n = 40$) were transferred to fresh TSB at 37°C for about 2 h in a shaking incubator. Subsequently, 200 μl of cell suspension ($OD_{600} \approx 0.3$) was transferred into sterile 96-well flat bottom polystyrene microplates (Corning Inc., Corning, NY, USA). The plates were incubated statically at 37°C for 48 h. Then the microtiter plates were gently washed three times with 250 μl of sterile distilled water and dried at room temperature. The biofilm was stained with 200 μl of 0.1% crystal violet solution for 30 min and washed three times with 250 μl sterile water. After drying, the crystal violet was liberated by 200 μl of 95% ethanol following 10 min incubation at room temperature. Finally, the sterile TSB was used as negative control and the optical density (OD) value of each well was measured at 595 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). All the experiments were performed three times.

The cutoff OD (ODc) was defined as three standard deviations (SD) above the mean OD of the negative controls. Based on the ODc, the *Cronobacter* isolates were classified into four categories: (1) non-biofilm producers: OD of test isolate $\leq$ ODc; (2) weak biofilm producers: ODc $<$ OD of test isolate $\leq$ (2 $\times$ ODc); (3) moderate biofilm producers: (2 $\times$ ODc) $<$ of test isolate $\leq$ (4 $\times$ ODc); (4) strong biofilm producers: OD of test isolate $>$ (4 $\times$ ODc).

Statistical Analysis

Fisher's exact test was used to compare serotypes, antimicrobial susceptibility rates, or biofilm-formation abilities between *Cronobacter* isolates from spices and cereals. Statistical analysis was performed using the SPSS version 17.0 software package (SPSS Inc, Chicago, IL, USA). A P-value of < 0.05 was considered statistically significant.

RESULTS

Species Identification

A total of 40 *Cronobacter* strains previously isolated from spices and cereal food samples were characterized by the *fus*A allele sequences analysis, and then all the allele sequences were submitted to the *Cronobacter* PubMLST database. A total of 21 *fus*A alleles (1, 7–8, 12–14, 17–18, 20, 22, 26, 36, 40, 67–68, 100, 144, and 146–149) were identified using the *Cronobacter* PubMLST database, four of which (146–149) were previously unreported (**Table 1**). Based on the *fus*A allele sequences analysis, a high diversity of *Cronobacter* species was observed, with five species of *Cronobacter* identified (**Tables 1**, **3**). The most frequently observed isolates were *C. sakazakii* ($n = 23$), followed by *C. dublinensis* ($n = 6$), *C. malonaticus* ($n = 5$), *C. turicensis* ($n = 4$), and *C. universalis* ($n = 2$). No strains of *C. muytjensii* or *C. condimenti* were identified. The phylogenetic tree based on the *fus*A allele sequences demonstrates a very clear clustering across the genus *Cronobacter* with the 40 strains in five out of the seven species (**Figure 1**), which is in agreement with the results obtained from *fus*A allele sequences analysis.

Multilocus Sequence Typing

A total of 30 different STs among the 40 isolates were found, 14 (ST495, ST498, ST500-ST502, ST505, ST506-ST512, and ST570) of which were novel to the *Cronobacter* PubMLST database (**Tables 1**, **3**). The most frequent STs in our study were ST148, identified five times, followed by ST17, ST72, ST136, ST158, ST224, ST371, and ST524 that included two isolates each, while the remaining 22 STs were identified only once. Of these frequent STs, the ST136, ST148, ST158, ST224, and ST524 were found in both spices and cereal samples; whereas ST17 and ST371 could only be found in cereal samples and ST72 found in spices samples. MLST assigned 12 isolates into 7 different CCs: CC4 ($n = 1$), CC13 ($n = 1$), CC16 ($n = 1$), CC17 ($n = 1$), CC72 ($n = 1$), CC129 ($n = 1$), and CC143 ($n = 1$), while the remaining 28 isolates were not assigned (**Table 1**).

Serotyping by PCR

Of the 40 *Cronobacter* isolates, 33 (82.5%) were clearly identified by PCR-based O-antigen serotyping methods, while seven (17.5%) isolates were undefined since O-antigen gene could not be amplified. O-antigen serotyping classified these strains into 9 serotypes: *C. sakazakii* serotype O1 ($n = 9$), *C. sakazakii* serotype

TABLE 3 | Summary of *fus*A alleles, MLST sequence types, and serotypes among different *Cronobacter* species.

Bacterial species	No. of strains	*fus*A alleles[a]	MLST sequence types[a]	Serotypes
C. sakazakii	23	1, 8, 12, 14, 17, 18, 36, 67, 68, **149**	4, 13, 17, 50, 68, 134, 136, 143, 148, 158, 224, **495**, **500, 501, 508, 509**	CsakO1, CsakO2, CsakO3, CsakO7
C. malonaticus	5	7, 13, 22, 40	129, 371, **504, 511,**	CmalO1, CmalO2
C. dublinensis	6	20, 100, 144, **148**	175, 176, **498, 524, 570**	CdubO1
C. turicensis	4	22, 26, **147**	72, **502, 506**	CturO3
C. universalis	2	**146**	**510, 512**	CuniO1

[a]*New alleles and new STs are indicated in bold character.*

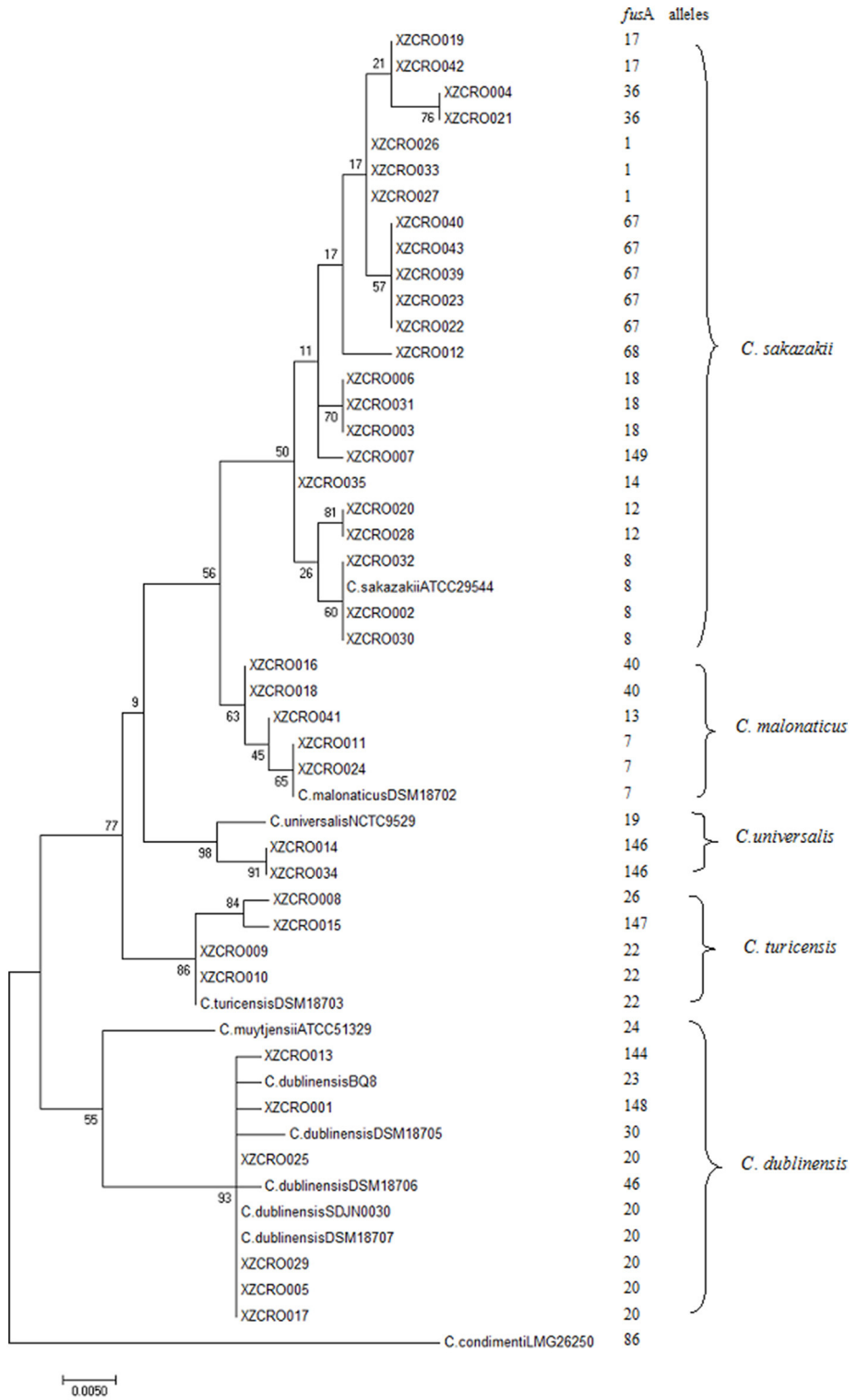


FIGURE 1 | Maximum likelihood tree based on the *fusA* alleles (438 bp) for the differentiation of *Cronobacter* species in this study. This tree is drawn to scale using the ClustalX (V.2.0) and the MEGA (v.7.02) with 1,000 bootstrap replicates.

O2 ($n = 9$), *C. sakazakii* serotype O3 ($n = 1$), *C. sakazakii* serotype O7 ($n = 3$), *C. dublinensis* O1 ($n = 3$), *C. malonaticus* O1 ($n = 1$), *C. malonaticus* O2 ($n = 2$), *C. turicensis* O3 ($n = 3$), and *C. universalis* O1 ($n = 2$) (**Tables 1**, **2**).

The serotype distribution of isolates from spices and cereals is shown in **Table 2.** A significant difference in the distribution of *Cronobacter* serotypes was observed between spices and cereals ($P < 0.05$). Analysis of the relationship between serotypes and MLST profiles revealed a connection between ST and serotype. For example, all strains genotyped as *C. sakazakii* ST158 were identified as *C. sakazakii* serotype O1, and *C. sakazakii* ST148 identified as *C. sakazakii* serotype O2. In contrast, isolates of the same serotype but different STs were found in this study. For example, isolates belonging to ST50, ST148, ST158, and ST495 were characterized as *C. sakazakii* serotype O1. Similarly, isolates belonging to ST4, ST13, ST17, ST68, ST136, and ST509 were characterized as *C. sakazakii* serotype O2.

Antimicrobial Susceptibility Testing

All of the 40 *Cronobacter* isolates were susceptible to 10 of the 13 antibiotic agents tested including ampicillin, cefixime, amikacin, gentamicin, tetracycline, ciprofloxacin, nitrofurantoin, chloramphenicol, trimethoprim, and ticarcillin-clavulanic acid. However, 70.0% (28/40) of the strains were resistant to ceftriaxone, among which 27.5% (11/40) of the strains were found in spices and 42.5% (17/40) of the strains were found in cereals. Besides ceftriaxone, 25.0% (10/40) of the strains were resistant to meropenem, eight (XZCRO006:ST500, XZCRO007:ST501, XZCRO011:ST504, XZCRO012:ST143, XZCRO013:ST570, XZCRO014:ST512, XZCRO015:ST506, and XZCRO042:ST158) of which were detected in spices, while the remaining 2 isolates (XZCRO019:ST158, and XZCRO020:ST17) in cereals. In addition, 2 isolates (XZCRO009:ST72, and XZCRO040:ST148) from spices and only 1 isolate (XZCRO027:ST508) from cereals were resistant to aztreonam (**Table 4**). No multidrug resistance (isolates resistant to three or more antimicrobial agents) strains were observed in both spices and cereals. Majority of *Cronobacter* isolates with the same ST showed a similar drug-resistance profile. However, isolates with the same ST sometimes showed different drug-resistance profile. For example, the 5 strains (XZCRO022, XZCRO023, XZCRO039, XZCRO040, and XZCRO043) of *Cronobacter* belonged to ST148, but only one strain was resistant to aztreonam (XZCRO040:ST148). When susceptibility results were compared according to their sources, there was no significant difference in the prevalence of antimicrobial resistance between isolates from spices and cereals for any of the agents tested ($P > 0.05$).

TABLE 4 | Antimicrobial susceptibility of the 40 *Cronobacter* strains recovered from spices and cereals by agar disc diffusion method.

Antibiotic	No. of resistant strains (%)		
	Spices ($n = 21$)	Cereals ($n = 19$)	Total ($n = 40$)
Ampicillin	0	0	0
Cefixime	0	0	0
Amikacin	0	0	0
Gentamicin	0	0	0
Tetracycline	0	0	0
Ciprofloxacin	0	0	0
Nitrofurantoin	0	0	0
Chloramphenicol	0	0	0
Trimethoprim	0	0	0
Ticarcillin-clavulanic acid	0	0	0
Aztreonam	2 (5.0)	1 (2.5)	3 (7.5)
Meropenem	8 (20.0)	2 (5.0)	10 (25.0)
Ceftriaxone	11 (27.5)	17 (42.5)	28 (70.0)

Biofilm-Formation Ability of *Cronobacter* spp.

The biofilm-formation ability among the 40 isolates was detected by the MPA, and the results were shown in **Table 1**. Overall, a wide variation was found among the *Cronobacter* strains in the quantity of biofilm produced. The results indicated that 15 (37.5%) of the 40 tested isolates, belonging to 12 of the 30 previously identified STs, were capable to produce biofilm on polystyrene microtiter plates (**Table 1**). Using the proposed cutoff criteria, a cutoff value of 0.149 at OD_{595} nm was used to categorize the test strains as non-biofilm, weak, moderate, and strong biofilm producers. According to the result of microtiter plate test, one isolate belonging to ST512 scored as the most efficient biofilm producer, one isolate belonging to ST148 as moderate biofilm producer, and the other 13 isolates as weak biofilm producers (**Table 1**). However, no correlation between biofilm formation and STs was observed. *Cronobacter* strains identified as the same ST sometimes showed different biofilm-formation ability. For example, 5 strains (XZCRO22, XZCRO23, XZCRO39, XZCRO040, and XZCRO043) of *Cronobacter* were identified as ST148 in our study, only 1 of which (XZCRO043) was categorized as moderate biofilm producer, and 2 (XZCRO39 and XZCRO040) as weak biofilm producer, whereas the other 2 isolates (XZCRO22 and XZCRO23) were categorized as non-biofilm producers. In addition, there was no significant difference ($p > 0.05$) in the amount of biofilm detected for *Cronobacter* spp. between spices and cereals.

DISCUSSION

Cronobacter spp. have been isolated from many kinds of foodstuffs including plant materials such as vegetables, flours, herbs, and spices (Huang et al., 2015; Brandão et al., 2017), however the prevalence of *Cronobacter* spp. in such foodstuffs varied greatly among different studies. In a study of the prevalence of *Cronobacter* spp., these bacteria were detected in 26.7% (12/45) of herbs and spices in India (Singh et al., 2015). In another study, the prevalence of *Cronobacter* spp. was particularly low in spices samples (3.6%, 1/28) and dry cereals (4.9%, 6/123) in Netherlands (Kandhai et al., 2010). *Cronobacter* spp. was detected in herbs and spices, cereal mixes for children in Brazil (Brandão et al., 2017), where its prevalence was 36.7% (11/30) and 23.3% (7/30), respectively. In our previous studies, the overall prevalence of *Cronobacter* spp. in spices and cereals

was determined to be 29.7% (19/64) (Li et al., 2017) and 21.0% (21/100) (Li Y. H. et al., 2016), respectively. However, in most of these studies, the MLST profiles of strains isolated from spices and cereals were not demonstrated. This study describes the genetic diversity, antimicrobial susceptibility, and biofilm formation of *Cronobacter* spp. recovered from spices and cereals in China during 2014–2015.

Based on the *fus*A sequence analysis, we found that the majority (57.5%) of *Cronobacter* isolates recovered from spices and cereals were *C. sakazakii*. The remaining strains were *C. dublinensis* (15.0%), *C. malonaticus* (12.5%), *C. turicensis* (20.0%), and *C. universalis* (5.0%). These findings are in agreement with previous studies which showed that *C. sakazakii* was the predominant *Cronobacter* species in different sources (Fei et al., 2015; Sulaiman et al., 2016; Brandão et al., 2017). Recent studies indicated that *C. sakazakii*, *C. malonaticus,* and *C. turicensis* were the three pathovars of *Cronobacter* spp. that associated with several neonatal infections and adult infections (Hunter and Bean, 2013; Ogrodzki and Forsythe, 2015). Unfortunately, these three pathovars of *Cronobacter* spp. were identified from spices and cereals in this study. These results underline the importance of sanitary-hygienic and epidemiological surveillance in spices and cereals to reduce the risk of *Cronobacter* infections.

The application of MLST analysis of *Cronobacter* isolates would be helpful to better understanding the genetic diversity, virulence, and epidemiology of genus *Cronobacter*. In this study, a total of 40 *Cronobacter* strains were genotyped with the 7-loci MLST scheme. MLST analysis revealed 16, 4, 5, 3, and 2 STs in *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. turicensis,* and *C. universalis*, respectively (**Table 3**). This finding was in agreement with previous studies reporting that the majority of STs were identified in *C. sakazakii* (Xu et al., 2015; Brandão et al., 2017). At the time of writing (August 2017), the *Cronobacter* PubMLST database contained 2097 isolates and consisted of 609 defined STs, with 225 clinical isolates belonging to 53 STs. The most frequent STs of clinical relevance in the *Cronobacter* PubMLST database were *C. sakazakii* ST4 (88/225), followed by *C. malonaticus* ST7 (30/225) and *C. sakazakii* ST8 (14/225). Among the 30 STs identified in our study, only 5 STs (ST4, ST13, ST50, ST129, and ST158) were of clinical origin, with 4 (ST4, ST13, ST50, and ST158) and 1 (ST129) ST(s) for *C. sakazakii*, and *C. malonaticus*, respectively. Among these 5 STs we identified, ST158, corresponding to *C. sakazakii*, was found in both spice (prickly ash powder) and cereal (soybean flour) samples, while ST4, ST13, ST50, and ST129 could only be found in cereal samples from mung bean flour, wheat flour, maize flour, and buckwheat flour, respectively. These findings underline that spices and cereals can also be potential sources of *Cronobacter* infections, which might pose great risks to human health.

Recent studies indicated a strong association between *C. sakazakii* CC4 (such as ST4, ST 15, ST97, and etc.) and neonatal infections as well as *C. malonaticus* CC7 (such as ST 7, ST 84, ST 159, and etc.) and adult infections (Joseph and Forsythe, 2011; Hariri et al., 2013; Forsythe et al., 2014). Moreover, a goeBURST analysis of 1007 *Cronobacter* isolates performed in 2014 indicated that 19.4% ($n = 195$) and 5.7% ($n = 58$) of strains in the *Cronobacter* PubMLST database were *C. sakazakii* CC4 and *C. malonaticus* CC7, with 45.1% (88/195) and 56.9% (33/58) strains obtained from clinical sources, respectively (Forsythe et al., 2014). These findings remark the importance of surveillance of *Cronobacter* belonging to *C. sakazakii* CC4 and *C. malonaticus* CC7, which are the dominant pathovars of *Cronobacter* associated with neonatal, pediatric and adult infections. However, these two CCs are not only found in powdered infant formula and related products but also in many other kinds of foodstuffs. For instance, in a study of the prevalence of *Cronobacter* contamination in 90 samples of retail foods in Brazil, two strains isolated from maize flour were characterized as *C. sakazakii* CC4 (Brandão et al., 2017). In another study, 4 *C. sakazakii* CC4 isolates were recovered from rice flour, noodle and potable water, and 10 *C. malonaticus* CC7 isolates from rice flour, dried shrimp, chocolate, cookie, and potable water (Cui et al., 2014). In our study, only one *C. sakazakii* CC4 isolate was obtained from cereals, and no strains of *C. malonaticus* CC7 were found in both cereals and spices.

For serotyping, a total of nine serotypes were found among the 40 isolates, including nine serotypes from spices and six from cereals. Among the nine serotypes found, *C. sakazakii* serotype O1 ($n = 9$) and O2 ($n = 9$) were the most two frequently observed serotypes, which was in accordance with previous studies (Alsonosi et al., 2015; Fei et al., 2015). Most *Cronobacter* isolates ($n = 33$) were clearly serotyped in this study, except for 3, 2, 1, and 1 isolate(s) in *C. dublinensis*, *C. malonaticus*, *C. sakazakii*, and *C. turicensis*, respectively. Previous studies also suggested that serotyping of *Cronobacter* strains were sometimes uncertain. For instance, 51 *Cronobacter* strains were isolated from hospitalized patients, one of which (identified as *C. muytjensii* ST28) could not be determined when the PCR serotyping scheme was carried out (Alsonosi et al., 2015). In another study, a total of 111 *Cronobacter* isolates from Chinese ready-to-eat foods were serotyped based on the O-antigen serotyping, two of which (one identified as *C. malonaticus* and the other as *C. dublinensis*) were uncertain (Xu et al., 2015). The appearance of unidentified serotypes may be due to the high genetic diversity of *Cronobacter* spp., which may result in a failure determination when the serotyping methods were performed in such studies. Recently, Ogrodzki and Forsythe established a new capsular typing scheme based on sequencing of *gnd* and *gal*E genes, which would be greatly helpful in distinguishing between *Cronobacter* species (Ogrodzki and Forsythe, 2015).

The increasing emergence of antibiotic resistant foodborne pathogens has been of great concern to public health in recent years. Results of the present study showed that frequency of antibiotic resistance in *Cronobacter* isolates recovered from spices and cereals was lower than strains of other foodborne pathogens such as *L. monocytogenes*, *C. jejuni,* and *Salmonella* spp. (Wang et al., 2013; Han et al., 2016; Komora et al., 2017). However, more attention should be paid to the inspection and control of strains of *Cronobacter* spp. because the resistance of these bacteria to many kinds of antimicrobial agents has been reported (Kilonzo-Nthenge et al., 2012; Li et al., 2014; Fei et al., 2017), even though the antimicrobial susceptibility profiles may

vary in different studies performed in various samples collected from different locations.

Antimicrobial susceptibility testing revealed that the 40 isolates were susceptible to most antibiotics tested, except for ceftriaxone, meropenem, and aztreonam. Cephalosporins, the commonly used antimicrobial agents worldwide, were sometimes categorized into "generations" by their antimicrobial properties. The results of the present study suggested that a high resistance (70%) of *Cronobacter* spp. particularly *C. sakazakii* to ceftriaxone (third generation), whereas all isolates were sensitive to cefixime (third generation). Compared to our study, a little lower incidence (65%) of resistance to ceftriaxone was reported by Zhang et al. (2013) in imported dairy products; in contrast, antimicrobial resistance was not observed in another study performed by Li Z. et al. (2016) in retail milk-based infant and baby foods. Besides ceftriaxone, resistance of *Cronobacter* spp. to other cephalosporins, including cefazolin (first generation), cephalothin (first generation), and cefoxitin (second generation), has been reported in Iraq (Mossawi and Joubori, 2015) and UK (Gosney, 2008). The different performance of antimicrobial resistance on *Cronobacter* spp. among various cephalosporins might be due to extensive use or misuse of these antimicrobial agents which increased drug resistance of these bacteria. In our study, a total of 10 (25%) *Cronobacter* isolates were resistant to meropenem; in contrast, all of the tested isolates from dairy products including powdered infant formula in China, Iraq, and Japan were susceptible to meropenem (Oonaka et al., 2010; Pan et al., 2014; Li Z. et al., 2016). Apart from isolates originating from food, several clinical isolates were found susceptible to meropenem in Taiwan (Tsai et al., 2013).

In contrast to previous studies whereas resistance of *Cronobacter* spp. to ampicillin has been reported (Oonaka et al., 2010; Li et al., 2014; Fei et al., 2017), ampicillin-resistant strains were not found in this study. Besides ampicillin, *Cronobacter* isolates showed 100% susceptibility to tetracycline, ciprofloxacin and chloramphenicol, whereas the other researchers reported a high resistance of *Cronobacter* spp. to these antibiotics (Kilonzo-Nthenge et al., 2012). In one study conducted in the USA, high resistance of *C. sakazakii* isolated from domestic kitchens to tetracycline (66.6% of isolates) and ciprofloxacin (57.1%) was observed. In another study in South Korea, Lee et al. (2012) reported that 3.4 and 1.8% of *Cronobacter* isolates recovered from various types of foods were resistant to chloramphenicol and tetracycline, respectively.

In the present study, 37.5% of the *Cronobacter* isolates from spices and cereals were able to form biofilm on polystyrene surfaces; however majority of these isolates (32.5%) were weak biofilm producers and less were moderate (2.5%) or strong (2.5%) biofilm producers. Similar results have been reported earlier in Mexico wherein 26% of *Cronobacter* spp. was capable of forming biofilms (Cruz et al., 2011). In contrast, a high proportion of biofilm-producing isolates of *Cronobacter* spp. recovered from various food in South Korea was observed (Lee et al., 2012). Differences in biofilm formation between various *Cronobacter* isolates could be due to strain variations that recovered from different sources and geographical locations. Moreover, the capacity of biofilm formation of *Cronobacter* strains is generally influenced by environmental conditions such as culture media and carbon source, and storage humidity levels (Jung et al., 2013).

CONCLUSION

In conclusion, the present study demonstrated a high genetic diversity of *Cronobacter* isolates recovered from spices and cereals, providing useful information on molecular epidemiology of *Cronobacter* infections. MLST analysis revealed that *C. sakazaki* was the most common species recovered from spices and cereals, followed by *C. dublinensis C. malonaticus*, *C. turicensis*, and *C. universalis*. The presence of isolates of clinical relevance including *C. sakazakii* ST4 (CC4) revealed that spices and cereals are likely to be the potential sources for human infection with *Cronobacter* spp. Although most *Cronobacter* strains were susceptible to the antimicrobial agents used in this study, further studies on the antimicrobial resistance of these foodborne pathogens are important to ensure effective treatment of human infections caused by *Cronobacter* spp.

AUTHOR CONTRIBUTIONS

YL and JS: Contributed to the conception of the study; YL and HY: Wrote the manuscript; YL and YZ: Analyzed and interpreted the data; YL, HJ, and YJ: Conducted the experiments; Each author substantially contributed to the work reported here.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31401595), the Scientific Research Foundation for Excellent Talents of Xuzhou Medical College (D2014002), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

REFERENCES

Alsonosi, A., Hariri, S., Kajsík, M., Oriešková, M., Hanulík, V., Röderová, M., et al. (2015). The speciation and genotyping of *Cronobacter* isolates from hospitalised patients. *Eur. J. Clin. Microbiol.* 34, 1979–1988. doi: 10.1007/s10096-015-2440-8

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., et al. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiol.* 9:223. doi: 10.1186/1471-2180-9-223

Brandão, M. L. L., Umeda, N. S., Jackson, E., Forsythe, S. J., and de Filippis, I. (2017). Isolation, molecular and phenotypic characterization, and antibiotic susceptibility of *Cronobacter* spp. from Brazilian retail foods. *Food Microbiol.* 63, 129–138. doi: 10.1016/j.fm.2016.11.011

Clinical Laboratory Standards Institute, (CLSI) (2012). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M100-S22*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cruz, A., Xicohtencatl-Jones, J., González-Pedrajo, B., Bobadilla, M., Eslava, C., and Rosas, I. (2011). Virulence traits in *Cronobacter* species isolated from different sources. *Can. J. Microbiol.* 57, 735–744. doi: 10.1139/w11-063

Cui, J. H., Du, X. L., Liu, H., Hu, G. C., Lv, G. P., Xu, B. H., et al. (2014). The genotypic characterization of *Cronobacter* spp. isolated in China. *PLoS ONE* 9:e102179. doi: 10.1371/journal.pone.0102179

Drudy, D., O'Rourke, M., Murphy, M., Mullane, N. R., O'Mahony, R., Kelly, L., et al. (2006). Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int. J. Food Microbiol.* 110, 127–134. doi: 10.1016/j.ijfoodmicro.2006.02.008

Fei, P., Jiang, Y. J., Feng, J., Forsythe, S. J., Li, R., Zhou, Y. H., et al. (2017). Antibiotic and desiccation resistance of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and processing environments. *Front. Microbiol.* 8:316. doi: 10.3389/fmicb.2017.00316

Fei, P., Man, C., Lou, B., Forsythe, S. J., Chai, Y. L., Li, R., et al. (2015). Genotyping and source tracking of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and an infant formula production factory in China. *Appl. Environ. Microbiol.* 81, 5430–5439. doi: 10.1128/AEM.01390-15

Forsythe, S. J., Dickins, B., and Jolley, K. A. (2014). Cronobacter, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. *BMC Genomics* 15:1121. doi: 10.1186/1471-2164-15-1121

Friedemann, M. (2009). Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *Eur. J. Clin. Microbiol.* 28, 1297–1304. doi: 10.1007/s10096-009-0779-4

Gosney, M. (2008). *Enterobacter sakazakii* bacteraemia with multiple splenic abscesses in a 75-year-old woman: a case report. *Age Ageing* 37, 236–238. doi: 10.1093/ageing/afn006

Han, X. F., Zhu, D. M., Lai, H. M., Zeng, H., Zhou, K., Zou, L. K., et al. (2016). Prevalence, antimicrobial resistance profiling and genetic diversity of *Campylobacter jejuni* and *Campylobacter coli* isolated from broilers at slaughter in China. *Food Control* 69, 160–170. doi: 10.1016/j.foodcont.2016.04.051

Hariri, S., Joseph, S., and Forsythe, S. J. (2013). *Cronobacter sakazakii* ST4 strains and neonatal meningitis, United States. *Emerg. Infect. Dis.* 19, 175–177. doi: 10.3201/eid1901.120649

Holý, O., PetrŽelová, J., Hanulík, V., Chromá, M., Matoušková, I., and Forsythe, S. J. (2014). Epidemiology of *Cronobacter* spp. isolates from patients admitted to the Olomouc University Hospital (Czech Republic). *Epidemiol. Mikrobiol. Imunol.* 63, 69–72.

Huang, Y., Pang, Y. H., Wang, H., Tang, Z. Z., Zhou, Y., Zhang, W. Y., et al. (2015). Occurrence and characterization of *Cronobacter* spp. in dehydrated rice powder from Chinese supermarket. *PLoS ONE* 10:e0131053. doi: 10.1371/journal.pone.0131053

Hunter, C. J., and Bean, J. F. (2013). *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *J. Perinatol.* 33, 581–585. doi: 10.1038/jp.2013.26

Iversen, C., Mullane, N., Mccardell, B., Tall, B. D., Lehner, A., Fanning, S., et al. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* 58, 1442–1447. doi: 10.1099/ijs.0.65577-0

Jarvis, K. G., Grim, C. J., Franco, A. A., Gopinath, G., Sathyamoorthy, V., Hu, L., et al. (2011). Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. *Appl. Environ. Microbiol.* 77, 4017–4026. doi: 10.1128/AEM.00162-11

Jarvis, K. G., Yan, Q. Q., Grim, C. J., Power, K. A., Franco, A. A., Hu, L., et al. (2013). Identification and characterization of five new molecular serogroups of *Cronobacter* spp. *Foodborne Pathog. Dis.* 10, 343–352. doi: 10.1089/fpd.2012.1344

Jo, S. H., Baek, S. B., Ha, J. H., and Ha, S. D. (2010). Maturation and survival of *Cronobacter* biofilms on silicone, polycarbonate, and stainless steel after UV light and ethanol immersion treatments. *J. Food. Prot.* 73, 952–956. doi: 10.4315/0362-028X-73.5.952

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., and Forsythe, S. J. (2012). *Cronobacter condimenti* sp nov., isolated from spiced meat, and *Cronobacter universalis* sp nov., a species designation for *Cronobacter* sp genomospecies 1, recovered from a leg infection, water and food ingredients. *Int. J. Syst. Evol. Micriobiol.* 62, 1277–1283. doi: 10.1099/ijs.0.032292-0

Joseph, S., and Forsythe, S. J. (2011). Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerg. Infect. Dis.* 17, 1713–1715. doi: 10.3201/eid1709.110260

Jung, J. H., Choi, N. Y., and Lee, S. Y. (2013). Biofilm formation and exopolysaccharide (EPS) production by *Cronobacter sakazakii* depending on environmental conditions. *Food Microbiol.* 34, 70–80. doi: 10.1016/j.fm.2012.11.008

Kandhai, M. C., Heuvelink, A. E., Reij, M. W., Beumer, R. R., Dijk, R., van Tilburg, J. J. H. C., et al. (2010). A study into the occurrence of *Cronobacter* spp. in the Netherlands between 2001 and 2005. *Food Control* 21, 1127–1136. doi: 10.1016/j.foodcont.2010.01.007

Kilonzo-Nthenge, A., Rotich, E., Godwin, S., Nahashon, S., and Chen, F. (2012). Prevalence and antimicrobial resistance of *Cronobacter sakazakii* isolated from domestic kitchens in middle Tennessee, United States. *J. Food Prot.* 75, 1512–1517. doi: 10.4315/0362-028X.JFP-11-442

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., and Teixeira, P. (2017). Survival of *Listeria monocytogenes* with different antibiotic resistance patterns to food-associated stresses. *Int. J. Food Microbiol.* 245, 79–87. doi: 10.1016/j.ijfoodmicro.2017.01.013

Lee, Y. D., Park, J. H., and Chang, H. (2012). Detection, antibiotic susceptibility and biofilm formation of *Cronobacter* spp. from various foods in Korea. *Food Control* 24, 225–230. doi: 10.1016/j.foodcont.2011.09.023

Li, Y. H., Chen, Q. M., Zhao, J. F., Jiang, H., Lu, F. X., Bie, X. M., et al. (2014). Isolation, identification and antimicrobial resistance of *Cronobacter* spp. isolated from various foods in China. *Food Control* 37, 109–114. doi: 10.1016/j.foodcont.2013.09.017

Li, Y. H., Jiang, H., Jiao, Y., Xu, W., Zou, X. Q., and Pei, S. F. (2017). Isolation and identification of *Cronobacter* spp. from dried spices and condiments. *Sci. Technol. Food Ind.* 38, 125–130. doi: 10.13386/j.issn1002-0306.2017.19.024

Li, Y. H., Zhang, Y. F., Zhang, Q. C., Xu, W., Zou, X. Q., Pei, S. F., et al. (2016). Isolation and identification of *Cronobacter* spp. from cereal foods. *Sci. Technol. Food Ind.* 37, 154–158, 164. doi: 10.13386/j.issn1002-0306.2016.15.022

Li, Z., Ge, W., Li, K., Gan, J., Zhang, Y., Zhang, Q., et al. (2016). Prevalence and characterization of *Cronobacter sakazakii* in retail milk-based infant and baby foods in Shaanxi, China. *Foodborne Pathog. Dis.* 13, 221–227. doi: 10.1089/fpd.2015.2074

Lou, X. Q., Si, G. J., Yu, H., Qi, J. J., Liu, T., and Fang, Z. G. (2014). Possible reservoir and routes of transmission of *Cronobacter* (*Enterobacter sakazakii*) via wheat flour. *Food Control* 43, 258–262. doi: 10.1016/j.foodcont.2014.03.029

Mossawi, M. A., and Joubori, Y. H. (2015). Detection of *Cronobacter sakazakii* (*Enterobacter sakazakii*) in powdered food infants (PIF) and raw milk in Iraq. *Baghdad Sci. J.* 12, 63–71.

Ogrodzki, P., and Forsythe, S. (2015). Capsular profiling of the *Cronobacter* genus and the association of specific *Cronobacter sakazakii* and *C. malonaticus* capsule types with neonatal meningitis and necrotizing enterocolitis. *BMC Genomics* 16:758. doi: 10.1186/s12864-015-1960-z

Oonaka, K., Furuhata, K., Hara, M., and Fukuyama, M. (2010). Powder infant formula milk contaminated with *Enterobacter sakazakii*. *Jpn. J. Infect. Dis.* 63, 103–107.

Pan, Z., Cui, J., Lyu, G., Du, X., Qin, L., Guo, Y., et al. (2014). Isolation and molecular typing of *Cronobacter* spp. in commercial powdered infant formula and follow-up formula. *Foodborne Pathog. Dis.* 11, 456–461. doi: 10.1089/fpd.2013.1691

Park, S. H., and Kang, D. H. (2014). Fate of biofilm cells of *Cronobacter sakazakii* under modified atmosphere conditions. *LWT Food Sci. Technol.* 57, 782–784. doi: 10.1016/j.lwt.2014.01.038

Patrick, M. E., Mahon, B. E., Greene, S. A., Rounds, J., Cronquist, A., Wymore, K., et al. (2014). Incidence of *Cronobacter* spp. infections, United States, 2003-2009. *Emerg. Infect. Dis.* 20, 1520–1523. doi: 10.3201/eid2009.140545

Reich, F., König, R., von Wiese, W., and Klein, G. (2010). Prevalence of *Cronobacter* spp. in a powdered infant formula processing environment. *Int. J. Food Microbiol.* 140, 214–217. doi: 10.1016/j.ijfoodmicro.2010.03.031

Sani, N. A., and Odeyemi, O. A. (2015). Occurrence and prevalence of *Cronobacter* spp. in plant and animal derived food sources: a systematic review and meta-analysis. *Springerplus* 4:545. doi: 10.1186/s40064-015-1324-9

Simoes, M., Simoes, L. C., and Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT Food Sci. Technol.* 43, 573–583. doi: 10.1016/j.lwt.2009.12.008

Singh, N., Goel, G., and Raghav, M. (2015). Prevalence and characterization of *Cronobacter* spp. from various foods, medicinal plants, and environmental samples. *Curr. Microbiol.* 71, 31–38. doi: 10.1007/s00284-015-0816-8

Song, X., Shukla, S., Lee, G., Park, S., and Kim, M. (2016). Detection of *Cronobacter* genus in powdered infant formula by enzyme-linked immunosorbent assayusing anti-*Cronobacter* antibody. *Front. Microbiol.* 7:1124. doi: 10.3389/fmicb.2016.01124

Sulaiman, I. M., Jacobs, E., Segars, K., Simpson, S., and Kerdahi, K. (2016). Genetic characterization of *Cronobacter sakazakii* recovered from the environmental surveillance samples during a sporadic case investigation of foodborne illness. *Curr. Microbiol.* 73, 273–279. doi: 10.1007/s00284-016-1059-z

Sun, Y., Arbatsky, N. P., Wang, M., Shashkov, A. S., Liu, B., Wang, L., et al. (2012a). Structure and genetics of the O-antigen of *Cronobacter turicensis* G3882 from a new serotype, *C. turicensis* O2, and identification of a serotype-specific gene. *FEMS Immunol. Med. Microbiol.* 66, 323–333. doi: 10.1111/j.1574-695X.2012.01013.x

Sun, Y., Wang, M., Liu, H., Wang, J., He, X., Zeng, J., et al. (2011). Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* 77, 2209–2214. doi: 10.1128/AEM.02229-10

Sun, Y., Wang, M., Wang, Q., Cao, B., He, X., Li, K., et al. (2012b). Genetic analysis of the *Cronobacter sakazakii* O4 to O7 O-antigen gene clusters and development of a PCR assay for identification of all *C. sakazakii* O serotypes. *Appl. Environ. Microbiol.* 78, 3966–3974. doi: 10.1128/AEM.07825-11

Tsai, H. Y., Liao, C. H., Huang, Y. T., Lee, P. I., and Hsueh, P. R. (2013). *Cronobacter* infections not from infant formula, Taiwan. *Emerg. Infect. Dis.* 19, 167–169. doi: 10.3201/eid1901.120774

Turcovský, I., Kuniková, K., Drahovská, H., and Kaclíková, E. (2011). Biochemical and molecular characterization of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) isolated from foods. *Antonie Van Leeuwenhoek* 99, 257–269. doi: 10.1007/s10482-010-9484-7

Vlach, J., Javurková, B., Karamonová, L., BlaŽková, M., and Fukal, L. (2017). Novel PCR-RFLP system based on rpoB gene for differentiation of *Cronobacter* species. *Food Microbiol.* 62, 1–8. doi: 10.1016/j.fm.2016.08.004

Wang, H. H., Ye, K. P., Wei, X. R., Cao, J. X., Xu, X. L., and Zhou, G. H. (2013). Occurrence, antimicrobial resistance and biofilm formation of *Salmonella* isolates from a chicken slaughter plant in China. *Food Control* 33, 378–384. doi: 10.1016/j.foodcont.2013.03.030

Xu, X., Li, C., Wu, Q., Zhang, J., Huang, J., and Yang, G. (2015). Prevalence, molecular characterization, and antibiotic susceptibility of *Cronobacter* spp. in Chinese ready-to-eat foods. *Int. J. Food Microbiol.* 204, 17–23. doi: 10.1016/j.ijfoodmicro.2015.03.003

Zhang, X. M., Zeng, J., Wei, H. Y., Fu, P. B., and Han, X. (2013). Study on antibiotic resistance of *Cronobacter sakazakii* isolated from imported dairy product. *Chin. J. Food Hyg.* 25, 320–323. doi: 10.13590/j.cjfh.2013.04.027

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Li, Yu, Jiang, Jiao, Zhang and Shao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 January 2018
doi: 10.3389/fmicb.2017.02697

Prevalence, Virulence Genes and Antimicrobial Resistance Profiles of *Salmonella* Serovars from Retail Beef in Selangor, Malaysia

Tze Y. Thung[1], Son Radu[1,2]*, Nor A. Mahyudin[1], Yaya Rukayadi[1], Zunita Zakaria[3], Nurzafirah Mazlan[4], Boon H. Tan[5], Epeng Lee[1,2], Soo L. Yeoh[1,2], Yih Z. Chin[1], Chia W. Tan[1], Chee H. Kuan[6], Dayang F. Basri[7] and Che W. J. Wan Mohamed Radzi[8]*

[1] Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Malaysia, [2] Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security (ITAFoS), Universiti Putra Malaysia, Serdang, Malaysia, [3] Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Malaysia, [4] Department of Diagnostic and Allied Science, Faculty of Health and Life Science, Management and Science University, Shah Alam, Malaysia, [5] Division of Applied Biomedical Sciences and Biotechnology, School of Health Sciences, International Medical University, Kuala Lumpur, Malaysia, [6] Department of Agricultural and Food Science, Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, Malaysia, [7] Novel Antibiotic Laboratory, School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, [8] Department of Science and Technology Studies, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Milan Zivko Baltić, Faculty of Veterinary Medicine, University of Belgrade, Serbia
Giorgia Perpetuini, Università di Teramo, Italy

***Correspondence:**
Tze Y. Thung
upmtty@yahoo.com
Son Radu
son@upm.edu.my

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *22 July 2017*
Accepted: *26 December 2017*
Published: *11 January 2018*

Citation:
Thung TY, Radu S, Mahyudin NA, Rukayadi Y, Zakaria Z, Mazlan N, Tan BH, Lee E, Yeoh SL, Chin YZ, Tan CW, Kuan CH, Basri DF and Wan Mohamed Radzi CWJ (2018) Prevalence, Virulence Genes and Antimicrobial Resistance Profiles of Salmonella Serovars from Retail Beef in Selangor, Malaysia. Front. Microbiol. 8:2697. doi: 10.3389/fmicb.2017.02697

The aim of the present study was to investigate the prevalence of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium in retail beef from different retail markets of Selangor area, as well as, to assess their pathogenic potential and antimicrobial resistance. A total of 240 retail beef meat samples (chuck = 60; rib = 60; round = 60; sirloin = 60) were randomly collected. The multiplex polymerase chain reaction (mPCR) in combination with the most probable number (MPN) method was employed to detect *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in the meat samples. The prevalence of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in 240 beef meat samples were 7.50, 1.25, and 0.83%, respectively. The microbial loads of total *Salmonella* was found in the range of <3 to 15 MPN/g. Eight different serovars of *Salmonella* were identified among the 23 isolates, and *S.* Agona was the predominant serovar (26.09%). Interestingly, all the *Salmonella* isolates were resistant to penicillin, erythromycin and vancomycin, but the sensitivity was observed for tetracycline, gentamicin and amoxicillin/clavulanic acid. All 23 isolates were resistant to at least three antibiotics. Two *S.* Typhimurium isolates (8.70%) exhibited the highest multiple antibiotic resistance (MAR) index value of 0.56 which shown resistance to nine antibiotics. PCR analysis of virulence genes showed that all *Salmonella* isolates (100%) were positive for the *invA* gene. Meanwhile, *pefA* was only identified in *S.* Enteritidis and *S.* Typhimurium. The findings in this study indicate that retail beef products tested were widely contaminated with multi-drug resistant (MDR) *Salmonella* and various virulence genes are present among the isolated *Salmonella* serovars.

Keywords: beef meat, *Salmonella*, multiplex PCR, prevalence, antimicrobial resistance, virulence gene

INTRODUCTION

The intestinal epithelium infection known as salmonellosis is caused by the genus *Salmonella*. The major pathogenic serovars of *Salmonella enterica* that infect humans from a variety of different food products include *Salmonella* Enteritidis and *Salmonella* Typhimurium (Kramarenko et al., 2014; Yang et al., 2016; Ed-dra et al., 2017). In the USA over 40,000 salmonellosis cases are reported each year and foods of animal origin are considered to be the most likely source of *Salmonella* (Finstad et al., 2012). It is well known that human *Salmonella* infections are associated with many different kinds of food, including beef meat and beef meat products (Brichta-Harhay et al., 2008; Sallam et al., 2014). Hence, the presence of *Salmonella* in beef at the slaughter level and at the market is a significant food safety risk.

Sensitive and specific methods with shorter turnaround time for the detection and identification of *Salmonella* are needed to reduce testing-related laboratory costs. Therefore, multiplex polymerase chain reaction (mPCR) uses few pairs of primers simultaneously detecting different pathogens in the same samples has the potential as a reliable and effective method (Pui et al., 2011). Previously, a detection method consist of mPCR and the most probable number (MPN) method has successfully been performed to detect and identify *S.* Enteritidis and *S.* Typhimurium in chicken meat (Thung et al., 2016). Indeed, the mPCR-MPN method has widely been used to detect and enumerate food-borne pathogens such as *Campylobacter* spp. (Chai et al., 2007), *Listeria monocytogenes* (Kuan et al., 2013) and *Vibrio parahaemolyticus* (Tan et al., 2017). Recently, gold nanoparticle-aptamer-based localized surface plasmon resonance (LSPR) sensing chip was developed to enable the ultra-sensitive and selective detection of *S.* Typhimurium in pork meat (Oh et al., 2017).

In general, bacterial virulence factors have a crucial role for systemic infections. The pathogenicity of *Salmonella* strains has been related to numerous virulence genes present in the chromosomal *Salmonella* pathogenicity islands (SPIs) (Nayak et al., 2004). Genes such as *invA* and *hilA*, found in SPI, allow *Salmonella* to invade epithelial cells (Cardona-Castro et al., 2002; Nayak et al., 2004). Besides, *Salmonella* outer proteins (sops) (SPI effector protein) encoded by *sop* gene have relevance to *Salmonella* virulence (Huehn et al., 2010). Meanwhile, the plasmid encoded fimbriae (*pefA*) gene contributes to the adhesion of *Salmonella* to epithelial cells (Murugkar et al., 2003). Other chromosomal gene like *stn*, codes for enterotoxin production has been shown to be a causative agent of diarrhea (Huehn et al., 2010). In addition, virulence plasmids carrying virulence genes such as the *spv* operon (*Salmonella* plasmid virulence) contribute to the colonization of deeper tissues among other functions (Swamy et al., 1996).

To date, the emergence and spread of antimicrobial resistance among zoonotic *Salmonella* has become a public health threat (Sallam et al., 2014). Importantly, *Salmonella* strains having "clinically important resistance" to some agents like extended-spectrum cephalosporins and fluoroquinolones, have been isolated from livestock (Li et al., 2013). In most developing countries, misuse and overuse of antibiotics has contributed to the increasing trend of multi-resistance in *Salmonella* (Ed-dra et al., 2017). In Selangor (center of Peninsular Malaysia), although some reports were found based on the prevalence of *Salmonella* in different types of foods, but limited information on the surveillance study of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in beef meat at retail level are available. Therefore, the aim of this study was to assess the prevalence, virulence genes and antimicrobial resistance of *Salmonella* serovars isolated from retail beef meat in Selangor area.

MATERIALS AND METHODS

Collection of Meat Sample

Four different parts of beef meat samples (chuck = 60; rib = 60; round = 60; sirloin = 60) with a total number of 240 were collected from retail markets (wet markets and hypermarkets) in Selangor area over 9 months period from September 2014 to May 2015 (approximately 25 to 27 samples per month). The retail meat samples were kept in sterile stomacher bags and transferred to the laboratory for further analysis.

Enrichment and Most Probable Number (MPN) Method

Ten gram of meat sample was homogenized for 50 s using a stomacher after added in 90 mL of sterile buffered peptone water (BPW) (Merck, Darmstadt, Germany). Then, the suspension was diluted up to 1,000-fold with 10-fold serial. Later, MPN method (three-tube) was performed by transferring 1 mL of each dilution into three replicate tubes with 10 mL of Rappaport-Vasiliadis (RV) broth (Merck, Darmstadt, Germany) each, and followed by overnight incubation at 37°C under aerobic conditions. The turbid MPN tubes were selected for subsequent DNA extraction using the boiled-cell method as described previously (Chai et al., 2007).

Multiplex PCR Conditions

For mPCR detection, three primer pairs were used to identify randomly selected-sequence of unknown function gene (429 bp) for *Salmonella* spp., *sdfI* gene (304 bp) for *S.* Enteritidis, and *fliC* gene (620 bp) for *S.* Typhimurium. The sequences of the primer pair used for targeting random sequence were 5′-GCCAACCATTGCTAAATTGGCGCA-3′ and 5′-GGTAGAAATTCCCAGCGGGTACTGG-3′ (Soumet et al., 1999), whereas the primer pair used for targeting the *sdfI* gene were 5′-TGTGTTTTATCTGATGCAAGAGG-3′ and 5′-TGAACTACGTTCGTTCTTCTGG-3′ (Alvarez et al., 2004), followed by the primer pair used for targeting the *fliC* gene were 5′-CGGTGTTGCCCAGGTTGGTAAT-3′ and 5′-ACTGGTAAAGATGGCT-3′ (Soumet et al., 1999). We optimized the mPCR reaction conditions by a series of preliminary experiments so that the three independent PCR reactions can be performed in the same tube with the detection limit of 10^5 cfu/mL (data not shown). The optimized mPCR reaction mixture (25 μL) contained 2 μL of DNA template, 5 μL of 5× PCR buffer, 2.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM deoxynucleotide triphosphate (dNTP), 0.5 μL of 1.2 μM primer mix and 14.2 μL of deionized water. The mixture was

then treated with 0.3 μL (1.5 U) *Taq* DNA polymerase. PCR amplification was performed in triplicate with the following conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The positive controls used were *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076. *Escherichia coli* ATCC 25922 was used as a negative control.

Isolation and Identification of *Salmonella*

The turbid MPN tubes were confirmed to be *Salmonella* by plating on selective CHROMagar *Salmonella* (CHROMagar Microbiology, Paris, France) and Xylose Lysine Deoxycholate (XLD) (Merck, Darmstadt, Germany) agar plates, and incubated at 37°C for 24 h. All the *Salmonella* isolates were then serotyped by slide agglutination using polyvalent "O" and "H" antisera (BD, Franklin Lakes, USA) at Veterinary Research Institute (VRI), Ipoh, Malaysia in accordance with the Kauffmann-White scheme.

Antimicrobial Resistance Profiles

The antimicrobial susceptibility was evaluated according to Clinical and Laboratory Standarts Institude (2012) by using disc diffusion method. Briefly, isolates were cultured aerobically in 10 mL Mueller-Hinton (MH) broth (Merck, Darmstadt, Germany) at 37°C for 24 h. Overnight cultures, grown on MH broth (OD adjusted to 0.5 MacFarland unit), were swabbed evenly with sterile non-toxic cotton swab on MH agar plates and left to dry for 2 to 4 min. Then, antimicrobial sensitivity discs were placed on the culture by using a disk dispenser and incubated at 37°C for 24 h. The tested antimicrobials were amoxicillin/clavulanic acid (AMC, 30 μg), amoxycillin (AML, 30 μg), ceftazidime (CAZ, 30 μg), cephazolin (KZ, 30 μg), ciprofloxacin (CIP, 5 μg), erythromycin (E, 15 μg), chloramphenicol (C, 30 μg), ampicillin (AMP, 10 μg), penicillin (P, 10 μg), streptomycin (S, 10 μg), tetracycline (TE, 30 μg), kanamycin (K, 30 μg), gentamicin (CN, 10 μg), vancomycin (VA, 30 μg), nalidixic acid (NA, 30 μg), and suphamethoxazole/trimethoprim (SXT, 25 μg) (Oxoid, Hamphire, United Kingdom). The multiple antibiotic resistance (MAR) index was calculated as "a/b," where "a" the number of antibiotics for a particular isolate was resistant and "b" the total number of antibiotics tested (Krumperman, 1983).

Detection of Virulence Genes

All *Salmonella* isolates collected in this study were screened for the presence of virulence genes using PCR. The primers, the size in base pairs of the respective amplification products and the references used for detection of six virulence genes are presented in **Table 1**. The virulence genes under study were *invA*, *pefA*, *hilA*, *sopB*, *stn,* and *spvC*. Positive (*S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076) and negative control (*E. coli* ATCC 25922) were conducted in the detection procedure. To evaluate the reproducibility of the experiments, PCR amplification and electrophoresis experiments were carried out in triplicate.

Statistical Analysis

All measurements were carried out in triplicate. Minitab (v. 14) statistical package (Minitab Inc., State College, PA) was used to determine if there was any significant difference between the prevalence of *Salmonella* in beef meat from wet market and hypermarket. For all analysis, $P < 0.05$ was considered significant.

RESULTS

Prevalence of *Salmonella* in Beef

The target genes specific to *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium produced amplicons at 429, 304, and 620 bp, respectively. **Figure 1** shows the result of gel electrophoresis comparing various combinations of the PCR primer sets and verifying the mPCR established for the current study consists of three independent and specific PCR reactions. Of the 240 retail beef meat samples tested, the contamination rates were 7.50% ($n = 18$), 1.25% ($n = 3$), and 0.83% ($n = 2$) for *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium, respectively (**Table 2**). Beef part round was the major reservoir for *Salmonella*, with prevalence rate of 16.67% ($n = 60$). The prevalence of *Salmonella* spp. in wet markets (10.00%) were significantly higher than hypermarkets (5.00%) ($P < 0.05$). As shown in **Table 3**, the highest microbial loads of *Salmonella* was found in *Salmonella* spp. (15.0 MPN/g), followed by *S.* Enteritidis (3.6 MPN/g) and *S.* Typhimurium (3.6 MPN/g).

Antimicrobial Resistance Profiles

Antibiotic sensitivity testing was performed for the 23 isolated *Salmonella* strains, which included *S.* Enteritidis ($n = 3$), *S.* Typhimurium ($n = 2$), *S.* Agona ($n = 6$), *S.* Anatum ($n = 3$), *S.* London ($n = 3$), *S.* Newport ($n = 3$), *S.* Stanley ($n = 1$), and *S.* Weltevreden ($n = 2$). As shown in **Table 4**, three antibiotics gentamicin, amoxicillin/clavulanic acid, and tetracycline were effective (100%) to all isolates. In this study, resistance to erythromycin, penicillin and vancomycin were seen in 100% of *S.* Enteritidis, *S.* Typhimurium, *S.* Agona, *S.* Anatum, *S.* London, *S.* Newport, *S.* Stanley and *S.* Weltevreden isolates. As shown in **Table 5**, the highest MAR index value of 0.56 was found in two *S.* Typhimurium isolates. Observation from the presence study indicated that all *Salmonella* isolates were multi-drug resistant (MDR) strains, which showed resistance to three antibiotics (penicillin, vancomycin and erythromycin) or more. Nine *Salmonella* isolates (39.13%) mainly from *S.* Agona and *S.* Newport were resistant to four antibiotics, and three *S.* Enteritidis (13.04%) were resistant to at least five of the antibiotics.

Distribution of Virulence Genes among *Salmonella* Isolates

All 23 *Salmonella* isolates were tested by PCR for the presence of virulence genes. The invasion gene operon *invA* was detected in all *Salmonella* isolates (**Table 6**). Regarding the different frequencies of *hilA*, *sopB*, and *stn* genes among different serovars, a clear difference was noticed in the occurrence of these genes among the isolates; *S.* London and *S.* Stanley did not show the

TABLE 1 | PCR primers used for amplification of virulence genes in *Salmonella* isolates.

Virulence genes	Primer sequence (5′-3′)	Size (bp)	Reference
invA	F- TATCGCCACGTTCGGCAA	275	Nayak et al., 2004
pefA	R- TCGCACCGTCAAAGGAACC	700	Murugkar et al., 2003
hilA	F- TGTTTCCGGGCTTGTGCT	854	Cardona-Castro et al., 2002
sopB	R- CAGGGCATTTGCTGATTCTTCC	517	Huehn et al., 2010
stn	F- CGGAAGCTTATTTGCGCCATGCTGAGGTAG	617	Murugkar et al., 2003
spvC	R- GCATGGATCCCCGCCGGCGAGAT TGTG	669	Swamy et al., 1996
	F- TCAGAAGRCGTCTAACCACTC		
	R- TACCGTCCTCATGCACACTC		
	F- TTGTGTCGCTATCACTGGCAACC		
	R- ATTCGTAACCCGCTCTCGTCC		
	F- CGGAAATACCATCAAATA		
	R- CCCAAACCCATACTTACTCTG		

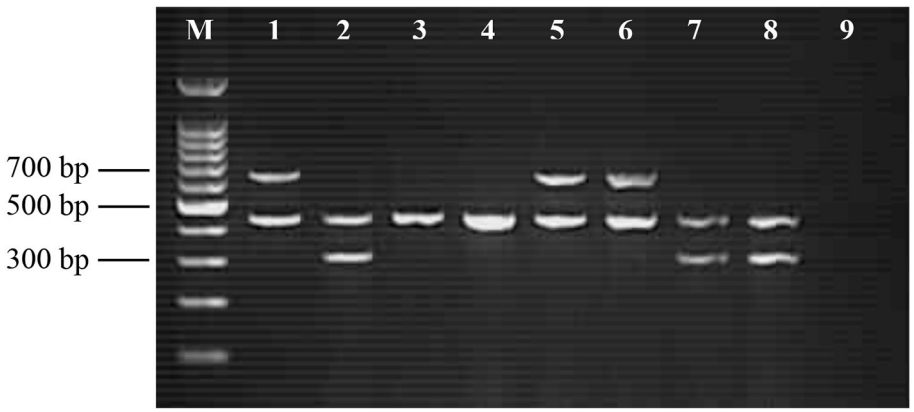


FIGURE 1 | Representative amplification of random sequence, *sdfI* gene and *fliC* gene for identification of *Salmonella* spp. (429 bp), *S.* Enteritidis (304 bp) and *S.* Typhimurium (620 bp), respectively. Lane M: 100-bp DNA ladder; Lane 1: mixture of *Salmonella* spp. and *S.* Typhimurium (positive); Lane 2: *Salmonella* spp. and *S.* Enteritidis (positive); Lane 3 to Lane 8: meat samples examined; and Lane 9: negative control.

presence of *sopB* gene. Furthermore, *pefA* gene was present in 3 of the 23 isolates tested (13.04%), comprising *S.* Enteritidis (one isolate) and *S.* Typhimurium (two isolates). Overall, the serovars tested showed at least three virulence-associated genes.

DISCUSSION

Results of investigations of retail beef meat samples do provide an estimate of the prevalence of *Salmonella* in retail shops. The high incidence of *Salmonella* in wet markets of the present study indicated poor sanitary condition in the food processing environment and lack of better personal hygiene of food handlers during product preparation. High incidence of *Salmonella* in wet markets was observed in the previous study of Thung et al. (2016), who found higher prevalence of *Salmonella* in retail chicken meat samples. An additional factor underlying potential differences in prevalence between wet markets and hypermarkets was that the storage temperature of the samples (Donado-Godoy et al., 2012). Meanwhile, the presence of *Salmonella* in retail beef meat might be due to the production system and conditions, hygienic slaughter, and transport before sale. In this study, the incidence of *Salmonella* in beef meat samples (9.58%, $n = 240$) was higher than the incidence (2.16%, $n = 417$) in Poland (Wieczorek and Osek, 2013) and less than the incidence (39.87%, $n = 158$) in North Vietnam (Thai et al., 2012). This could be due to the geographical variation such as climate and feed. On the other hand, prevalence of *Salmonella* has been reported in other food products in Malaysia. For example, *Salmonella* spp. and *S.* Typhimurium were detected in sliced fruits (such as papaya, watermelon, mango, sapodilla, jackfruit, dragon fruit and honeydew) (Pui et al., 2011), and vegetables (such as cabbage, carrot, capsicum, cucumber, lettuce and tomato) (Elexson et al., 2011). Besides, Najwa et al. (2015) have shown that *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis were detected in different types of local salad known as *ulam* (such as *kacang botol*, *kacang panjang*, *pegaga nyonya,* and *selom*).

In the present study, a combined MPN-mPCR was used to quantify the microbial load (MPN/g) which can facilitate the enumeration of *Salmonella* spp., *S.* Enteritidis and *S.* Typhimurium in the meat samples within a short period. The

TABLE 2 | Prevalence of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium in beef meat samples using MPN-mPCR method.

Beef part	Wet markets				Hypermarkets			
	No. of positive sample				No. of positive sample			
	n[a]	*Salmonella* spp.	*S.* Enteritidis	*S.* Typhimurium	*n*	*Salmonella* spp.	*S.* Enteritidis	*S.* Typhimurium
Chuck	30	3 (10.0%)[b]	0 (0.0%)	0 (0.0%)	30	2 (6.7%)	0 (0.0%)	0 (0.0%)
Rib	30	3 (10.0%)	0 (0.0%)	0 (0.0%)	30	0 (0.0%)	0 (0.0%)	0 (0.0%)
Round	30	4 (13.3%)	1 (3.3%)	1 (3.3%)	30	2 (6.7%)	1 (3.3%)	1 (3.3%)
Sirloin	30	2 (6.7%)	1 (3.3%)	0 (0.0%)	30	2 (6.7%)	0 (0.0%)	0 (0.0%)
Total	120	12 (10.0%)	2 (1.7%)	1 (0.8%)	120	6 (5.0%)	1 (0.8%)	1 (0.8%)

[a]*Number of samples.*
[b]*Percentage of positive samples.*

TABLE 3 | Microbial loads of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium (MPN/g) in beef meat samples using MPN-mPCR method.

Beef part	Wet markets									Hypermarkets								
	Salmonella spp.			*S.* Enteritidis			*S.* Typhimurium			*Salmonella* spp.			*S.* Enteritidis			*S.* Typhimurium		
	Min[a]	Med[b]	Max[c]	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
Chuck	<3	<3	7.4	<3	<3	<3	<3	<3	<3	<3	<3	3.6	<3	<3	<3	<3	<3	<3
Rib	<3	<3	7.4	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3
Round	<3	<3	15	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6	<3	<3	3.6
Sirloin	<3	<3	3.6	<3	<3	3.6	<3	<3	<3	<3	<3	3.6	<3	<3	<3	<3	<3	<3

[a]*Minimum MPN/g value.*
[b]*Median MPN/g value.*
[c]*Maximum MPN/g value.*

TABLE 4 | Antimicrobial susceptibility pattern of *Salmonella* isolates.

Antimicrobial agent	No. of isolates tested	Antibiogram pattern of *Salmonella* isolates		
		Resistant (%)	Intermediate (%)	Sensitive (%)
Amoxycillin (30 μg)	23	4 (17.39)	4 (17.39)	15 (65.22)
Ampicillin (10 μg)	23	11 (47.83)	8 (34.78)	4 (17.39)
Amoxicillin/Clavulanic acid (30 μg)	23	–	–	23 (100)
Cephazolin (30 μg)	23	2 (8.70)	2 (8.70)	19 (82.60)
Ceftazidime (30 μg)	23	–	5 (21.74)	18 (78.26)
Chloramphenicol (30 μg)	23	7 (30.43)	13 (56.52)	3 (13.05)
Ciprofloxacin (5 μg)	23	5 (21.74)	5 (21.74)	13 (56.52)
Erythromycin (15 μg)	23	23 (100)	–	–
Gentamicin (10 μg)	23	–	–	23 (100)
Kanamycin (30 μg)	23	–	2 (8.70)	21 (91.30)
Penicillin (10 μg)	23	23 (100)	–	–
Nalidixic acid (30 μg)	23	–	2 (8.70)	21 (91.30)
Streptomycin (10 μg)	23	–	2 (8.70)	21 (91.30)
Suphamethoxazole/trimethoprim (25 μg)	23	4 (17.39)	6 (26.09)	13 (56.52)
Tetracycline (30 μg)	23	–	–	23 (100)
Vancomycin (30 μg)	23	23 (100)	–	–

molecular amplification techniques can overcome the limitation of detecting viable but non-culturable (VBNC) cells with providing high specificity and sensitivity (Pui et al., 2011). The procedure of the MPN-mPCR should be familiar to laboratory personnel since it has been extensively used in academic research as well as in industrial settings. Previous studies have

TABLE 5 | The antibiotic resistance profile patterns and multiple antibiotic resistance (MAR) index of *Salmonella* isolates.

Isolate no.	Retail market	Source	*Salmonella* serovar	Antibiotic resistance profiles[a]	MAR index
1	Wet market	Round	*S.* Enteritidis	AMP, C, E, P, SXT, VA	0.38
2	Wet market	Sirloin	*S.* Enteritidis	AMP, C, E, P, VA	0.31
3	Wet market	Round	*S.* Typhimurium	AML, AMP, KZ, C,CIP, E, P, SXT, VA	0.56
4	Wet market	Chuck	*S.* Agona	AMP, E, P, VA	0.25
5	Wet market	Rib	*S.* Agona	AMP, E, P, VA	0.25
6	Wet market	Rib	*S.* Agona	AMP, E, P, VA	0.25
7	Wet market	Rib	*S.* Agona	AMP, E, P, VA	0.25
8	Wet market	Round	*S.* Anatum	E, P, VA	0.19
9	Wet market	Round	*S.* Anatum	E, P, VA	0.19
10	Wet market	Sirloin	*S.* Anatum	E, P, VA	0.19
11	Wet market	Chuck	*S.* London	E, P, VA	0.19
12	Wet market	Chuck	*S.* London	E, P, VA	0.19
13	Wet market	Round	*S.* Newport	CIP, E, P, VA	0.25
14	Wet market	Round	*S.* Newport	CIP, E, P, VA	0.25
15	Wet market	Sirloin	*S.* Stanley	E, P, VA	0.19
16	Hypermarket	Round	*S.* Enteritidis	AMP, C, E, P, SXT, VA	0.38
17	Hypermarket	Round	*S.* Typhimurium	AML, AMP, KZ, C, CIP, E, P, SXT, VA	0.56
18	Hypermarket	Chuck	*S.* Agona	AMP, E, P, VA	0.25
19	Hypermarket	Round	*S.* Agona	AMP, E, P, VA	0.25
20	Hypermarket	Chuck	*S.* London	E, P, VA	0.19
21	Hypermarket	Round	*S.* Newport	CIP, E, P, VA	0.25
22	Hypermarket	Sirloin	*S.* Weltevreden	AML, C, E, P, VA	0.31
23	Hypermarket	Sirloin	*S.* Weltevreden	AML, C, E, P, VA	0.31

[a]*AML, Amoxycillin; AMP, Ampicillin; KZ, Cephazolin; C, Chloramphenicol; CIP, Ciprofloxacin; E, Erythromycin; P, Penicillin; SXT, Suphamethoxazole/trimethoprim; VA, Vancomycin.*

TABLE 6 | Virulence genes profiles of *Salmonella* isolates.

Gene	Number of *Salmonella* serovars positive for virulence genes								Total (%) (23)
	S. Enteritidis (3)	*S.* Typhimurium (2)	*S.* Agona (6)	*S.* Anatum (3)	*S.* London (3)	*S.* Newport (3)	*S.* Stanley (1)	*S.* Weltevreden (2)	
invA	3	2	6	3	3	3	1	2	23 (100)
pefA	1	2	0	0	0	0	0	0	3 (13.04)
hilA	3	2	4	3	3	1	1	2	19 (82.61)
sopB	2	1	6	1	0	2	0	2	14 (60.87)
stn	3	1	3	1	2	3	1	2	16 (69.57)
spvC	0	0	0	0	0	0	0	0	0 (0)

described detection methods that successfully combined MPN with mPCR to enumerate bacteria such as *Campylobacter*, *Listeria monocytogenus*, and *Vibrio parahaemolyticus* in samples (Kuan et al., 2017; Premarathne et al., 2017; Tan et al., 2017). Worth to note that beef meat products by their nature, undergo extensive processing and handling during their production, may also increase the risk of contamination (Thung et al., 2016). Typically, improper or ineffective cleaning of chopping boards, tables and knives does play a role in harboring and multiplying the organism. Cross-contamination may occur when microorganisms are transferred from one surface to another, possibly leading to contamination of other safe meat or clean equipment. *Salmonella* contamination was common in retail meats such as beef, pork and lamb, which could be a potential vehicle for transmitting *Salmonella* to humans (Yang et al., 2010). Thus, implementation and maintenance of some control measures like the good manufacturing practices (GMP) and hazard analysis and critical control point (HACCP), as well as further strengthening the education of food processors will be necessary, for reducing *Salmonella* contamination.

Due to clinical significance, determining or *Salmonella* resistance or otherwise to antimicrobial agents is critical for

treatment during outbreaks. High resistance of *Salmonella* isolates to erythromycin, penicillin and vancomycin in this finding are of clinical concern and could be the result of widespread use of these antibiotics in Selangor area. Similarly, high percentage of penicillin and erythromycin resistance were observed in different *Salmonella* serovars which isolated from retail meat products such as beef burger, ground beef and fresh beef (Sallam et al., 2014). Interestingly, there were no *Salmonella* serovars resistant to amoxicillin/clavulanic acid, ceftazidime, gentamicin, kanamycin, nalidixic acid, streptomycin, and tetracycline. In contrast, resistance to tetracycline was observed among the serovars of *S.* Enteritidis and *S.* Typhimurium isolated from retail beef meat samples (Yang et al., 2010). Previously, a study in China demonstrated that all the *Salmonella* strains ($n = 83$) were sensitive to amoxicillin/clavulanic acid, while 98.80 and 92.77% were observed for gentamicin and tetracycline, respectively (Dong et al., 2014). In fact, MDR *Salmonella* serovars are considered to be more virulent than non-MDR *Salmonella* (Nayak et al., 2004; Dong et al., 2014). High percentages of antimicrobial resistant *Salmonella* serovars from retail meat products have been reported worldwide by several researchers (Yang et al., 2010; Thai et al., 2012; Sallam et al., 2014). In Malaysia, such observation was also reported by Geidam et al. (2012), where they were able to detect MDR *Salmonella* in the poultry environment in Selangor region. In this study, MDR *Salmonella* isolates are prevalent in both retail markets. Hence, more attention should be focused on the supervision and control of antimicrobial use, typically in the agriculture and human health care sectors in Malaysia.

Accordingly, the virulence of bacteria is influenced by both antimicrobial resistance and the presence of virulence genes (Huehn et al., 2010; Dong et al., 2014). The emergence of MDR strains of *Salmonella* are mainly based on the factors of genetic and biochemical mechanisms in order to enhance their survivability by preserving their drug resistance genes (Yang et al., 2010). Regarding the virulence factors that were analyzed, *S.* Enteritidis, *S.* Typhimurium, *S.* Agona, *S.* Anatum, *S.* Newport, and *S.* Weltevreden isolates showed a broader range of pathogenicity determinants as compared to other serovars. The most common virulence gene which present in *Salmonella*, *invA* gene, was used as PCR target gene for detection of *Salmonella* (Nayak et al., 2004; Dong et al., 2014). On the other hand, an OmpR/ToxR transcriptional regulator encoded by the *hilA* gene to activate the expression of invasion genes was shown to play an important role in *Salmonella* virulence (Cardona-Castro et al., 2002). In this study, the PCR screening using *hilA*-targeted *Salmonella*-specific primers showed a clear abundance of this virulence gene was detected in 19 of 23 analyzed strains (82.61%), irrespective of their serovars. In addition, similar results have been reported by Murugkar et al. (2003) who found that the chromosomally encoded virulent *stn* gene was widely distributed in all the isolated serovars. This strengthens to our present finding; the *stn* gene was prevalent among the isolated *Salmonella* serovars by PCR-based assay (69.57%). Several studies have shown that the *Salmonella* virulence plasmid plays an important role in human disease (Swamy et al., 1996).

In summary, this study has shown that a combined MPN-mPCR method was a reliable and useful for rapid screening of *Salmonella* from retail beef meat. Our results indicate that retail beef meat act as reservoirs in harboring multiple *Salmonella* serovars, where cross-contamination might be occurred during processing and at the retail level. *S.* Agona was the most common serotype found in retail beef. Moreover, the recovered *Salmonella* isolates exhibiting multi-drug resistant and multiple virulence genes, which constitute a possible risk to humans from consumption of these products. Therefore, it is important to manage the use of antimicrobial agents in livestock now, to prevent the acquisition and increased resistance to recent molecules in order to fight against the vertical and horizontal transfer of MDR strains. Alternatively, it is necessary for developing more effective intervention strategies such as green control method using bacteriophages as controlling measure in the food chain in order to reduce the risk of food-borne diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This research was funded by Fundamental Research Grant Scheme from the Ministry of Education, Malaysia (FRGS 5524559) and the Putra Grant of Universiti Putra Malaysia (GP-IPS 9438703).

REFERENCES

Alvarez, J., Sota, M., Vivanco, A. B., Perales, I., Cisterna, R., Rementeria, A., et al. (2004). Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. *J. Clin. Microbiol.* 42, 1734–1738. doi: 10.1128/JCM.42.4.1734-1738.2004

Brichta-Harhay, D. M., Guerini, M. N., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Shackelford, S. D., et al. (2008). *Salmonella* and *Escherichia coli* O157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States: an evaluation of prevalence and bacterial loads by immunomagnetic separation and direct plating methods. *Appl. Environ. Microbiol.* 74, 6289–6297. doi: 10.1128/AEM.00700-08

Cardona-Castro, N., Restrepo-Pineda, E., and Correa-Ochoa, M. (2002). Detection of hilA gene sequences in serovars of *Salmonella enterica* subspecies *enterica*. *Mem. Inst. Oswaldo Cruz* 97, 1153–1156. doi: 10.1590/S0074-02762002000800016

Chai, L. C., Tunung, R., Usha, M. R., Jurin, W. G., Bakar, F. A., Ghazali, F. M., et al. (2007). Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. *Int. J. Food Microbiol.* 117, 106–111. doi: 10.1016/j.ijfoodmicro.2007.02.014

Clinical and Laboratory Standarts Institude (CLSI) (2012). *Performance Standards for Antimicrobial disk Susceptibility Tests*. Wayne, PA: CLSI.

Donado-Godoy, P., Clavijo, V., Leon, M., Tafur, M. A., Gonzales, S., Hume, M., et al. (2012). Prevalence of *Salmonella* on retail broiler chicken meat carcasses in Colombia. *J. Food Prot.* 75, 1134–1138. doi: 10.4315/0362-028X.JFP-11-513

Dong, P., Zhu, L., Mao, Y., Liang, R., Niu, L., Zhang, Y., et al. (2014). Prevalence and profile of *Salmonella* from samples along the production line in Chinese beef processing plants. *Food Control.* 38, 54–60. doi: 10.1016/j.foodcont.2013.09.066

Ed-dra, A., Filali, F. R., Karraouan, B., El Allaoui, A., Aboulkacem, A., and Bouchrif, B. (2017). Prevalence, molecular and antimicrobial resistance of *Salmonella* isolated from sausages in Meknes, Morocco. *Microb. Pathog.* 105, 340–345. doi: 10.1016/j.micpath.2017.02.042

Elexson, N., Chai, L. C., Pui, C. F., Tunung, R., Ubong, A., Tuan Zainazor, T. C., et al. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw salad vegetables and vegetarian burger patties. *Food Nutr. Sci.* 2, 1077–1081. doi: 10.4236/fns.2011.210144

Finstad, S., O'Bryan, C. A., Marcy, J. A., Crandall, P. G., and Ricke, S. C. (2012). *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Res. Int.* 45, 789–794. doi: 10.1016/j.foodres.2011.03.057

Geidam, Y. A., Zakaria, Z., Aziz, S. A., Bejo, S. K., Abu, J., and Omar, S. (2012). High prevalence of multi-drug resistant bacteria in selected poultry farms in Selangor, Malaysia. *Asian J. Anim. Vet. Adv.* 7, 891–897. doi: 10.3923/ajava.2012.891.897

Huehn, S., La Ragione, R. M., Anjum, M., Saunders, M., Woodward, M. J., Bunge, C., et al. (2010). Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. *Foodborne Pathog. Dis.* 7, 523–535. doi: 10.1089/fpd.2009.0447

Kramarenko, T., Nurmoja, I., Karssin, A., Meremae, K., Horman, A., and Roasto, M. (2014). The prevalence and serovar diversity of *Salmonella* in various food products in Estonia. *Food Control.* 42, 43–47. doi: 10.1016/j.foodcont.2014.01.032

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to indentify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* 46, 165–170.

Kuan, C. H., Rukayadi, Y., Ahmad, S. H., Wan Mohamed Radzi, C. W. J., Thung, T. Y., Premarathne, J. M. K. J. K., et al. (2017). Comparison of the microbiological quality and safety between conventional and organic vegetables sold in Malaysia. *Front. Microbiol.* 8:1433. doi: 10.3389/fmicb.2017.01433

Kuan, C. H., Wong, W. C., Pui, C. F., Mahyudin, N. A., Tang, J. Y. H., Nishibuchi, M., et al. (2013). Prevalence and quantification of Listeria monocytogenes in beef offal at retail level in Selangor, Malaysia. *Braz. J. Microbiol.* 44, 1169–1172. doi: 10.1590/S1517-83822014005000002

Li, R., Lai, J., Wang, Y., Liu, S., Li, Y., Liu, K., et al. (2013). Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. *Int. J. Food Microbiol.* 163, 14–18. doi: 10.1016/j.ijfoodmicro.2013.01.020

Murugkar, H. V., Rahman, H., and Dutta, P. K. (2003). Distribution of virulence genes in *Salmonella* serovars isolated from man and animals. *Indian J. Med. Res.* 117, 66–70.

Najwa, M. S., Rukayadi, Y., Ubong, A., Loo, Y. Y., Chang, W. S., Lye, Y. L., et al. (2015). Quantification and antibiotic susceptibility of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw vegetables (ulam). *Int. Food Res. J.* 22, 1761–1769.

Nayak, R., Stewart, T., Wang, R. F., Lin, J., Cerniglia, C. E., and Kenney, P. B. (2004). Genetic diversity and virulence gene determinants of antibiotic-resistance *Salmonella* isolated from preharvest turkey production sources. *Int. J. Food Microbiol.* 91, 51–62. doi: 10.1016/S0168-1605(03)00330-1

Oh, S. Y., Heo, N. S., Shukla, S., Cho, H. J., Vilian, A. T. E., Kim, J., et al. (2017). Development of gold nanoparticle-aptamer-based LSPR sensing chips for the rapid detection of *Salmonella* Typhimurium in pork meat. *Sci. Rep.* 7:10130. doi: 10.1038/s41598-017-10188-2

Premarathne, J. M. K. J. K., Anuar, A. S., Thung, T. Y., Satharasinghe, D. A., Jambari, N. N., Abdul Mutalib, N. A., et al. (2017). Prevalence and antibiotic resistance against tetracycline in *Campylobacter jejuni* and *C. coli* in cattle and beef meat from Selangor, Malaysia. *Front. Microbiol.* 8:2254. doi: 10.3389/fmicb.2017.02254

Pui, C. F., Wong, W. C., Chai, L. C., Elexson, N., Farinazleen, M. G., Cheah, Y. K., et al. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. *Food Control.* 22, 337–342. doi: 10.1016/j.foodcont.2010.05.021

Sallam, K. I., Mohammed, M. A., Hassan, M. A., and Tamura, T. (2014). Prevalence, molecular identification and antimicrobial resistance profile of *Salmonella* serovars isolated from retail beef products in Mansoura, Egypt. *Food Control.* 38, 209–214. doi: 10.1016/j.foodcont.2013.10.027

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G., et al. (1999). Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. *Lett. Appl. Microbiol.* 28, 113–117. doi: 10.1046/j.1365-2672.1999.00488.x

Swamy, S. C., Barnhart, H. M., Lee, M. D., and Dreesen, D. W. (1996). Virulence determinantsinvA and spvC in *Salmonellae* isolated from poultry products, wastewater, and human sources. *Appl. Environ. Microbiol.* 62, 3768–3771.

Tan, C. W., Malcolm, T. T. H., Kuan, C. H., Thung, T. Y., Chang, W. S., Loo, Y. Y., et al. (2017). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from Short Mackerels (*Rastrelliger brachysoma*) in Malaysia. *Front. Microbiol.* 8:1087. doi: 10.3389/fmicb.2017.01087

Thai, T. H., Hirai, T., Lan, N. T., Shimada, A., Ngoc, P. T., and Yamaguchi, R. (2012). Antimicrobial resistance of *Salmonella* serovars isolated from beef at retail markets in the North Vietnam. *J. Vet. Med. Sci.* 74, 1163–1169. doi: 10.1292/jvms.12-0053

Thung, T. Y., Mahyudin, N. A., Basri, D. F., Wan Mohamed Radzi, C. W., Nakaguchi, Y., Nishibuchi, M., et al. (2016). Prevalence and antibiotic resistance of *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw chicken meat at retail markets in Malaysia. *Poult. Sci.* 95, 1888–1893. doi: 10.3382/ps/pew144

Wieczorek, K., and Osek, J. (2013). Prevalence and characterisation of *Salmonella* in slaughtered cattle and beef in Poland. *Bull. Vet. Inst. Pulawy.* 57, 607–611. doi: 10.2478/bvip-2013-0103

Yang, B., Qu, D., Zhang, X., Shen, J., Cui, S., Shi, Y., et al. (2010). Prevalence and characterization of *Salmonella* serovars in retail meats of marketplace in Shaanxi, China. *Int. J. Food Microbiol.* 141, 63–72. doi: 10.1016/j.ijfoodmicro.2010.04.015

Yang, X., Huang, J., Wu, Q., Zhang, J., Liu, S., Guo, W., et al. (2016). Prevalence, antimicrobial resistance and genetic diversity of *Salmonella* isolated from retail ready-to-eat foods in China. *Food Control.* 60, 50–56. doi: 10.1016/j.foodcont.2015.07.019

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GP and handling Editor declared their shared affiliation.

Copyright © 2018 Thung, Radu, Mahyudin, Rukayadi, Zakaria, Mazlan, Tan, Lee, Yeoh, Chin, Tan, Kuan, Basri and Wan Mohamed Radzi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 January 2018
doi: 10.3389/fmicb.2017.02674

Comprehensive Proteomic Analysis of Lysine Acetylation in the Foodborne Pathogen *Trichinella spiralis*

Yong Yang[1,2†], Mingwei Tong[3†], Xue Bai[1†], Xiaolei Liu[1], Xuepeng Cai[4,5], Xuenong Luo[5], Peihao Zhang[2], Wei Cai[2], Isabelle Vallée[6], Yonghua Zhou[7] and Mingyuan Liu[1]**

[1] Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis/College of Veterinary Medicine, Jilin University, Changchun, China, [2] Wu Xi Medical School, Jiangnan University, Affiliated Hospital of Jiangnan University, Wuxi, China, [3] State Key Laboratory for Molecular Biology of Special Economic Animals, Institute of Special Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, China, [4] China Institute of Veterinary Drug Control, Beijing, China, [5] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China, [6] JRU BIPAR, ANSES, École Nationale Vétérinaire d'Alfort, INRA, Université Paris-Est, Animal Health Laboratory, Maisons-Alfort, France, [7] Jiangsu Institute of Parasitic Disease, Wuxi, China

OPEN ACCESS

Edited by:
Maria Schirone, Università di Teramo, Italy

Reviewed by:
Zhao Chen, University of California, Davis, United States
M. Guadalupe Ortega-Pierres, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Mexico

***Correspondence:**
Mingyuan Liu
liumy36@163.com
Yonghua Zhou
toxo2001@163.com

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 August 2017*
Accepted: *21 December 2017*
Published: *11 January 2018*

Citation:
Yang Y, Tong M, Bai X, Liu X, Cai X, Luo X, Zhang P, Cai W, Vallée I, Zhou Y and Liu M (2018) Comprehensive Proteomic Analysis of Lysine Acetylation in the Foodborne Pathogen Trichinella spiralis. Front. Microbiol. 8:2674. doi: 10.3389/fmicb.2017.02674

Lysine acetylation is a dynamic and highly conserved post-translational modification that plays a critical role in regulating diverse cellular processes. *Trichinella spiralis* is a foodborne parasite with a considerable socio-economic impact. However, to date, little is known regarding the role of lysine acetylation in this parasitic nematode. In this study, we utilized a proteomic approach involving anti-acetyl lysine-based enrichment and highly sensitive mass spectrometry to identify the global acetylated proteome and investigate lysine acetylation in *T. spiralis*. In total, 3872 lysine modification sites were identified in 1592 proteins that are involved in a wide variety of biological processes. Consistent with the results of previous studies, a large number of the acetylated proteins appear to be involved in metabolic and biosynthetic processes. Interestingly, according to the functional enrichment analysis, 29 acetylated proteins were associated with phagocytosis, suggesting an important role of lysine acetylation in this process. Among the identified proteins, 15 putative acetylation motifs were detected. The presence of serine downstream of the lysine acetylation site was commonly observed in the regions surrounding the sites. Moreover, protein interaction network analysis revealed that various interactions are regulated by protein acetylation. These data represent the first report of the acetylome of *T. spiralis* and provide an important resource for further explorations of the role of lysine acetylation in this foodborne pathogen.

Keywords: lysine acetylation, post-translational modification, lysine acetylation motif, interaction network, *Trichinella spiralis*

INTRODUCTION

Trichinellosis is a common food-borne parasitic zoonosis worldwide. Infection occurs by consuming raw or inadequately cooked meat containing muscle larvae of the *Trichinella* parasite. This parasite can infect a wide variety of hosts, including humans. *Trichinella* infection has been reported in 66 countries around the world. *Trichinella spiralis* is the major causative agent of

trichinellosis. Outbreaks of trichinellosis in humans have been regularly reported worldwide, and serological analysis has indicated that more than 11 million people may be infected with *Trichinella* (Murrell and Pozio, 2011). Trichinellosis is regarded as an emerging or re-emerging infectious disease, particularly in developing countries, where cooking habits, poor sanitary conditions, and a lack of veterinary inspection facilitate infection (Gottstein et al., 2009). This zoonosis is both a public health hazard and an economic issue in terms of the porcine breeding industry and food safety (Bai et al., 2017). Therefore, in order to control and eradicate this disease, it is necessary to develop novel diagnostic and therapeutic methods for trichinellosis, which will require a comprehensive understanding of the parasite's biology.

In recent years, the genome, transcriptome, epigenome, and proteome of *T. spiralis* have been reported and released in public databases, providing a greater understanding of the molecular basis of *T. spiralis* biology, host–parasite interactions, and pathogenesis (Mitreva et al., 2011; Gao et al., 2012; Liu et al., 2012, 2016). However, to the best of our knowledge, no post-translational modifications (PTMs), which occur during or after protein biosynthesis, have been described in *T. spiralis*, limiting further functional studies. PTMs regulate diverse protein properties, including folding, stability, localization, binding affinities, and activity, and they play important roles in a variety of cellular pathways and processes. Because of their substantial effects on protein functions and cellular processes, studies of protein PTMs have become more important in the post-genomic era (Walsh et al., 2005; Witze et al., 2007). To date, more than 461 different PTMs, such as phosphorylation, methylation, ubiquitination, malonylation, succinylation, glycosylation, and acetylation, have been identified to play a role in the functional regulation of many prokaryotic and eukaryotic proteins. These PTMs form a complex regulatory system for the dynamic control of cellular processes under various conditions (Yang and Seto, 2008).

Among these PTMs, lysine acetylation (Kac) is a highly dynamic and regulated modification that is defined as the reversible transfer of an acetyl group from the acetyl donor acetyl-CoA to the ε-amino group of a lysine residue of a protein molecule. The Kac process is regulated by lysine acetyltransferases and deacetylases (KDACs), resulting in a regulatory range that is extensive and comparable with those of other major PTMs (Choudhary et al., 2009; Drazic et al., 2016). KDACs have been identified as drug target to develop novel anti-parasite treatments, and some selective KDAC ligands showed the potential for the treatment of parasitic diseases (Wang et al., 2015).

Recent studies have indicated that non-enzymatic chemical acetylation occurs broadly in eukaryotic cells, suggesting that this PTM may have various roles in protein function and cellular processes (Wagner and Hirschey, 2014; Olia et al., 2015). Kac was first identified in eukaryotic histones, where it is involved in regulating chromatin structure and transcription. In addition to its role in histones, Kac were found in a number of non-nuclear proteins which are located in almost every compartment of the cell (Friedmann and Marmorstein, 2013; Drazic et al., 2016). Protein acetylation outside the nucleus participates in diverse cellular physiological processes, including cell-cycle regulation, cytoskeletal dynamics, apoptosis, autophagy, and metabolism (Kim et al., 2006; Close et al., 2010; Wang et al., 2010; Zhao et al., 2010; Sun et al., 2015). In addition, acetylation has also been implicated to influence the persistence, virulence and antibiotic resistance of pathogenic bacteria (Xie et al., 2015).

Recent advances in antibody-based affinity purification of acetylated peptides and highly sensitive mass spectrometry (MS) have made significant contributions to the study of Kac at the whole-proteome level. Global Kac has been characterized in a number of eukaryotic and prokaryotic organisms (Jeffers and Sullivan, 2012; Zhang et al., 2013; Pan et al., 2014; Nie et al., 2015; Xie et al., 2015). These proteome-wide analyses of Kac have mapped large datasets of lysine-acetylated proteins and have confirmed that Kac is involved in various cellular events, especially in modulating metabolic processes. Based on these studies, it is proposed that the functional roles of acetylation in these processes are evolutionarily conserved in both eukaryotes and prokaryotes, and acetylation may thus rival phosphorylation.

Although Kac has been characterized in many organisms, the study of the lysine acetylome in helminths has been relatively limited, with only one nematode species (*Schistosoma japonicum*) being previously characterized (Hong et al., 2016). Given the general use of acetylation as a regulatory mechanism in different organisms, it can be speculated that *T. spiralis* also employs acetylation as a modification to modulate various aspects of its biology. Lending evidence to this hypothesis, *T. spiralis* appears to contain a great number of acetyltransferases and deacetylases in its genome (Mitreva et al., 2011).

To bridge this knowledge gap, in this study, proteomics was used in combination with immunoprecipitation in the first large-scale analysis of lysine-acetylated proteins in *T. spiralis*, identifying 3872 Kac sites in 1592 proteins. These lysine-acetylated proteins are involved in a variety of biological functions and are localized to multiple cellular compartments. Bioinformatics analysis showed that acetylation in *T. spiralis* modulates a wide range of cell processes. This study provides a global picture of the *T. spiralis* acetylome, which not only greatly expands our knowledge of helminth protein acetylation but also provides important information for the design of new effective drugs or vaccines for controlling trichinellosis.

MATERIALS AND METHODS

Parasite Collection and Protein Extraction

Animal experiments were performed according to the guidelines of the National Institute of Health (publication No. 85–23, revised 1996) and Animal Experimentation Guidelines of Jilin University and were approved by the Animal Experimental Ethics Committee of Jilin University, China. *T. spiralis* (ISS534) parasites were maintained by serial passage in female BALB/c mice. *T. spiralis* muscle larvae (ML) were recovered from infected BALB/c mice at 35 days post-infection (dpi) using a standard HCl-pepsin digestion method. ML were washed manually in

phosphate-buffered saline (PBS) at 37°C to remove any residual host proteins and were then collected for further lysine acetylome analysis.

Total proteins were extracted as previously described (Zhou et al., 2016) with minor modifications. In brief, parasites were lysed with lysis buffer containing 8 M urea, 10 mM dithiothreitol (DTT) and 0.1% protease inhibitor cocktail, followed by sonication. Cellular debris was then removed by centrifugation at 20,000 × *g* and 4°C for 10 min. Proteins contained in the supernatant were precipitated with cold 15% trichloroacetic acid (TCA) for 2 h at −20°C. After centrifugation, the pellet was washed three times with cold acetone, followed by dissolution in buffer containing 8 M urea and 100 mM NH_4CO_3 (pH 8.0). Protein concentration was measured using a 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions.

Trypsin Digestion and High-Performance Liquid Chromatography (HPLC) Fractionation

Proteins were reduced with 10 mM DTT, alkylated with 20 mM iodoacetamide (IAA), and digested with trypsin (Promega) overnight at a 1:50 trypsin:protein mass ratio. To ensure complete digestion, additional trypsin was added at a 1:100 trypsin:protein mass ratio for 4 h for a second digestion. Tryptic peptides were separated into six fractions using high-pH reverse-phase HPLC, as previously described (Zhou et al., 2016). Separated peptides were then dried completely in a SpeedVac (Thermo Scientific) and stored at −80°C for further enrichment of acetylated peptides.

Affinity Enrichment and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI–MS/MS) Analysis

Fractionated peptides were dissolved in NETN buffer containing 100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, and 0.5% NP-40 (pH 8.0) and incubated with anti-acetyl lysine antibody beads (PTM Biolabs) at 4°C overnight with gentle shaking. After washing with NETN buffer and deionized H_2O, the bound peptides were eluted with 0.1% trifluoroacetic acid, dried in a SpeedVac, and cleaned using C18 ZipTips (Millipore) according to the manufacturer's instructions.

The obtained peptides were then analyzed by LC-ESI–MS/MS on a Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled online to the EASY-nLC 1000 ultra-performance liquid chromatography (UPLC) system. The resulting MS/MS data were processed using MaxQuant with an integrated Andromeda search engine (v.1.5). Tandem mass spectra were queried against the UniProt *T. spiralis* database using the following parameters (Zhou et al., 2016): trypsin/P was specified as the cleavage enzyme and allowed up to four missed cleavages, five modifications per peptide, and five charges. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on Cys was specified as a fixed modification, and oxidation on Met, acetylation on Lys, and acetylation on the protein N-terminus were specified as variable modifications. False discovery rate (FDR) thresholds for the protein, peptide, and modification sites were set at 1%. The minimum peptide length was set at 7. The site localization probability was set as > 0.75.

Bioinformatics Analysis

Gene Ontology (GO) annotation of the proteome was derived from the UniProt-GOA database[1] according to Zhou et al. (2016). All identified acetylated proteins were classified using three categories of GO annotations: biological process, cellular component, and molecular function. The subcellular localization predication program, Wolfpsort (Horton et al., 2007), was used to analyze cellular localization data, and the InterPro database[2] and InterProScan were used to compile functional descriptions of protein domains. Protein pathways were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2004). Amino acid sequence motifs in identified protein were analyzed using Soft motif-x, and a position-specific heat map was generated using WebLogo 3.4[3]. Secondary structures of all acetylated proteins were predicted using NetSurfP software. Fisher's exact test was used to analyze GO and KEGG pathway and domain enrichment. Terms with adjusted *p*-values < 0.05 were considered significantly enriched. The Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database[4] was used for analysis of the protein–protein interaction (PPI) network. The interaction network form STRING was visualized using Cytoscape (version 3.0) software (Bauer-Mehren, 2013).

RESULTS AND DISCUSSION

Identification and Analysis of Lysine-Acetylated Proteins in *T. spiralis*

Sequencing of the *T. spiralis* genome revealed the existence of a number of acetyltransferase and deacetylase orthologs, suggesting that Kac may play a vital role in *T. spiralis* development and metabolism (Mitreva et al., 2011). To comprehensively characterize protein acetylation in *T. spiralis*, analysis of Kac was carried out at the proteome level using tryptic digestion, anti-acetyl lysine antibody enrichment, and high-resolution LC-MS/MS. Generally, the distribution of mass errors was near zero, and most were ≤ 10 ppm (**Figure 1A**). Moreover, all lysine-acetylated peptides were between 7 and 37 amino acids in length, which is consistent with tryptic peptides (**Figure 1B**). These results indicated that the sample preparation method was adequate and that the modified peptide data obtained from MS was highly accurate.

In total, 3872 Kac sites distributed across 1592 acetylated proteins were identified (Supplementary Table S1). This exceeds the level of Kac that has so far been reported in *S. japonicum* (Hong et al., 2016), indicating the essential role of Kac in

[1]http://www.ebi.ac.uk/GOA/

[2]http://www.ebi.ac.uk/interpro/

[3]http://weblogo.threeplusone.com/create.cgi

[4]http://string-db.org/

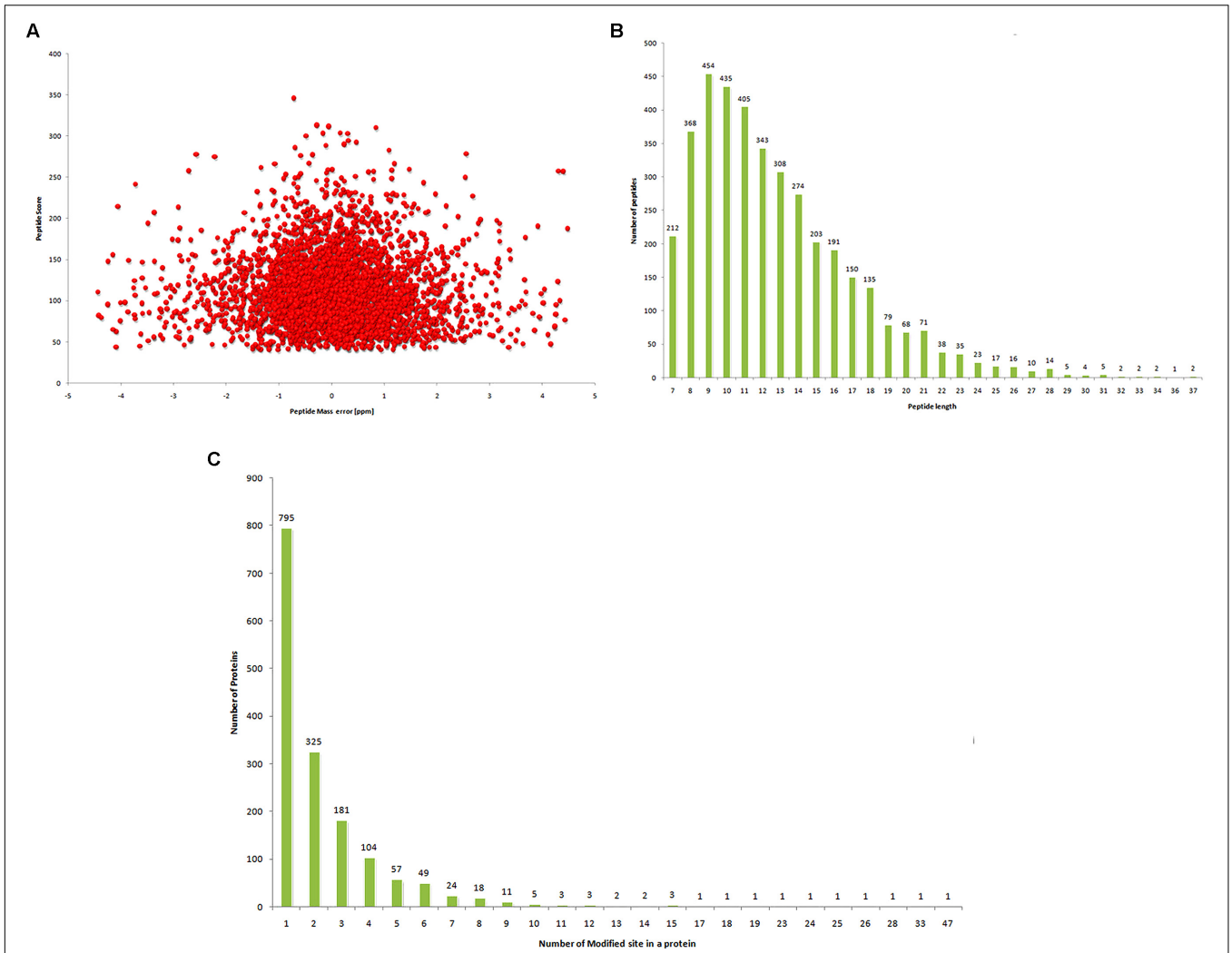


FIGURE 1 | Proteome-wide identification of lysine acetylation sites in *T. spiralis*. **(A)** Mass error distribution of all identified peptides. **(B)** Distribution of acetylated peptides based on length. **(C)** Distribution of acetylated proteins based on number of acetylation sites.

T. spiralis. The acetylated proteins of *T. spiralis* contained various numbers of acetylation sites, ranging from 1 to 47 in the putative myosin head protein (E5SJK9) (**Figure 1C**), with an average frequency of 2.4 sites per protein. Among all acetylated proteins, approximately half (795/1592) contained only one acetylated site, and the percentages of proteins with two, three, four, or > 4 modification sites were 20.4, 11.4, 6.5, and 11.7%, respectively. Acetylation of non-histone proteins at multiple sites have shown to modulate every step of cellular processes from signaling to transcription to protein degradation through various molecular mechanisms (Huang and Berger, 2008; Spange et al., 2009). The role of multiple acetylation sites in regulating protein function remains to be elucidated.

Analysis of Kac Motifs

Previous studies in both eukaryotic and prokaryotic cells have shown preferences for amino acid residues at specific positions surrounding acetylated lysines (Nie et al., 2015; Zhou et al., 2016). In our study, a total of 15 conserved amino acid sequence motifs from −10 to +10 surrounding the acetylated lysine were defined from among 3440 peptides (accounting for 88.8% of the total identified peptides). These motifs included KacS, KacR, KacH, KacN, E**KacK, KacK, KacT, K*Kac, Kac*R, TKacV, Kac*D, Kac**R, Kac*E, KacV, and KacD, an asterisk indicates a random amino acid residue. These motifs exhibited different abundances (**Figure 2A** and Supplementary Table S2, with the KacS, KacR, KacH, and KacN motifs being the most frequent and accounting for 18.7, 16.1, 11.4, and 11.3% of all identified peptides, respectively (**Figure 2B**). In addition, most of the conserved residues were located at the ± 1 or ± 2 positions of the Kac sites. The motif analysis results suggest that two particular types of residues are found near Kac sites. Enrichment of residues with major hydrophilic side chain groups, such as serine (S), arginine (R), and aspartic acid (D), was observed at the +1 position, and enrichment of threonine (T) with hydrophobic side chain groups was observed at the −1

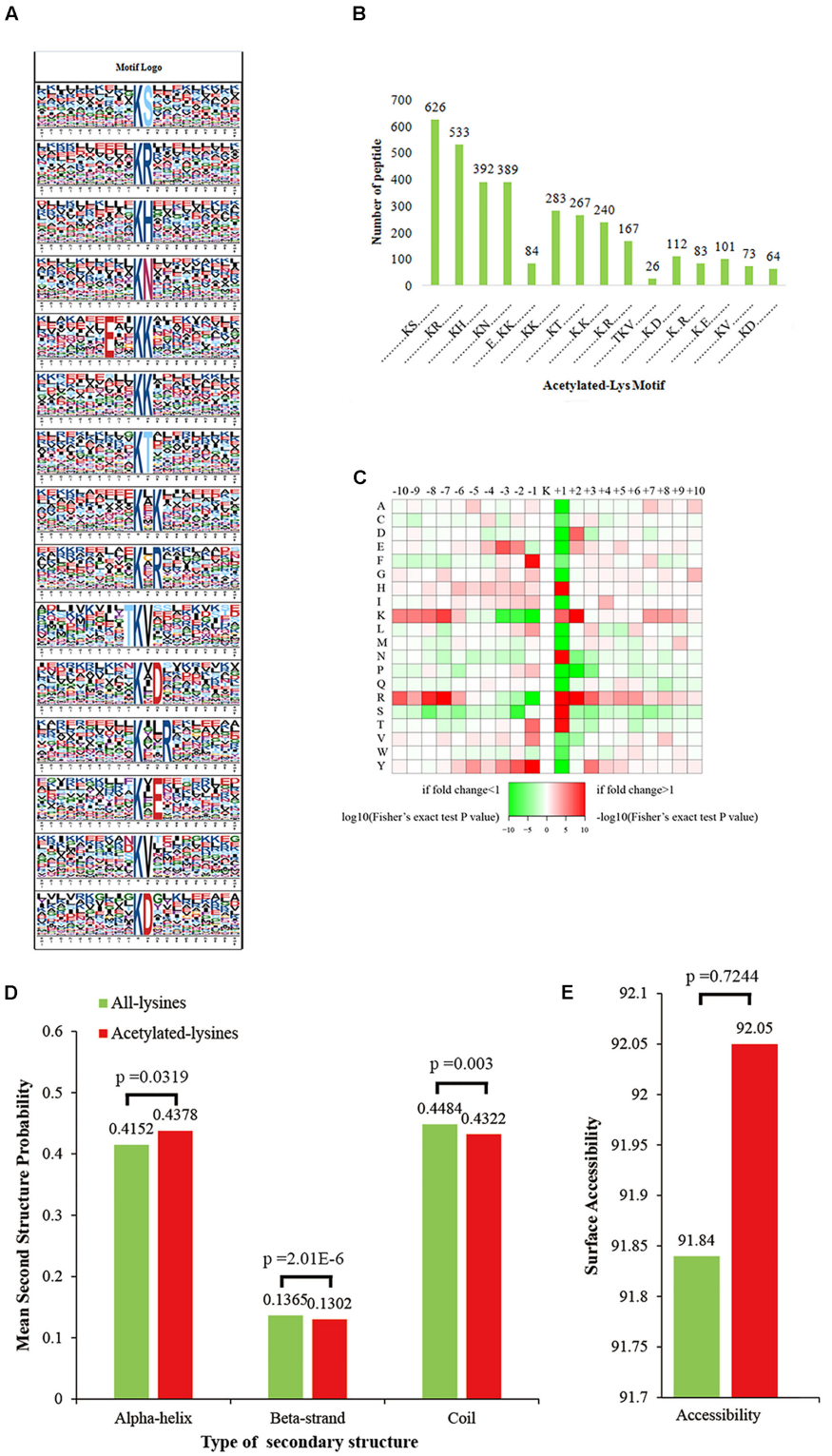


FIGURE 2 | Bioinformatics study of lysine-acetylated sites. **(A)** Probable sequence motifs of *T. spiralis* acetylation sites consisting of 20 residues surrounding the targeted lysine residue, identified using Motif-X. **(B)** Number of identified peptides containing acetylated lysines and each motif. **(C)** Heat map showing the relative frequencies of amino acids in specific positions, including enrichment (red) or depletion (green) of amino acids flanking the acetylated lysine in *T. spiralis* proteins. **(D)** Probabilities of lysine acetylation in different protein secondary structures (alpha-helix, beta-strand, and coil). **(E)** Predicted surface accessibility of acetylation sites. All lysine sites are shown in green, and acetylated lysine sites are in red.

position. These results suggest that amino acid residues with hydrophilic and hydrophobic side chains may be more easily attacked by Kac.

Studies of acetylation motifs have shown that aromatic amino acids frequently surround modification sites (Hong et al., 2016; Wang et al., 2017). Unexpectedly, we noticed only one aromatic amino acid (histidine, H) surrounding acetylated lysines, suggesting that the lysine acetyltransferases of *T. spiralis* might not prefer polypeptides with aromatic amino acid residues as substrates. Furthermore, some acetylated lysine motifs found in *T. spiralis* have also been identified in other organisms, such as KacH in humans (Shao et al., 2012) and *Mycobacterium tuberculosis* (Xie et al., 2015); Kac**R in humans (Shao et al., 2012); Kac*E in rats (Lundby et al., 2012); and KacR, KacT, and KacV in *Toxoplasma gondii* (Jeffers and Sullivan, 2012), confirming that Kac is a highly conserved modification among various species. Moreover, LceLogo heat maps of the amino acid compositions surrounding the acetylation sites showed that the frequencies of serine (S), threonine (T), and histidine (H) in positions −2 to + 2 were the highest, while the frequency of tryptophan (W) was the lowest (**Figure 2C**) in regions surrounding these sites. Based on these findings, we infer that proteins with S, T, and H but not W around lysine residues are the favored targets of lysine acetyltransferases in *T. spiralis*. A structural analysis of the acetylated proteins was carried out using NetSurfP software in order to understand the relationship between acetylation and the presence of specific protein secondary structures in *T. spiralis*. As shown in **Figure 2D**, acetylation sites (56.8%) were more common in regions with ordered secondary structures.

Among the acetylated sites, 43.8% of the acetylated sites were located in alpha-helices, and 13.2% were located in beta-strands. The remaining 43.2% of the acetylated sites were distributed in unstructured protein regions. These results suggest that acetylases may prefer ordered structural regions to disordered regions in *T. spiralis* proteins, which differs from findings of the acetyl proteomes of humans and *S. japonicum* (Hong et al., 2016). The acetylation sites in these species have been found to be located primarily in disordered protein regions, with less acetylation in ordered protein structures, suggesting that secondary structure preferences at lysine-acetylated sites may vary among species. However, a similar distribution pattern of secondary structures was observed for non-acetylated lysines in the proteome of *T. spiralis*, indicating that there is no tendency toward acetylation in *T. spiralis*. Finally, the surface accessibility of the acetylated lysine sites was also studied. The results showed that the exposure of acetylated residues on the protein surface is very similar to that of non-acetylated lysine residues (**Figure 2E**). Therefore, Kac may not be affected by the surface properties of proteins in *T. spiralis*.

Evolutionary Conservation of Kac in *T. spiralis*

Increasing evidence has indicated that Kac maybe evolutionarily conserved among various species (Zhou et al., 2016). To further improve our understanding of the evolution of acetylation in *T. spiralis*, we compared the acetylated proteins in *T. spiralis* with sacetylated proteins that have been reported in other organisms based on the quantity of orthologs. The published acetylated proteins of *Escherichia coli* (Zhang et al., 2013), budding yeast (*Saccharomyces cerevisiae*) (Henriksen et al., 2012), *Candida albicans* (Zhou et al., 2016), *T. gondii* (Jeffers and Sullivan, 2012), *S. japonicum* (Hong et al., 2016), mouse (liver tissue), and human [cervical cancer (HeLa) cells] (Weinert et al., 2013) were selected. These acetylome data are comparable, as similar methods were used for the systematic identification of Kac in each of these species. Among the 1592 acetylated proteins in *T. spiralis*, 408 (35.5%) proteins had orthologs in the *S. japonicum* acetylome, 479 (35.2%) had orthologs in the mouse acetylome, 471 (36.3%) had orthologs in the human acetylome, 159 (31.6%) had orthologs in the *S. cerevisiae* acetylome, 190 (39.8%) had orthologs in the *C. albicans* acetylome, 87 (30%) had orthologs in the *T. gondii* acetylome, and 43 (12.3%) had orthologs in the *E. coli* acetylome (**Figure 3** and Supplementary Table S3). The lowest ortholog rate was between *T. spiralis* and *E. coli,* and this could reflect differences in cellular structures between prokaryotes and eukaryotes. The highest overlap was between *T. spiralis* and *C. albicans*, which can be explained by the genetic relatedness between them and is likely due to a similar subcellular distribution of acetylated proteins. These results indicate that acetylation occurs frequently in both eukaryotes and prokaryotes with certain species.

Functional Annotation and Cellular Localization of Acetylated Proteins in *T. spiralis*

To functionally characterize the identified lysine-acetylated proteins, we first investigated the GO functional classifications of all acetylated proteins (**Figure 4** and Supplementary Table S4). As shown in **Figure 4A**, acetylated proteins were distributed among a variety of cellular compartments, primarily in cells (37%), organelles (24%), macromolecular complexes (22%), and membranes (13%). According to the molecular function classification, the largest group of acetylated proteins was composed of those involved in the binding of various targets, accounting for 40% of all identified acetylated proteins (**Figure 4B**). This result suggests that acetylation may play essential roles in regulating PPIs in *T. spiralis*. The second largest group in the molecular function category included proteins related to catalytic activity (37%). Enzymes play important roles in regulating biochemical reactions in organisms because of their catalytic activity. Studies have indicated that reversible Kac can regulate metabolic enzymes by increasing or decreasing enzyme quantity, catalytic activity, or substrate accessibility (Xiong and Guan, 2012). Our results are consistent with those of previous studies, in which a considerable number of lysine-acetylated proteins with catalytic activity were enzymes involved in metabolic processes (Jeffers and Sullivan, 2012; Hong et al., 2016).

In the biological process classification of the GO annotation of acetylated proteins, two main classes of acetylated proteins

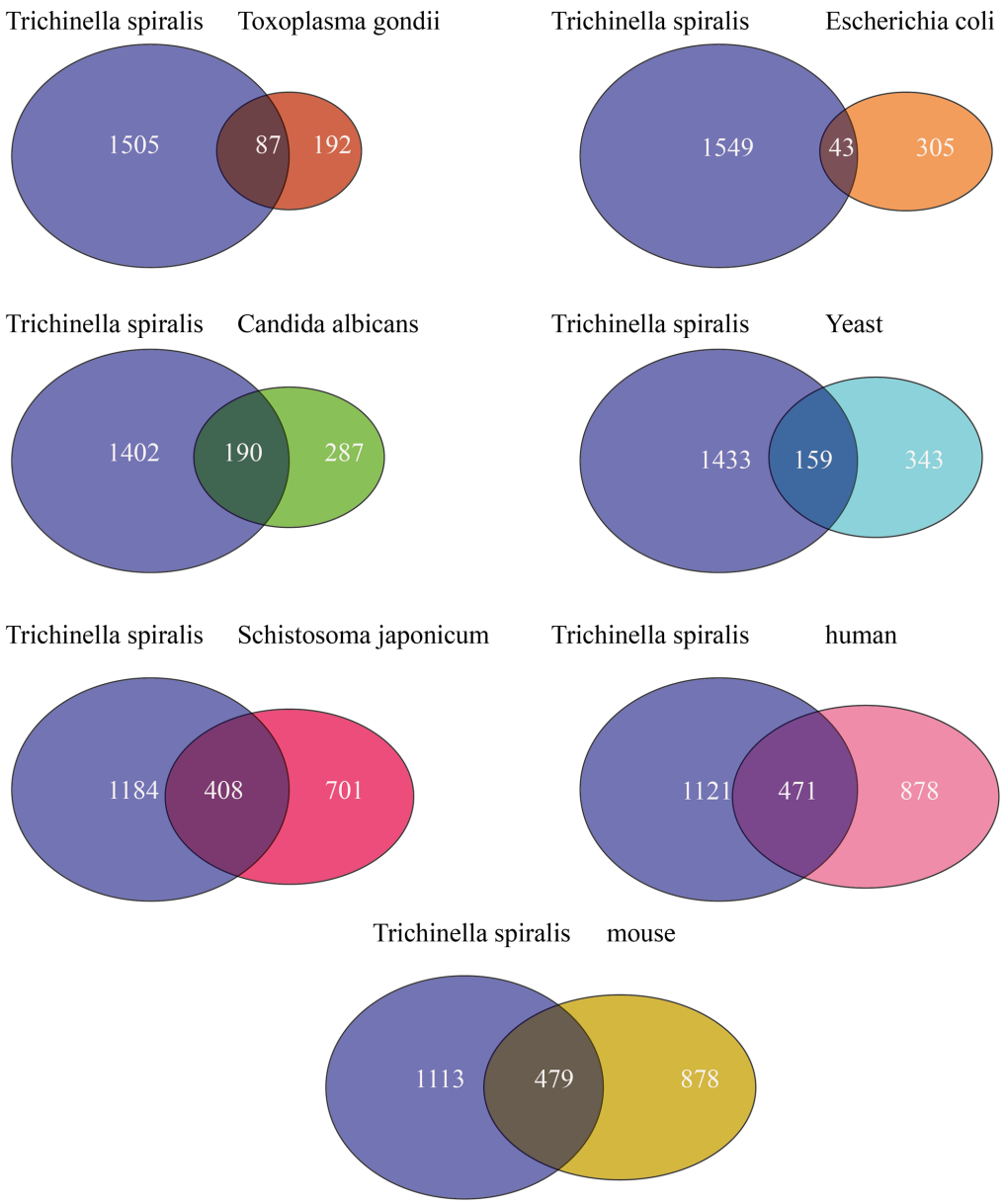


FIGURE 3 | Analysis comparing the *T. spiralis* acetyl proteome with those of *E. coli,* budding yeast (*S. cerevisiae*), *C. albicans*, *T. gondii*, *S. japonicum*, mouse, and human. Venn diagram describing the relationships among lysine-acetylated proteins from these species.

were involved in cellular and metabolic processes, accounting for 29 and 28% of identified proteins, respectively (**Figure 4C**). Previous studies have demonstrated that reversible Kac is an emerging regulatory mechanism for proteins involved in metabolism and cellular processes in bacteria (Pan et al., 2014), parasites (Jeffers and Sullivan, 2012), animals, and humans (Weinert et al., 2013). Here, our results indicate that Kac may also play a critical role in *T. spiralis* metabolism and cellular regulation.

The localization of proteins to various subcellular compartments is important for allowing proteins to perform their necessary functions. To obtain a profile of acetylated proteins throughout the cell, we analyzed the subcellular localization of the 1592 acetylated proteins in *T. spiralis* (**Figure 4D**). Analysis of the acetylated proteins showed that the majority of identified Kac proteins were localized to the cytoplasm (38%), followed by the nucleus (17%), and mitochondria (12%). This result is consistent with those of the biological process analysis, which showed that most acetylated proteins were involved in protein cellular metabolism and protein synthesis. Importantly, we also found that a number of acetylated proteins were cytoplasmic membrane (12%) or extracellular (12%) proteins, which could function in regulating cuticular layer-related proteins, impacting the interactions between *T. spiralis* and host cells. In addition,

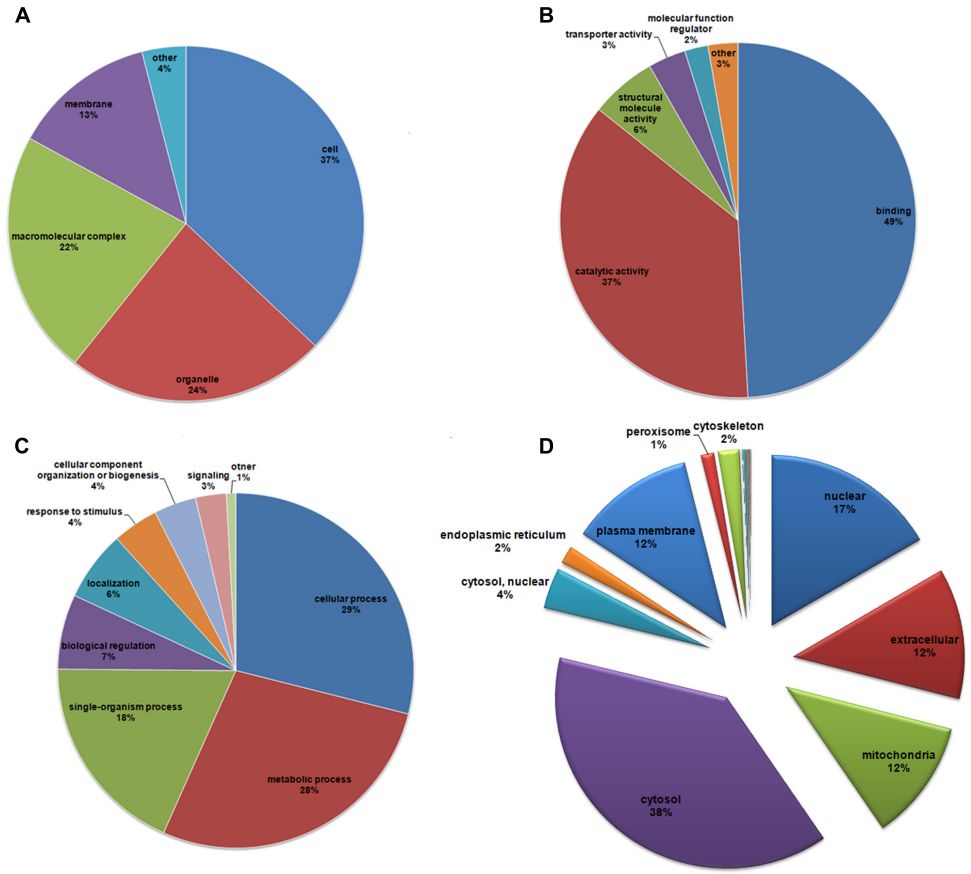


FIGURE 4 | Gene Ontology functional classification and subcellular location information of identified acetylated proteins. **(A)** Acetylated proteins classified according to cellular components. **(B)** Acetylated proteins classified according to biological process. **(C)** Acetylated proteins classified according to molecular function. **(D)** Subcellular localizations of identified acetylated proteins.

1% of the acetylated proteins in this study were located in the peroxisome, where fatty acids are activated and converted into acyl-CoA before being transformed into sucrose.

Functional Enrichment Analysis

In order to reveal the preferred targets of Kac, we performed a GO enrichment analysis of the acetylation data. The GO enrichment analysis of the molecular function category further demonstrated that acetylated proteins were significantly enriched in those related to binding activity and the structural constituents of ribosomes (**Figure 5A**). GO enrichment analysis based on the biological process category showed that acetylated proteins were enriched in those involved in biosynthetic and metabolic processes (**Figure 5A**), suggesting that these processes may be precisely regulated by protein acetylation. As acetylated proteins may be involved in both of these fundamental processes, Kac appears to be a pivotal regulatory mechanism in *T. spiralis*.

To gain in-depth insights into the functions of Kac proteins in *T. spiralis*, KEGG enrichment analysis was also carried out (**Figure 5B**). Our data showed that the acetyl proteome of *T. spiralis* was highly enriched in proteins involved in protein biosynthesis and the metabolism of three types of biological macromolecules (**Figure 5B**). The significant enrichment of proteins involved in various pathways, including translation, transcription, and metabolism, among acetylated proteins has also been reported in other eukaryotes and prokaryotes (Nie et al., 2015; Hong et al., 2016; Zhou et al., 2016), confirming the essential role of Kac in various organisms.

Small protein motifs involved in protein interactions are also regulated by PTMs. Thus, we performed a domain enrichment analysis. In total, acetylated proteins were significantly enriched for 25 protein domains, including the NAD(P)-binding domain, AAA+ ATPase domain, and nucleic acid-binding (OB-fold) domain (**Figure 5C**). Proteins with these functional domains are involved in a variety of metabolic pathways. A previous study indicated that enzymes with an NAD(P)-binding domain catalyze redox reactions, suggesting that Kac plays an important role in the stress response (Hua et al., 2014). The OB-fold is a compact structural motif that recognizes single-stranded and unusually structured nucleic acids, and it plays a critical role in DNA replication, DNA repair, transcription, translation, and telomere maintenance (Theobald et al., 2003), making these proteins attractive anti-parasitic drug targets. In our study, a large

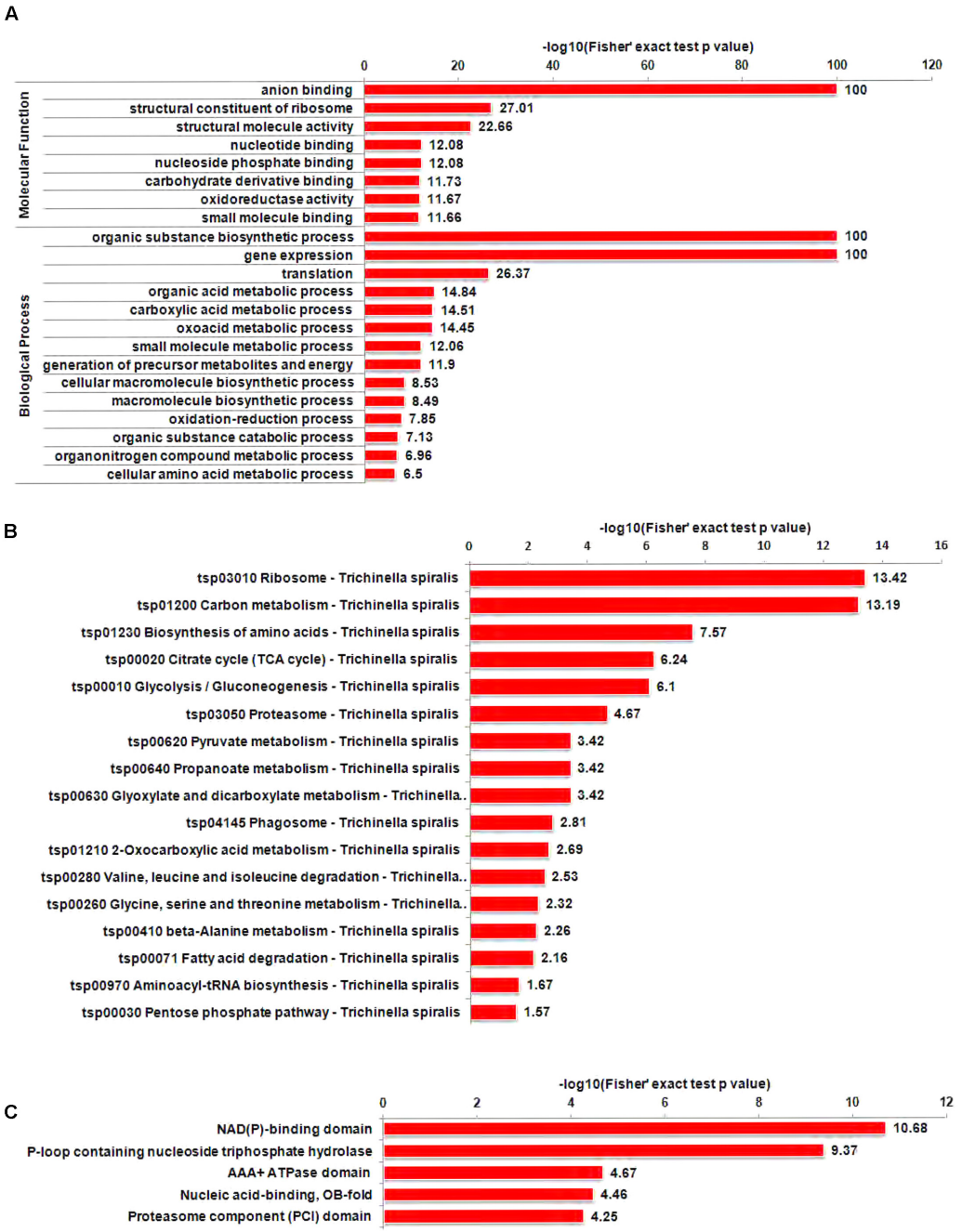


FIGURE 5 | Enrichment analysis of acetylated proteins in *T. spiralis*. **(A)** GO-based enrichment analysis of acetylated proteins in terms of molecular function, cellular component, and biological process. **(B)** KEGG pathway enrichment analysis. **(C)** Protein domain enrichment analysis.

number of proteins containing nucleic acid-binding domains, such as helicase, tyrosine–tRNA ligase, 40S ribosomal protein S11, GTP-binding protein, asparaginyl–tRNA synthetase, and nuclease domain-containing protein 1, were found to have different numbers of acetylation sites. Prevalent acetylation of proteins containing nucleic acid-binding domains may play an important role in directing the recognition of single-stranded and unusually structured nucleic acids. Overall, our results indicate that acetylated proteins are widely distributed throughout the cell, with important effects on a variety of processes in *T. spiralis*.

PPI Analysis

Protein–protein interactions occur widely in all cell types and are important in regulating cellular processes. To better understand how interactions among acetylated proteins may affect the regulation of *T. spiralis* physiology and development,

we assembled a PPI network containing all of the identified acetylated proteins using Cytoscape. The results showed that 666 acetylated proteins represented network nodes that were connected by 9446 direct physical interactions, with a combined score > 0.90 obtained from the STRING database. The majority of proteins in this interaction network contain multiple Kac sites, and the network suggests that the physiological interactions among these acetylated proteins are involved in multiple protein interaction networks and control various signaling pathways in *T. spiralis*. A complete *T. spiralis* network of acetylated proteins is shown in Supplementary Table S5 and represents the first high-quantity interaction network of acetylated proteins in a foodborne parasite.

On the basis of the interaction network, we retrieved four highly interconnected interaction clusters of acetylated proteins using the MCODE algorithm in Cytoscape.

The top cluster, with 73 acetylated proteins (cluster I), consisted of ribosome-associated proteins that were connected in a PPI network with a relatively high density (**Figure 6A**). Clusters II and III consisted of proteins associated with the proteasome and RNA transport, whereas the fourth highest-ranking complex consisted of proteins involved in the tricarboxylic acid (TCA) cycle (**Figures 6B–D**). These subnetworks also have relatively high densities, and many of the acetylated proteins in the network contain multiple Kac sites. These findings suggest that the protein metabolic metabolite machinery is probably regulated by Kac in *T. spiralis*; the identified proteins were also involved in central metabolic pathways.

Analysis of Histone Kac

Histone acetylation occurs in diverse organisms and is now known to play a critical role in chromatin remodeling, DNA repair, and the epigenetic regulation of gene expression (Campos and Reinberg, 2009). The acetylation of histones 3 and 4 is commonly associated with active transcription, and histone acetylation at H3K27 play critical roles in regulating enhancer activities in cooperation with histone methylation (Hyun et al., 2017; Shen et al., 2017; Youn, 2017) Histone acetylation is also essential for parasite survival. In *S. mansoni*, histone deacetylase inhibitors induce mortality and apoptosis, which are associated with the increased expression of the caspase 3 and 7 transcripts due to histone H4 hyperacetylation. Thus, the histone deacetylase of *S. mansoni* may be an important target for schistosomiasis chemotherapy (Dubois et al., 2009). Many specific histone acetylation sites have been characterized in various parasites. The acetylation of H4K8 and H4K16 in *Giardia lamblia* plays a crucial role in regulating encystation, which protects the parasite from harsh environmental conditions (Carranza et al., 2016). Similarly, variations in the levels of H4K8 acetylation are important in regulating stage-specific gene expression throughout the *Plasmodium falciparum* life cycle (Carranza et al., 2016). In the present study, we identified six histone proteins with multiple Kac sites (Supplementary Table S6), with H2B (E5RY73) being heavily acetylated at eight lysine residues. Some acetylated lysine sites identified in our study have also been found in other parasites, such as *T. gondii* (Jeffers and Sullivan, 2012) and *P. falciparum* (Miao et al., 2013). However, further research is required to determine the significance of these modifications in *T. spiralis* and their roles in development and infection.

Protein Acetylation Regulates Diverse Metabolic Pathways in *T. spiralis*

Recently, proteomic studies into acetylation have indicated that abundant proteins are acetylated in various organisms, suggesting that acetylation is a common mechanism of metabolic regulation (Hong et al., 2016; Zhou et al., 2016). Consistent with previous results, our study showed that 138 acetylated proteins were involved in multiple metabolic pathways. Here, we focused on two of these pathways: glycometabolism and protein metabolism.

Glycometabolism

The acetylation of proteins involved in glycometabolism (mainly enzymes) has been showed to play a crucial regulatory role in processes such as parasite development and growth (Miao et al., 2013; Hong et al., 2016). In our study, a large number of enzymes in these pathways were observed to undergo acetylation in *T. spiralis* (**Figure 7**), indicating that acetylation also participates in regulating glycometabolism in *T. spiralis*.

Trichinella spiralis larvae ferment glycogen under aerobic or anaerobic conditions via phosphorylative glycolysis, converting it to a mixture of volatile fatty acids (Agosin and Aravena, 1959). We found that almost all glycolytic enzymes involved in the conversion of glucose to pyruvate were substrates of Kac (15 proteins). Previous studies have found that seven out of ten glycolytic enzymes are acetylated in the *E. coli* acetylome (Zhang et al., 2013), while only glucose-6-phosphate isomerase is not acetylated in *P. falciparum* (Miao et al., 2013). Similarly, all enzymes involved in glycolysis are acetylated in the *S. japonicum* acetylome (Hong et al., 2016). In our study, only glyceraldehyde-phosphate dehydrogenase (GAPDH) was not found to be acetylated. In glycolysis, GAPDH acetylation increases glycolytic activity. Thus, the acetylation of GAPDH is important for controlling carbon flux, which is beneficial for glycolysis in cells supplied with glucose (Li et al., 2014). As the GAPDH protein of *T. spiralis* was not acetylated in our study, other enzymes, such as fructose-bisphosphate aldolase or phosphoglycerate kinase (PGK), with six and eight Kac sites, respectively, may be the main targets for the regulation of glycolysis in *T. spiralis*. Previous study have indicated that acetylation of fructose 1,6-bisphosphate aldolase at K14 increased enzymatic activity (Kim et al., 2006). PGK is another central enzyme in the glycolysis pathway, catalyzing the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate and generating a molecule of ATP. The proliferation of cancer cells demands a lot of energy and is mainly dependent on anaerobic glycolysis for the conversion of glucose, a phenomenon known as the "Warburg effect." Glycolysis is much less potent than mitochondrial oxidative phosphorylation in terms of ATP production, as only two enzymes, PGK and pyruvate kinase, control the production of ATP during anaerobic glycolysis in cancer cells. A recent study found that the acetylation of PGK at K323 enhances PGK activity and promotes cancer cell proliferation and tumorigenesis

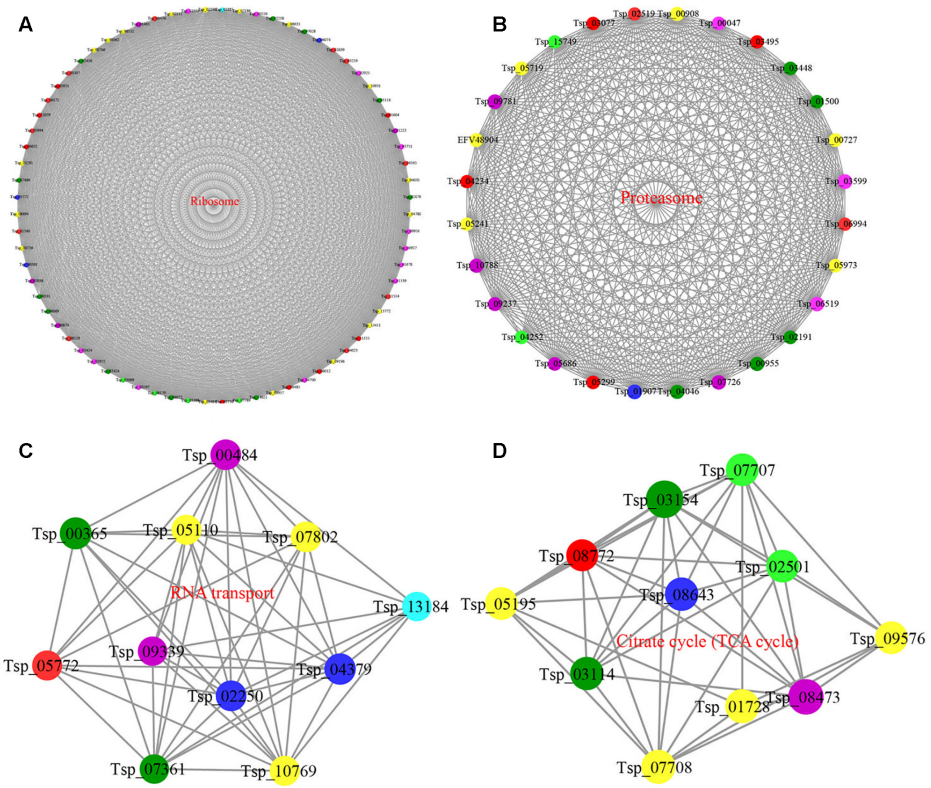


FIGURE 6 | Interaction networks of acetylated proteins in *T. spiralis*. **(A)** Interaction network of acetylated proteins associated with the ribosome. **(B)** Interaction network of acetylated proteins associated with the proteasome. **(C)** Interaction network of acetylated proteins associated with RNA transport. **(D)** Interaction network of acetylated proteins associated with the TCA cycle.

(Hu et al., 2017). In our study, the lysine sites in PGK were heavily acetylated, which may facilitate nutrient absorption and *T. spiralis* growth.

In addition, in our study, enolase exhibited the greatest number of acetylated sites (12 sites). In addition to their glycolytic function, enolases were recently shown to interact with plasminogen to induce fibrinolytic activity and to promote the migration of larval stages through tissues by plasmin-mediated proteolysis, such as the degradation of the host's extracellular matrix. Enolase can induce some protection against schistosomal infection and also inhibits egg production in schistosomes (Yang et al., 2010). Therefore, enolase is considered a potential target for the development of new anti-parasitic vaccines and drugs. However, more work is required to fully elucidate the role of acetylation in controlling the function of enolase.

Another interesting pathway, the pentose phosphate pathway contained 10 acetylated proteins according to our results. This pathway provides reductive potential by producing NADPH, a cellular reductant that protects against antioxidant enzyme activity. It has also been shown to play an essential protective role against metalloid-mediated oxidative stress by stringently regulating the accumulation of reactive oxygen species (ROS) (Ghosh et al., 2017). Thus, the pentose phosphate pathway is considered to play an essential role in protecting cells against oxidative stress (Ghosh et al., 2015). Based on our results, we speculate that Kac plays a vital role in protecting the biomolecules of *T. spiralis* from oxidative damage.

Carbon is metabolized continuously in parasites and is essential for energy circulation and parasite survival. The TCA cycle is essential for supplying the energy needed for numerous cellular functions. Unlike *Schistosoma,* which completely relies upon glucose fermentation and the formation of lactate to meet its energy requirements, *T. spiralis* utilizes the oxidation of carbohydrates to CO_2 and water via the TCA cycle as its major energy source (Goldberg, 1957).

A key entry point for carbon into the TCA cycle for energy production is the pyruvate dehydrogenase complex (PDC), which catalyzes the overall conversion of the glycolytic degradation products of carbohydrate metabolism and pyruvate to acetyl-CoA and CO_2. The PDC is composed of three different subunits: pyruvate decarboxylase (E1, PDH), dihydrolipoamide transacetylase (E2, DLAT), and dihydrolipoamide dehydrogenase (E3, DLD). All of these subunits are required to form NADH from the oxidation of pyruvate. In our study, we identified five acetylated proteins in this complex. Of note, the PDC in glycolysis was acetylated at 22 sites among its three subunits, with six and nine lysine sites on DLAT and DLD, respectively, modified by Kac. In human cancer cells, K321 acetylation of PDHA1 and K202 acetylation of PDH phosphatase (PDP1) inhibit PDC activity (Fan et al., 2014). Thus, the intensive acetylation of acetyl-CoA

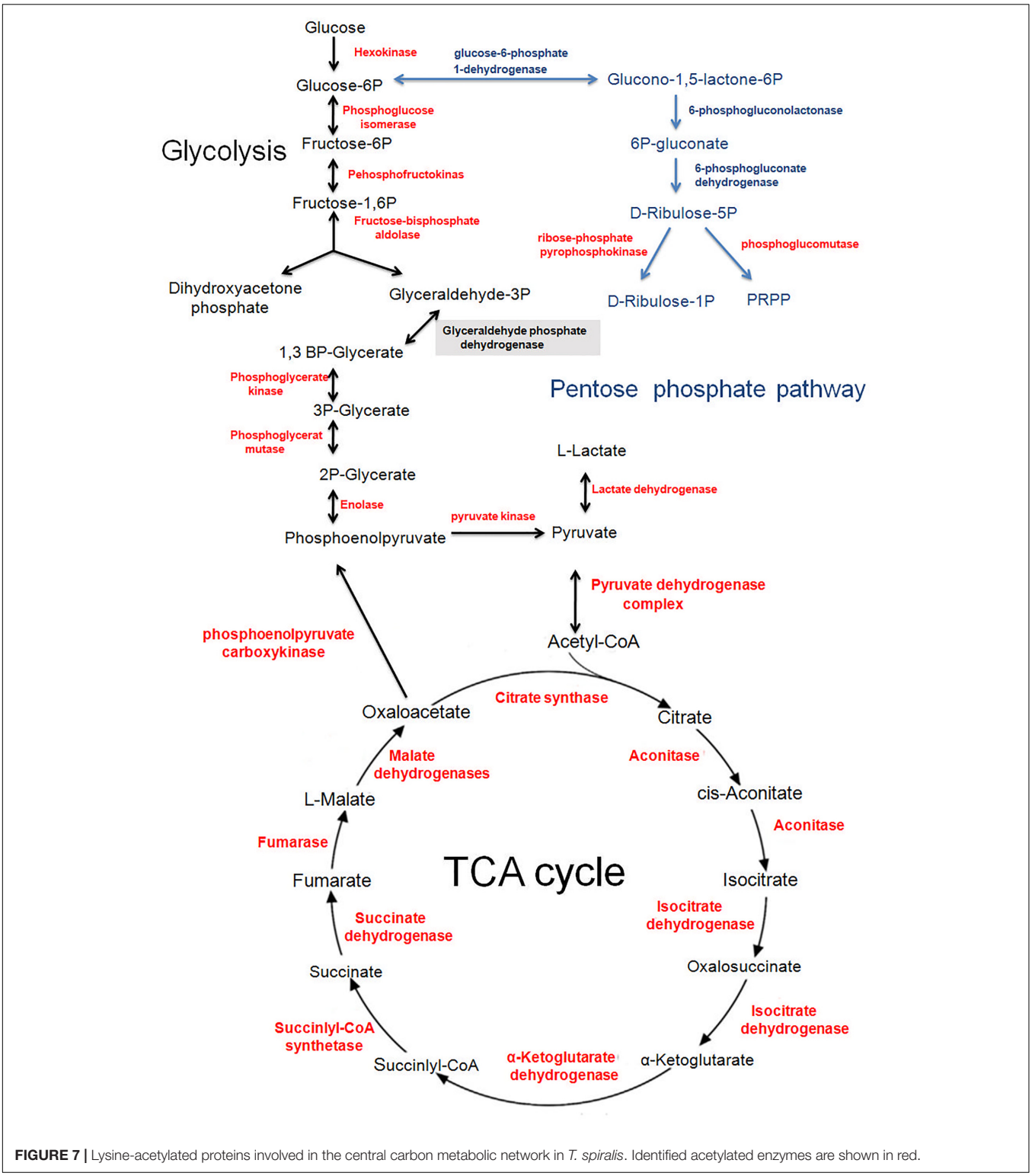


FIGURE 7 | Lysine-acetylated proteins involved in the central carbon metabolic network in *T. spiralis*. Identified acetylated enzymes are shown in red.

metabolism-related enzymes in *T. spiralis* may regulate the catalytic activity of the complex, especially PDH, and control its functional balance in *T. spiralis*.

Many mitochondrial proteins that play an essential role in energy metabolism exhibit a high level of acetylation in many organisms (Hong et al., 2016; Zhou et al., 2016). Previous studies have shown that the acetylation of malate dehydrogenase 1 (MDH1), a key enzyme in the TCA cycle, significantly enhanced its enzymatic activity. This led to a subsequent increase in intracellular NADPH levels, promoting

adipogenic differentiation in preadipocyte cells (Kim et al., 2012). We identified eight acetylation sites in malate dehydrogenase (MDH) in this study, including the acetylation of lysines 108, 119, 145, 168, 178, 228, 312, and 314. This protein was also found to be acetylated in *S. japonicum* (Hong et al., 2016). In our study, other enzymes involved in the TCA cycle were also identified as Kac targets, confirming that acetylation has a potential conserved function in the regulation of the TCA cycle in both eukaryotic and prokaryotic cells.

Protein Metabolism

In addition its regulation of glycometabolism, Kac was also observed in proteins regulating protein expression and proteolysis. In this study, we identified 120 lysine-acetylated proteins related to protein synthesis, accounting for 7.5% of the total lysine-acetylated proteins (Supplementary Table S1). Our results are consistent with those of previous studies showing that ribosomal proteins and aminoacyl–tRNA synthetases are the main targets of acetylation in both prokaryotes and eukaryotes. In *E. coli,* 22% of the acetylated proteins are involved in the translational machinery or processes (Zhang et al., 2013), while this figure is 15% in *Toxoplasma* (Jeffers and Sullivan, 2012*)*. Among these proteins, elongation factor 1-α of *T. spiralis* was heavily acetylated at 15 lysine residues, similar to the acetylation of its orthologs in other eukaryotes (humans, rats, and *Toxoplasma*) (Jeffers and Sullivan, 2012; Lundby et al., 2012; Shao et al., 2012). tRNA synthetases represent potential drug targets for the prevention and control of parasitic infections. For example, inhibitors of isoleucine–tRNA synthetase and seryl–tRNA synthetase can suppress *P. falciparum* growth (Miao et al., 2013). In this study, 20 aminoacyl–tRNA synthetases were found to have acetylated lysine residues, reflecting a higher frequency of acetylation than among some other eukaryotes.

The ubiquitin–proteasome system provides a major mechanism for regulating the degradation of cell-cycle regulators and potentially toxic misfolded proteins, and it is required for maintaining cellular protein homeostasis in eukaryotic cells. The core of this large protease is the 20S proteasome, a barrel-shaped structure made of a stack of four heptameric rings. Kac has dual roles in regulating protein degradation in the proteasome. A direct role of protein acetylation involves competing with protein ubiquitination for lysine sites, preventing the proteasomal degradation of target proteins. In addition, in some cases, the acetylation of a specific lysine can unexpectedly create a high-affinity binding site for other proteins, leading to its subsequent ubiquitination and degradation (Caron et al., 2005). In our study, the 20S core particle of the proteasome was frequently modified by Kac (eight proteins), particularly the α subunits (Supplementary Figure S1). Accordingly, three types of amino acid degradation-related enzymes associated with the degradation of valine, leucine, and isoleucine were acetylated (Supplementary Table S1), suggesting that acetylation plays a direct regulatory role in the ubiquitin–proteasome system and influences proteolytic processes in *T. spiralis*.

Phagocytosis, a multistep process that is completely dependent on cytoskeletal complexes (Radulovic and Godovac-Zimmermann, 2011; Drazic et al., 2016), has been shown to play crucial roles in the acquisition of nutrients, cell proliferation, and virulence in parasites (Mansuri et al., 2014). After binding to a cell surface receptor, the actin cytoskeleton is locally remodeled to provide the necessary force for the formation of phagocytic cups and phagosomes. The spatial and temporal regulation of actin dynamics is required for controlling the various stages of phagocytosis, and interference with actin dynamics reduces phagocytosis (Mansuri et al., 2014). Kac have been suggested to play a crucial role in maintaining actin structure and dynamics and facilitating stress fiber formation and actomyosin interactions (Choudhary et al., 2009). Another group of important cytoskeleton-associated proteins are microtubules, which are formed from α- and β-tubulin heterodimers. The acetylation of α-tubulin has been shown to affect the kinetics of tubulin polymerization and the localization of the motor protein kinesin. The hyperacetylation of microtubules causes the misregulation of transport by kinesin motors (Yang and Seto, 2008; Friedmann and Marmorstein, 2013). In addition, investigations into the antitumor efficacies of histone acetyltransferase inhibitors have shown that the acetylation level of tubulin may be a predictive biomarker of its sensitivity to Histone acetyltransferase inhibition (Di Martile et al., 2016). In our study, the cytoskeleton-associated proteins actin and tubulin, which are also involved in phagocytosis, were found to be acetylated (Supplementary Figure S2), indicating that the acetylation of cytoskeleton-associated proteins may participate in the regulation of phagocytosis in *T. spiralis*.

Recently, it has become evident that two major endoplasmic reticulum (ER) proteins, calnexin and calreticulin, are crucial regulators of particle uptake into phagosomes, controlling the opening and closing of the cups in *Dictyostelium discoideum* (Muller-Taubenberger et al., 2001). During phagocytosis, an acidic phagosomal environment generated by V-ATPases is of pivotal importance for the degradation of macromolecules. Unexpectedly, we found that these proteins important in phagocytosis were heavily acetylated in our study. Among these, eight ATPases were found to be acetylated at multiple sites (Supplementary Figure S2). To the best of our knowledge, this is the first time that abundant proteins involved in phagocytosis have been found to be acetylated in an organism, confirming that Kac is of great significance in the regulation of phagocytosis.

The complete life-cycle of *T. spiralis* occurs in a single host and can be divided into three main stages: ML, adult worms, and newborn larvae (NBL). These occupy two distinct intracellular niches: the intestinal epithelium and skeletal muscle cells. Among these developmental stages, *T. spiralis* has developed fascinating strategies for adapting to different environments and successfully establishing infection, such as the switching of parasite surface antigens. Studies have indicated that acetylation can regulate antigenic variation and developmental transitions in parasites, and these are principally determined by strong selective pressures (Coleman et al., 2014; Carranza et al., 2016). Thus, it would be of great interest to investigate the global acetyl-proteome of *T. spiralis* in other developmental stages and compare the levels of acetylation of the proteomes. This will facilitate the dissection of the role of the lysine acetylome in regulating the developmental transitions of *T. spiralis* and its establishment

of infection under the selective pressures of different living environments.

CONCLUSION

We have produced the first large-scale acetyl-proteome of *T. spiralis*, an important foodborne parasite, showing abundant Kac among *T. spiralis* proteins. The lysine acetylome in the present study provides a good foundation for in-depth studies of the functions of reversible Kac in the growth and development of *T. spiralis* and other nematodes. Identifying the functions of the acetylation of target proteins may also help to design specific and effective drugs or vaccines to prevent and control trichinellosis.

AUTHOR CONTRIBUTIONS

ML and YZ designed the research. YY, MT, XB, XiL, and IV carried out the data acquisition, analysis, and interpretation. PZ and WC performed the *T. spiralis* infection experiments. YY, MT, and XB contributed to the writing of the manuscript. XC and XuL contributed to improvement of the manuscript. All authors contributed to the study concept and critical revision of the manuscript for important intellectual content.

ACKNOWLEDGMENTS

This study was supported by the National Key Research and Development Program of China (2017YFD0501302 and 2016YFD0500707), Guangdong Innovative and Entrepreneurial Research Team Program (No. 2014ZT05S123), the scientific fund from Health and Family Planning Commission of Wuxi (No. Q201640), and the Fundamental Research Funds for the Central Universities (1282050205162430).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02674/full#supplementary-material

REFERENCES

Agosin, M., and Aravena, L. C. (1959). Anaerobic glycolysis in homogenates of *Trichinella spiralis* larvae. *Exp. Parasitol.* 8, 10–30. doi: 10.1016/0014-4894(59)90003-7

Bai, X., Hu, X. X., Liu, X. L., Tang, B., and Liu, M. Y. (2017). Current research of trichinellosis in China. *Front. Microbiol.* 8:1472. doi: 10.3389/fmicb.2017.01472

Bauer-Mehren, A. (2013). Integration of genomic information with biological networks using Cytoscape. *Methods Mol. Biol.* 1021, 37–61. doi: 10.1007/978-1-62703-450-0_3

Campos, E. I., and Reinberg, D. (2009). Histones: annotating chromatin. *Annu. Rev. Genet.* 43, 559–599. doi: 10.1146/annurev.genet.032608.103928

Caron, C., Boyault, C., and Khochbin, S. (2005). Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. *Bioessays* 27, 408–415. doi: 10.1002/bies.20210

Carranza, P. G., Gargantini, P. R., Prucca, C. G., Torri, A., Saura, A., Svard, S., et al. (2016). Specific histone modifications play critical roles in the control of encystation and antigenic variation in the early-branching eukaryote *Giardia lamblia*. *Int. J. Biochem. Cell Biol.* 81(Pt A), 32–43. doi: 10.1016/j.biocel.2016.10.010

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., et al. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325, 834–840. doi: 10.1126/science.1175371

Close, P., Creppe, C., Gillard, M., Ladang, A., Chapelle, J. P., Nguyen, L., et al. (2010). The emerging role of lysine acetylation of non-nuclear proteins. *Cell. Mol. Life. Sci.* 67, 1255–1264. doi: 10.1007/s00018-009-0252-7

Coleman, B. I., Skillman, K. M., Jiang, R. H. Y., Childs, L. M., Altenhofen, L. M., Ganter, M., et al. (2014). A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. *Cell Host Microbe* 16, 177–186. doi: 10.1016/j.chom.2014.06.014

Di Martile, M., Desideri, M., De Luca, T., Gabellini, C., Buglioni, S., Eramo, A., et al. (2016). Histone acetyltransferase inhibitor CPTH6 preferentially targets lung cancer stem-like cells. *Oncotarget* 7, 11332–11348. doi: 10.18632/oncotarget.7238

Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016). The world of protein acetylation. *Biochim. Biophys. Acta* 1864, 1372–1401. doi: 10.1016/j.bbapap.2016.06.007

Dubois, F., Caby, S., Oger, F., Cosseau, C., Capron, M., Grunau, C., et al. (2009). Histone deacetylase inhibitors induce apoptosis, histone hyperacetylation and up-regulation of gene transcription in *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 168, 7–15. doi: 10.1016/j.molbiopara.2009.06.001

Fan, J., Shan, C. L., Kang, H. B., Elf, S., Xie, J. X., Tucker, M., et al. (2014). Tyr phosphorylation of PDP1 toggles recruitment between ACAT1 and SIRT3 to regulate the pyruvate dehydrogenase complex. *Mol. Cell* 53, 534–548. doi: 10.1016/j.molcel.2013.12.026

Friedmann, D. R., and Marmorstein, R. (2013). Structure and mechanism of non-histone protein acetyltransferase enzymes. *FEBS J.* 280, 5570–5581. doi: 10.1111/febs.12373

Gao, F., Liu, X., Wu, X. P., Wang, X. L., Gong, D., Lu, H., et al. (2012). Differential DNA methylation in discrete developmental stages of the parasitic nematode *Trichinella spiralis*. *Genome Biol.* 13:R100. doi: 10.1186/gb-2012-13-10-r100

Ghosh, A. K., Saini, S., Das, S., Mandal, A., Sardar, A. H., Ansari, M. Y., et al. (2017). Glucose-6-phosphate dehydrogenase and Trypanothione reductase interaction protects *Leishmania donovani* from metalloid mediated oxidative stress. *Free Radic. Biol. Med.* 106, 10–23. doi: 10.1016/j.freeradbiomed.2017.02.008

Ghosh, A. K., Sardar, A. H., Mandal, A., Saini, S., Abhishek, K., Kumar, A., et al. (2015). Metabolic reconfiguration of the central glucose metabolism: a crucial strategy of *Leishmania donovani* for its survival during oxidative stress. *FASEB J.* 29, 2081–2098. doi: 10.1096/fj.14-258624

Goldberg, E. (1957). Studies on the intermediary metabolism of *Trichinella spiralis*. *Exp. Parasitol.* 6, 367–382. doi: 10.1016/0014-4894(57)90028-0

Gottstein, B., Pozio, E., and Nockler, K. (2009). Epidemiology, diagnosis, treatment, and control of trichinellosis. *Clin. Microbiol. Rev.* 22, 127–145. doi: 10.1128/CMR.00026-08

Henriksen, P., Wagner, S. A., Weinert, B. T., Sharma, S., Bacinskaja, G., Rehman, M., et al. (2012). Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in *Saccharomyces cerevisiae*. *Mol. Cell. Proteomics* 11, 1510–1522. doi: 10.1074/mcp.M112.017251

Hong, Y., Cao, X., Han, Q., Yuan, C., Zhang, M., Han, Y., et al. (2016). Proteome-wide analysis of lysine acetylation in adult *Schistosoma japonicum* worm. *J. Proteomics* 148, 202–212. doi: 10.1016/j.jprot.2016.08.008

Horton, P., Park, K. J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C. J., et al. (2007). WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* 35, W585–W587. doi: 10.1093/nar/gkm259

Hu, H. G., Zhu, W. W., Qin, J., Chen, M., Gong, L. Y., Li, L., et al. (2017). Acetylation of PGK1 promotes liver cancer cell proliferation and tumorigenesis. *Hepatology* 65, 515–528. doi: 10.1002/hep.28887

Hua, Y. H., Wu, C. Y., Sargsyan, K., and Lim, C. (2014). Sequence-motif detection of NAD(P)-binding proteins: discovery of a unique antibacterial drug target. *Sci. Rep.* 4:6471. doi: 10.1038/srep06471

Huang, J., and Berger, S. L. (2008). The emerging field of dynamic lysine methylation of non-histone proteins. *Curr. Opin. Genet. Dev.* 18, 152–158. doi: 10.1016/j.gde.2008.01.012

Hyun, K., Jeon, J., Park, K., and Kim, J. (2017). Writing, erasing and reading histone lysine methylations. *Exp. Mol. Med.* 49:e324. doi: 10.1038/emm.2017.11

Jeffers, V., and Sullivan, W. J. (2012). Lysine acetylation is widespread on proteins of diverse function and localization in the protozoan parasite *Toxoplasma gondii*. *Eukaryot. Cell* 11, 735–742. doi: 10.1128/Ec.00088-12

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277–D280. doi: 10.1093/nar/gkh063

Kim, E. Y., Kim, W. K., Kang, H. J., Kim, J. H., Chung, S. J., Seo, Y. S., et al. (2012). Acetylation of malate dehydrogenase 1 promotes adipogenic differentiation via activating its enzymatic activity. *J. Lipid Res.* 53, 1864–1876. doi: 10.1194/jlr.M026567

Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., et al. (2006). Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol. Cell.* 23, 607–618. doi: 10.1016/j.molcel.2006.06.026

Li, T. T., Liu, M. X., Feng, X., Wang, Z., Das, I., Xu, Y. P., et al. (2014). Glyceraldehyde-3-phosphate dehydrogenase is activated by lysine 254 acetylation in response to glucose signal. *J. Biol. Chem.* 289, 3775–3785. doi: 10.1074/jbc.M113.531640

Liu, J. Y., Zhang, N. Z., Li, W. H., Li, L., Yan, H. B., Qu, Z. G., et al. (2016). Proteomic analysis of differentially expressed proteins in the three developmental stages of *Trichinella spiralis*. *Vet. Parasitol.* 231, 32–38. doi: 10.1016/j.vetpar.2016.06.021

Liu, X. L., Song, Y. X., Jiang, N., Wang, J. L., Tang, B., Lu, H. J., et al. (2012). Global gene expression analysis of the zoonotic parasite *Trichinella spiralis* revealed novel genes in host parasite interaction. *PLOS Negl. Trop. Dis.* 6:e1794. doi: 10.1371/journal.pntd.0001794

Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., et al. (2012). Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep.* 2, 419–431. doi: 10.1016/j.celrep.2012.07.006

Mansuri, M. S., Bhattacharya, S., and Bhattacharya, A. (2014). A novel alpha kinase EhAK1 phosphorylates actin and regulates phagocytosis in *Entamoeba histolytica*. *PLOS Pathog.* 10:e1004411. doi: 10.1371/journal.ppat.1004411

Miao, J., Lawrence, M., Jeffers, V., Zhao, F., Parker, D., Ge, Y., et al. (2013). Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during *Plasmodium falciparum* intraerythrocytic development. *Mol. Microbiol.* 89, 660–675. doi: 10.1111/mmi.12303

Mitreva, M., Jasmer, D. P., Zarlenga, D. S., Wang, Z., Abubucker, S., Martin, J., et al. (2011). The draft genome of the parasitic nematode *Trichinella spiralis*. *Nat. Genet.* 43, 228–235. doi: 10.1038/ng.769

Muller-Taubenberger, A., Lupas, A. N., Li, H. W., Ecke, M., Simmeth, E., and Gerisch, G. (2001). Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *EMBO J.* 20, 6772–6782. doi: 10.1093/emboj/20.23.6772

Murrell, K. D., and Pozio, E. (2011). Worldwide occurrence and impact of human trichinellosis, 1986-2009. *Emerg. Infect. Dis.* 17, 2194–2202. doi: 10.3201/eid1712.110896

Nie, Z., Zhu, H., Zhou, Y., Wu, C., Liu, Y., Sheng, Q., et al. (2015). Comprehensive profiling of lysine acetylation suggests the widespread function is regulated by protein acetylation in the silkworm, *Bombyx mori*. *Proteomics* 15, 3253–3266. doi: 10.1002/pmic.201500001

Olia, A. S., Barker, K., McCullough, C. E., Tang, H. Y., Speicher, D. W., Qiu, J., et al. (2015). Non-enzymatic protein acetylation detected by NAPPA protein arrays. *Acs Chem. Biol.* 10, 2034–2047. doi: 10.1021/acschembio.5b00342

Pan, J. Y., Ye, Z. C., Cheng, Z. Y., Peng, X. J., Wen, L. Y., and Zhao, F. K. (2014). Systematic analysis of the lysine acetylome in *Vibrio parahemolyticus*. *J. Proteome Res.* 13, 3294–3302. doi: 10.1021/pr500133t

Radulovic, M., and Godovac-Zimmermann, J. (2011). Proteomic approaches to understanding the role of the cytoskeleton in host-defense mechanisms. *Expert Rev. Proteomics* 8, 117–126. doi: 10.1586/epr.10.91

Shao, J. L., Xu, D., Hu, L. D., Kwan, Y. W., Wang, Y. F., Kong, X. Y., et al. (2012). Systematic analysis of human lysine acetylation proteins and accurate prediction of human lysine acetylation through bi-relative adapted binomial score Bayes feature representation. *Mol. Biosyst.* 8, 2964–2973. doi: 10.1039/c2mb25251a

Shen, H., Xu, W., and Lan, F. (2017). Histone lysine demethylases in mammalian embryonic development. *Exp. Mol. Med.* 49:e325. doi: 10.1038/emm.2017.57

Spange, S., Wagner, T., Heinzel, T., and Kramer, O. H. (2009). Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int. J. Biochem. Cell Biol.* 41, 185–198. doi: 10.1016/j.biocel.2008.08.027

Sun, T., Li, X., Zhang, P., Chen, W. D., Zhang, H. L., Li, D. D., et al. (2015). Acetylation of Beclin 1 inhibits autophagosome maturation and promotes tumour growth. *Nat. Commun.* 6:7215. doi: 10.1038/ncomms8215

Theobald, D. L., Mitton-Fry, R. M., and Wuttke, D. S. (2003). Nucleic acid recognition by OB-fold proteins. *Annu. Rev. Biophys. Biomol. Struct.* 32, 115–133. doi: 10.1146/annurev.biophys.32.110601.142506

Wagner, G. R., and Hirschey, M. D. (2014). Non-enzymatic protein acylation as a carbon stress regulated by sirtuin deacylases. *Mol. Cell.* 54, 5–16. doi: 10.1016/j.molcel.2014.03.027

Walsh, C. T., Garneau-Tsodikova, S., and Gatto, G. J. (2005). Protein posttranslational modifications: the chemistry of proteome diversifications. *Angew. Chem. Int. Ed.* 44, 7342–7372. doi: 10.1002/anie.200501023

Wang, Q., Rosa, B. A., Nare, B., Powell, K., Valente, S., Rotili, D., et al. (2015). Targeting lysine deacetylases (KDACs) in parasites. *PLOS Negl. Trop. Dis.* 9:e0004026. doi: 10.1371/journal.pntd.0004026

Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., et al. (2010). Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327, 1004–1007. doi: 10.1126/science.1179687

Wang, Z. K., Cai, Q., Liu, J., Ying, S. H., and Feng, M. G. (2017). Global insight into lysine acetylation events and their links to biological aspects in *Beauveria bassiana*, a fungal insect pathogen. *Sci. Rep.* 7:44360. doi: 10.1038/srep44360

Weinert, B. T., Scholz, C., Wagner, S. A., Iesmantavicius, V., Su, D., Daniel, J. A., et al. (2013). Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep.* 4, 842–851. doi: 10.1016/j.celrep.2013.07.024

Witze, E. S., Old, W. M., Resing, K. A., and Ahn, N. G. (2007). Mapping protein post-translational modifications with mass spectrometry. *Nat. Methods* 4, 798–806. doi: 10.1038/nmeth1100

Xie, L., Wang, X., Zeng, J., Zhou, M., Duan, X., Li, Q., et al. (2015). Proteome-wide lysine acetylation profiling of the human pathogen *Mycobacterium tuberculosis*. *Int. J. Biochem. Cell Biol.* 59, 193–202. doi: 10.1016/j.biocel.2014.11.010

Xiong, Y., and Guan, K. L. (2012). Mechanistic insights into the regulation of metabolic enzymes by acetylation. *J. Cell Biol.* 198, 155–164. doi: 10.1083/jcb.201202056

Yang, J. M., Qiu, C. H., Xia, Y. X., Yao, L. X., Fu, Z. Q., Yuan, C. X., et al. (2010). Molecular cloning and functional characterization of *Schistosoma japonicum* enolase which is highly expressed at the schistosomulum stage. *Parasitol. Res.* 107, 667–677. doi: 10.1007/s00436-010-1913-z

Yang, X. J., and Seto, E. (2008). Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol. Cell.* 31, 449–461. doi: 10.1016/j.molcel.2008.07.002

Youn, H. D. (2017). Methylation and demethylation of DNA and histones in chromatin: the most complicated epigenetic marker. *Exp. Mol. Med.* 49:e321. doi: 10.1038/emm.2017.38

Zhang, K., Zheng, S. Z., Yang, J. S., Chen, Y., and Cheng, Z. Y. (2013). Comprehensive profiling of protein lysine acetylation in *Escherichia coli*. *J. Proteome Res.* 12, 844–851. doi: 10.1021/pr300912q

Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., et al. (2010). Regulation of cellular metabolism by protein lysine acetylation. *Science* 327, 1000–1004. doi: 10.1126/science.1179689

Zhou, X., Qian, G., Yi, X., Li, X., and Liu, W. (2016). Systematic analysis of the lysine acetylome in *Candida albicans*. *J. Proteome Res.* 15, 2525–2536. doi: 10.1021/acs.jproteome.6b00052

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Yang, Tong, Bai, Liu, Cai, Luo, Zhang, Cai, Vallée, Zhou and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 January 2018
doi: 10.3389/fmicb.2017.02680

Effect of Various Inoculum Levels of Multidrug-Resistant *Salmonella enterica* Serovar Heidelberg (2011 Ground Turkey Outbreak Isolate) on Cecal Colonization, Dissemination to Internal Organs, and Deposition in Skeletal Muscles of Commercial Turkeys after Experimental Oral Challenge

Divek V. T. Nair[1], Jijo Vazhakkattu Thomas[1], Sally Noll[1], Robert Porter Jr.[2] and Anup Kollanoor Johny[1]*

[1] Department of Animal Science, University of Minnesota, Saint Paul, MN, United States, [2] Department of Veterinary Population Medicine, University of Minnesota, Saint Paul, MN, United States

OPEN ACCESS

Edited by:
Maria Schirone,
Università di Teramo, Italy

Reviewed by:
Michael J. Rothrock,
Agricultural Research Service (USDA), United States
Bradley L. Bearson,
Agricultural Research Service (USDA), United States

***Correspondence:**
Anup Kollanoor Johny
anupjohn@umn.edu;
anupkollanoor@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *04 October 2017*
Accepted: *22 December 2017*
Published: *12 January 2018*

Citation:
Nair DVT, Vazhakkattu Thomas J, Noll S, Porter R Jr. and Kollanoor Johny A (2018) Effect of Various Inoculum Levels of Multidrug-Resistant Salmonella enterica Serovar Heidelberg (2011 Ground Turkey Outbreak Isolate) on Cecal Colonization, Dissemination to Internal Organs, and Deposition in Skeletal Muscles of Commercial Turkeys after Experimental Oral Challenge. Front. Microbiol. 8:2680. doi: 10.3389/fmicb.2017.02680

Salmonella enterica serovar Heidelberg (*S*. Heidelberg) is a major foodborne pathogen colonizing poultry. The pathogen is associated with a significant number of foodborne outbreaks through contaminated poultry meat, including turkeys. Recently, multidrug-resistant (MDR) strains of *S*. Heidelberg have emerged as a threat to human public health in the United States. The objective of this study was to determine the cecal colonization, dissemination to internal organs, and the potential for skeletal muscle deposition of an MDR *S.* Heidelberg isolate from the 2011 ground turkey outbreak in the United States after the experimental oral challenge of poults (young turkeys) and adult turkey hens. In the poult study, two separate experiments using day-old, straight-run, commercial hybrid converter poults were randomly assigned to five challenge groups (0, 10^2, 10^4, 10^6, 10^8 CFU groups; 12 poults/group; $N = 60$/experiment) and a week after, treatment groups were challenged separately with 0-, 2-, 4-, 6-, and 8- $\log_{10}$ CFU of *S.* Heidelberg orally. After 14 days post-challenge, the poults were euthanized, and samples were collected to determine MDR *S.* Heidelberg colonization in the cecum, dissemination to liver and spleen, and deposition in the thigh, drumstick, and breast muscles. A similar experimental design was followed for the adult turkey hens. In two separate experiments, 11-week-old commercial Hybrid Converter turkey hens (4 hens/group; $N = 20$/experiment) were challenged with MDR *S*. Heidelberg and on day 16 post-challenge, birds were euthanized and samples were collected to determine *Salmonella* populations in the samples. The results indicated that, in turkey poults, the recovery of MDR *S.* Heidelberg was highest in the cecum followed by spleen, liver, thigh,

drumstick, and breast. All tested inoculum levels resulted in more than 3.5 $\log_{10}$ CFU/g colonization in the poult cecum. The cecal colonization, dissemination to internal organs, and tissue deposition of MDR *S.* Heidelberg were high in poults. The pathogen recovery from the cecum of adult turkey hens ranged from 37.5 to 62.5% in the challenge groups. The results signify the importance of controlling MDR *S*. Heidelberg in turkeys at the farm level to improve the safety of turkey products.

Keywords: *Salmonella* Heidelberg, turkeys, challenge, colonization, dissemination, muscle, multidrug-resistant

INTRODUCTION

Gastrointestinal (GI) illness caused by foodborne pathogens is a major public health concern resulting in significant loss to the United States economy (Scallan et al., 2011; Scharff, 2012; Marder et al., 2017). Poultry meat and eggs contribute to >50% foodborne outbreaks associated with non-typhoidal *Salmonella* (CDC, 2013). Poultry, including turkeys, are a common reservoir host for *Salmonella* and are commonly implicated contamination vehicles for human infections (Mead et al., 2010). The colonization of the GI tract of poultry with the pathogen results in the excretion through droppings, subsequently contaminating the farm environment and the poultry carcasses during processing. Prevalence of *Salmonella* in poultry-derived foods, including turkeys, along with increased consumer taste for poultry products in the United States are potential risk factors for foodborne outbreaks (Foley et al., 2008; NCC, 2017).

Salmonella has > 2500 serovars, and among these, 7% are associated with foodborne outbreaks through poultry. The predominant serovars such as *S.* Enteritidis and *S.* Typhimurium are continuing as major pathogens whereas emerging serovars such as *S.* Heidelberg, *S.* Infantis, and *S.* Oranienburg are new threats to the United States food industry (Foley et al., 2011; CDC, 2016; Hindermann et al., 2017). Among these, *S.* Heidelberg is highly invasive affecting humans and is among the top five *Salmonella* serovars frequently associated with human salmonellosis (CDC, 2013). In addition, *S.* Heidelberg is among the top three serovars of *Salmonella* commonly isolated from chickens under pathogen reduction and HACCP (PR: HACCP) verification samples for broiler meat and outbreaks associated with chickens (FSIS, 2010; Finstad et al., 2012). Moreover, *S.* Heidelberg is commonly isolated from turkey production facilities and has been accountable for 14% of foodborne outbreaks through turkeys in the previous years (1998–2008) (Jackson et al., 2013). Furthermore, *S.* Heidelberg is only second to *S.* Enteritidis in causing foodborne outbreaks through eggs (Jackson et al., 2013).

Development of antibiotic resistance in *S.* Heidelberg and the involvement of resistant strains in foodborne outbreaks through poultry is a serious concern. In 2010, 65% *S.* Heidelberg isolated from ground turkey were resistant to multiple drugs, including ceftriaxone, the drug of choice for treating human salmonellosis, and many other clinically relevant antibiotics such as streptomycin, tetracycline, sulfamethoxazole, chloramphenicol, and trimethoprim-sulfamethoxazole (Hoffmann et al., 2012, 2014). In 2011, *S.* Heidelberg caused a foodborne outbreak through contaminated ground turkey products resulting in 136 reported cases from 34 states. Some of the isolates implicated in the outbreak were resistant to common antibiotics such as ampicillin, streptomycin, gentamicin, and tetracycline. These isolates carry five plasmid-encoded resistance genes such as *bla*TEM-1, *aac (3)-IIa*, *aadA1*, *ant (3_)-Ia*, and *tetA*. The encoded plasmids are the IncI1 type that are common poultry associated plasmids (Folster et al., 2012). In addition, foodborne outbreaks linked to multidrug-resistant (MDR) *S.* Heidelberg were reported from poultry products from a leading producer in California and to the mechanically separated chicken from a Tennessee correctional facility in 2013 and 2014, respectively (CDC, 2014a,b). Moreover, Rothrock et al. (2015) reported the recovery of MDR *S.* Heidelberg from water used in scalding tanks, underscoring the necessity of understanding this pathogen as a public health threat emerging from the poultry farms.

Although the current literature presents some evidence on the use of antibacterials against *S.* Heidelberg, since the 2011 ground turkey outbreak, studies that determine the response of turkeys to the MDR clones of this pathogen, are only emerging. The objectives of the current study were to determine (1) the colonization potential of a 2011 ground turkey outbreak isolate in poults (young turkeys) and adult turkey hens, and (2) the dose required for effective cecal colonization, dissemination of the pathogen to liver and spleen, and the risk of deposition in skeletal muscle tissues, after the experimental oral challenge.

MATERIALS AND METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee, and the use of MDR *S.* Heidelberg (infectious agent) in turkeys was approved by the Institutional Biosafety Committee at the University of Minnesota.

Pathogen, Growth Conditions, and Inoculum Preparation

One of the 2011 ground turkey outbreak isolates of MDR *S.* Heidelberg was used in the current study (Donators – Dr. Irene Hanning, College of Genome Sciences and Technology, University of Tennessee, and Dr. Kumar Venkitanarayanan, University of Connecticut; Identity of the isolate at Dr. Kollanoor Johny's lab: GT2011). The glycerol stocks of MDR *S.* Heidelberg was prepared and stored at −80°C before the experiment. Working cultures were prepared by transferring 100 μl of MDR *S.* Heidelberg from glycerol stock to 10 ml tryptic soy broth (TSB; catalog no. C7141, Criterion, Hardy Diagnostics, Santa Maria,

CA, United States) and were incubated at 37°C for 24 h with agitation (100 rpm). For selective enumeration, the pathogen was made resistant to 50 μg/ml nalidixic acid sodium salt (NA; CAS. no. 3374-05-8, Alfa Aesar, Haverhill, MA, United States). Growth of NA resistant *S.* Heidelberg (Identity of the isolate at Dr. Kollanoor Johny's lab: GT2011NAL) in overnight broth cultures (24 h) was determined by plating appropriate dilutions of *S.* Heidelberg on xylose lysine desoxycholate agar plates (XLD; catalog no. C 7322, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) containing 50 μg/ml NA and incubating at 37°C for 24 h. For inoculating birds, *S.* Heidelberg was grown in 100 ml TSB containing 50 μg/ml NA. The pathogen inoculum was prepared from 24 h broth culture after centrifuging at 3,600 × *g* for 15 min at 4°C and resuspending the pellets in phosphate-buffered saline (PBS; pH 7.2). Five different inoculum levels of *S.* Heidelberg: 0, 10^2, 10^4, 10^6, and 10^8 CFU/ml were used in poults and adult turkey hens, in separate studies.

Experimental Design

Turkey Poult Study

Day-old poults were purchased from a commercial turkey hatchery in Minnesota and were housed in the BSL2 Veterinary Isolation Facility of the Research Animal Resources (RAR) at the University of Minnesota. Two separate experiments were conducted. In each experiment, 60 poults were randomly distributed to 5 treatment groups (12 poults/treatment group): a negative control (Negative Control; 0 CFU *S.* Heidelberg), challenge group 1 (2-log; 10^2 CFU *S.* Heidelberg), challenge group 2 (4-log; 10^4 CFU *S.* Heidelberg), challenge group 3 (6-log; 10^6 CFU *S.* Heidelberg) and challenge group 4 (8-log; 10^8 CFU *S.* Heidelberg). On day 0, the incoming flock was tested for any inherent *Salmonella* by enriching the fecal samples in Selenite Cysteine Broth (SCB, Hardy Diagnostics; $n = 6$). On day 7, the poults were challenged with appropriate levels of MDR *S.* Heidelberg as crop gavage. *S.* Heidelberg recovery was determined in the cecum (for colonization), spleen and liver (for dissemination), and skeletal muscles [drumstick (*Peroneus longus*), thigh (*Semimembranosus*) and breast (*Pectoralis major*)] for potential muscle deposition, after euthanizing poults on days 9 (2 poults/group) and 21 (10 poults/group) of the study. *S.* Heidelberg colonization was ensured on day 9. Tissue samples (liver, spleen, and muscles; 2 samples/study) were also collected on day 21 for histopathology and immunohistochemistry examination.

Turkey Hen Study

Adult turkey hens (11-weeks old) were housed in the BSL2 Veterinary Isolation Barn of the RAR at the University of Minnesota. Two separate experiments were conducted. In each experiment, 20 hens were randomly distributed to 5 treatment groups (4 hens/treatment group) as mentioned for the poult study (Negative Control, 2-log, 4-log, 6-log, and 8-log). Turkeys were given a week for acclimatization in the isolation barn pens and were tested for any inherent *Salmonella* by enriching the fecal samples ($n = 6$). A week after, the birds were challenged with appropriate levels of MDR *S.* Heidelberg orally. *S.* Heidelberg recovery rates were determined in the cecum, spleen, liver, and skeletal muscles after euthanizing birds on day 16 post-challenge (4 hens/group) of the study. Tissue samples (liver, spleen, and muscles; 4 samples/group) were collected for histopathology and immunohistochemistry examination.

Determination of *S.* Heidelberg in Cecum, Liver, Spleen, and Skeletal Muscles

The samples were collected in 50 ml sterile PBS tubes on the day of *S.* Heidelberg recovery (necropsy days). Samples were homogenized, 10-fold serially diluted in PBS and 200 μl of appropriate dilutions were surface plated on XLD + NA plates. *S.* Heidelberg enumeration was conducted after incubating the plates at 37°C for 24 h. In addition, all samples from the poult and adult turkey hen studies were enriched in 10 ml SCB on the day of collection. After incubation for 8–12 h at 37°C, cultures from SCB was streaked on XLD and XLD + NA plates. The plates were incubated for 24 h at 37°C to detect the presence of *S.* Heidelberg in the enriched samples (Hammack et al., 1999). In adult turkey hens, the fecal samples were also enriched daily for 16 days post-challenge to detect fecal shedding of *S.* Heidelberg. This step was to determine if the adult turkey hens remained positive for the pathogen despite their maturity.

Histopathology Examination

Histopathological examination of tissue samples from liver, spleen, and muscles [drum stick (*Peroneus longus*), thigh (*Semimembranosus*) and breast (*Pectoralis major*)] of the birds was conducted. Tissue sections of 5 mm thickness were collected in 10% neutral buffered formalin from both challenged and non-challenged groups. Histological examinations were carried out after processing and staining the samples using a standard hematoxylin and eosin staining (Gu et al., 2015; Tavakkoli et al., 2015) at the Veterinary Diagnostic Laboratory at the University of Minnesota.

Immunohistochemistry

The formalin-fixed tissue samples were also used for detection of *Salmonella* antigens in the tissues using antibodies specific for *Salmonella* by conducting immunohistochemistry as described previously (Campero et al., 2002). Briefly, the paraffin-embedded sections of tissues were deparaffinized by placing in a slide rack at 60–70°C for 30–45 min. Then the tissue sections were rehydrated by passing through descending grades of alcohol in a chemical hood. After washing, the slides were treated with Proteinase K (Dako Agilent Pathology Solutions, Santa Clara, CA, United States) enzyme for the retrieval of antigen and incubated in a humidity chamber for 5 min. The slides were then immersed in 0.05M TBS/Tween 20 buffer after washing with distilled water. The endogenous peroxidase enzyme was blocked by adding 3.0% H_2O_2 and incubated for 15 min. Then the slides were incubated adding mouse anti-*Salmonella* LPS core antibody (ViroStat, Inc., Portland, ME, United States) for 45 min at room temperature. Positive and negative samples were included. After incubating with primary antibody, the slides were incubated for 45 min at room temperature with

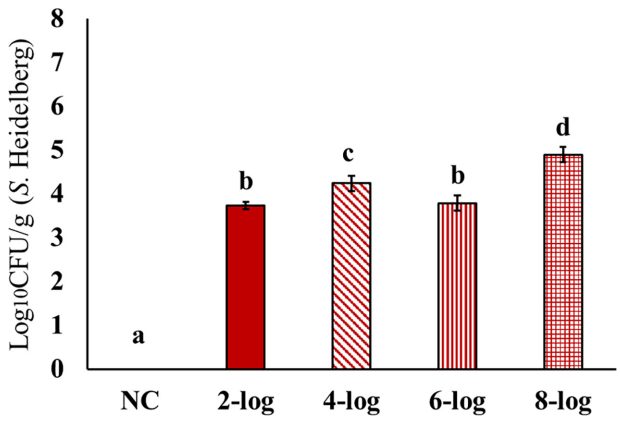


FIGURE 1 | Effect of different inoculum levels on MDR *S.* Heidelberg colonization in the ceca of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 20, 20, 18, 19, and 20 cecum samples/group, respectively, for the final analysis. [a–d] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

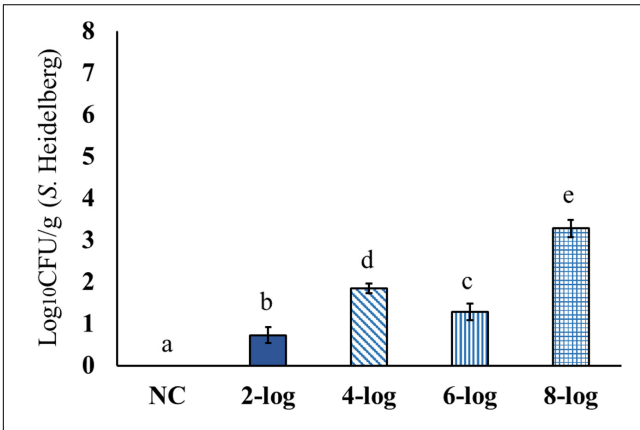


FIGURE 2 | Effect of different inoculum levels on the dissemination of MDR *S.* Heidelberg to spleen of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 18, 18, 16, 17, and 18 spleen samples/group, respectively, for the final analysis. [a–e] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

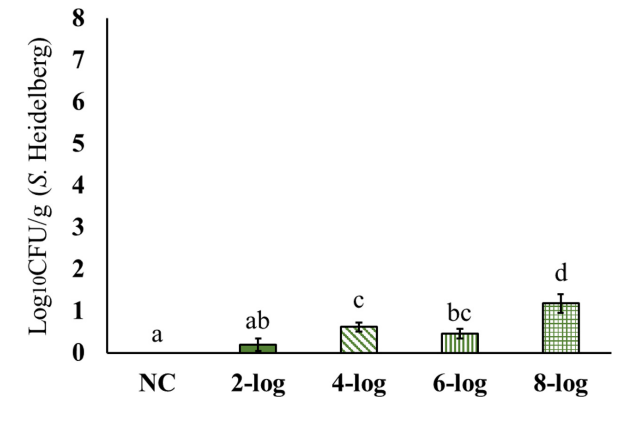


FIGURE 3 | Effect of different inoculum levels on the dissemination of MDR *S.* Heidelberg to liver of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 20, 20, 18, 18, and 20 liver samples/group, respectively, for the final analysis. [a–d] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

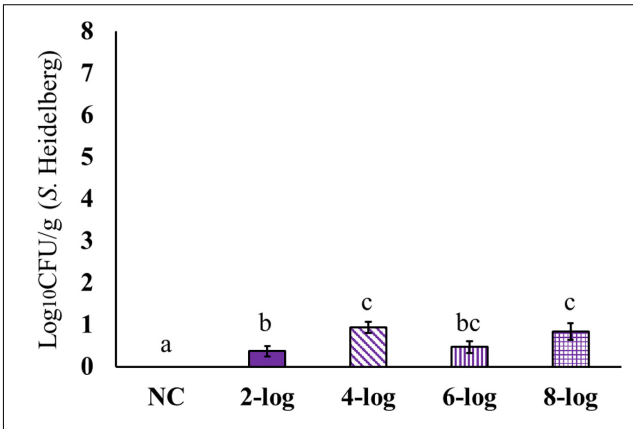


FIGURE 4 | Effect of different inoculum levels on MDR *S.* Heidelberg deposition in the thigh (*Semimembranosus* muscle) of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 20, 20, 18, 19, and 20 thigh samples/group, respectively, for the final analysis. [a,b,c] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

goat anti-mouse IgG (H + L), HRP conjugate (Dako Agilent Pathology Solutions, Carpinteria, CA, United States) which served as secondary antibody. A chromogen, 3-Amino-9-Ethylcarbazole (Dako Agilent Pathology Solutions, Carpinteria, CA, United States) was added to the slides and incubated for 15 min to detect the immune reactivity. The slides were washed and stained with Mayer's Hematoxylin (counterstain) for 5 min. The slides were then rinsed with tap water, mounted and observed under the microscope.

Statistical Analysis

Data from the poult and hen studies were evaluated separately due to the difference in the colonization of *S.* Heidelberg in different age groups. A completely randomized design with a 2X5X6 factorial treatment structure was used for both studies. The factors included two experiments/study, five inoculum levels (0, 10^2, 10^4, 10^6, and 10^8 CFU/ml), and six tissue samples (cecum, liver, spleen, breast, thigh, drumstick). An isolator (poult study) or an isolator room (adult turkey hen study) was the experimental unit, and each study were repeated (two experiments per age group; a total of four experiments discussed in the manuscript). The number of birds used in the study was sufficient to detect significant mean differences in the pathogen counts between the groups with a two-sided alpha = 0.05 and statistical power of 0.99 and 0.91, respectively, for the poult and hen studies. Normally distributed data were analyzed using the PROC-MIXED procedure of the SAS software (version 9.4, SAS Institute, Cary, NC, United States). Whenever a qualitative analysis was required (presence and absence), we used PROC-GENMODE procedure of the SAS software. A *P* value of

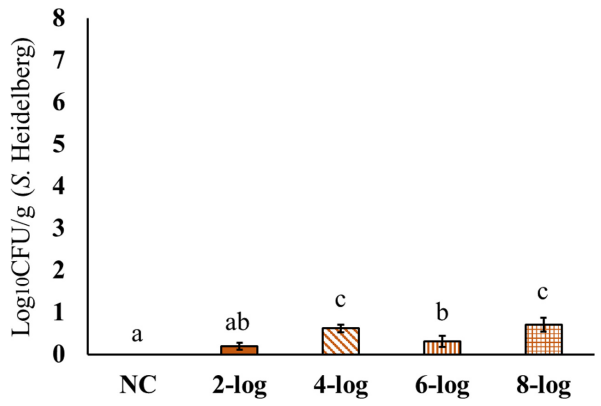


FIGURE 5 | Effect of different inoculum levels on MDR *S.* Heidelberg deposition in the drumstick (*Peroneus longus* muscle) of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 20, 20, 18, 19, and 20 drumstick samples/group, respectively, for the final analysis. [a,b,c] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

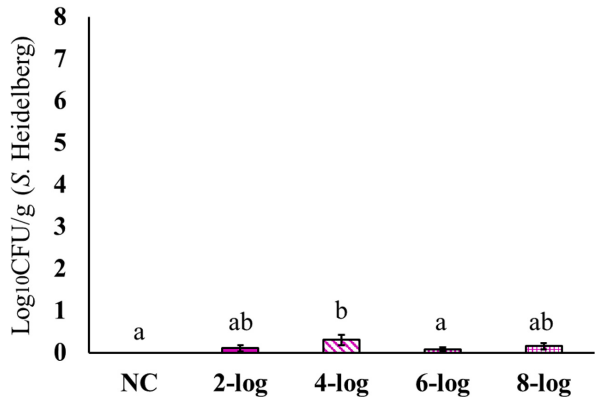


FIGURE 6 | Effect of different inoculum levels on MDR *S.* Heidelberg deposition in the breast (*Pectoralis major* muscle) of poults (Means ± SE; NC, 2-log, 4-log, 6-log, and 8-log treatments had a total of 20, 20, 18, 19, and 20 breast samples/group, respectively, for the final analysis. [a,b] Bars with different superscripts differ significantly from each other at $P < 0.05$). NC, Negative Control.

0.05 was considered statistically significant. For histopathology and immunohistochemistry, presence or absence of bacteria by visual analysis was carried out (Gonzalez-Escobedo et al., 2013).

RESULTS AND DISCUSSION

Non-typhoidal *Salmonella* is the leading bacterial cause of foodborne illness in the United States. Poultry and poultry products, including turkeys, are epidemiologically linked to human outbreaks and remain major contributors of foodborne infections. Although carcass processing plays a significant role in the cross-contamination, farms are the focal points of *Salmonella* persistence and distribution to poultry. Several sources such as litter, feed, water, transportation equipment and vectors including insects, humans, and rodents exist on farms (Jones et al., 1991; Suzuki, 1994; Hoover et al., 1997; Foley et al., 2008). In addition, poultry serves as the natural reservoir host for several *Salmonella* serovars (Hopper and Mawer, 1988; Foley et al., 2011; Kollanoor Johny and Kumar, 2016). *Salmonella* enters the intestinal tract of poultry after ingestion and establishes colonization in the cecum. The pathogen uses various virulence mechanisms to cross the intestinal barriers, multiplies in the reticuloendothelial system and invades liver, spleen, ovary, and oviduct resulting in the systemic spread of the infection (Suzuki, 1994; Kollanoor-Johny et al., 2012a,b; Kollanoor Johny and Kumar, 2016). In the current study, *S.* Heidelberg was selected since it is an emerging serovar of *Salmonella* that contributes to human infections and *Salmonella*-related deaths in the United States (Kennedy et al., 2004; Gokulan et al., 2013). In addition, there is a recent interest in studying the MDR strains of *S.* Heidelberg involved in the 2011 ground turkey outbreak (Bearson et al., 2017; Nair and Kollanoor Johny, 2017). In the present study, different inoculum levels of *S.* Heidelberg were administered in poults and adult turkey hens orally. Then the colonization efficacy of the MDR isolates in the cecum, dissemination to liver and spleen, and potential risk of the pathogen deposition in the muscle tissues [drum stick (*Peroneus longus*), thigh (*Semimembranosus*) and breast (*Pectoralis major*)] were studied.

Turkey Poult Study

Salmonella Heidelberg showed high colonization potential in the cecum of poults resulting in an efficient colonization for all the tested inoculum levels (2-, 4-, 6-, and 8- log) (**Figure 1**). All cecal samples tested positive for *Salmonella*. In both experiments, 2-log, 4-log, and 6-log of MDR *S.* Heidelberg resulted in 3.7–4.9 $\log_{10}$ CFU/g colonization in the cecum of poults. However, 8-log inoculum resulted in the maximum colonization among the tested inoculum levels ($P \leq 0.05$; 5.1 and 4.7 $\log_{10}$ CFU/g in experiments 1 and 2, respectively). The results indicated that the inoculation level as low as 2-log is capable of effective colonization of MDR *S.* Heidelberg in the cecum of poults (**Figure 1**).

Results showed significant dissemination of MDR *S.* Heidelberg to spleen for all the tested inoculum levels (**Figure 2**). The highest counts were observed with 8-log inoculum of *S.* Heidelberg that resulted in ∼3 $\log_{10}$ CFU/g in the spleen of 21 days old poults. The 4-log, and 6-log inoculation levels resulted in 1.0 to 2.0 $\log_{10}$ CFU/g *S.* Heidelberg in the spleen (**Figure 2**). However, dissemination of MDR *S.* Heidelberg to the liver was less compared to that of the spleen (**Figure 3**). Majority of the spleen (61 positive/69 total), and liver (46 positive/76 total) samples were positive by surface plating. Samples that yielded no colonies by surface plating were confirmed negative by enrichment method.

Interestingly, MDR *S.* Heidelberg evidently reached muscle samples in poults (**Figures 4–6**). However, the deposition of the isolate was less in the muscles. The inoculum levels administered to the poults could not be related to the recovery of the pathogen from the muscle samples in both experiments. For different

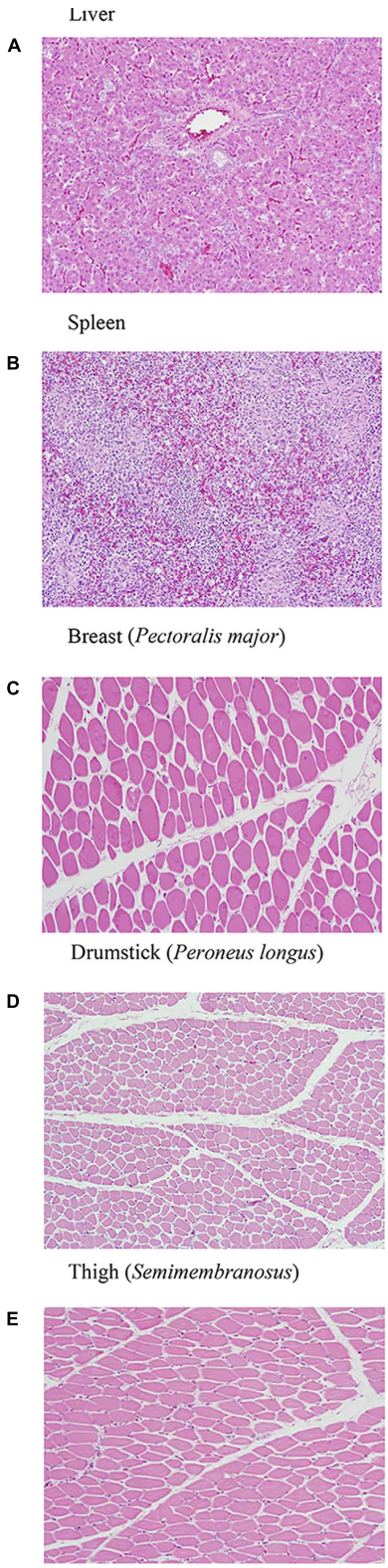


FIGURE 7 | (A–E) H&E staining of liver, spleen, and muscle samples after 14 days of MDR *S.* Heidelberg challenge in poults did not reveal any pathological changes signifying an infection [only the representative samples from the highest inoculum (8 $\log_{10}$ CFU) are included].

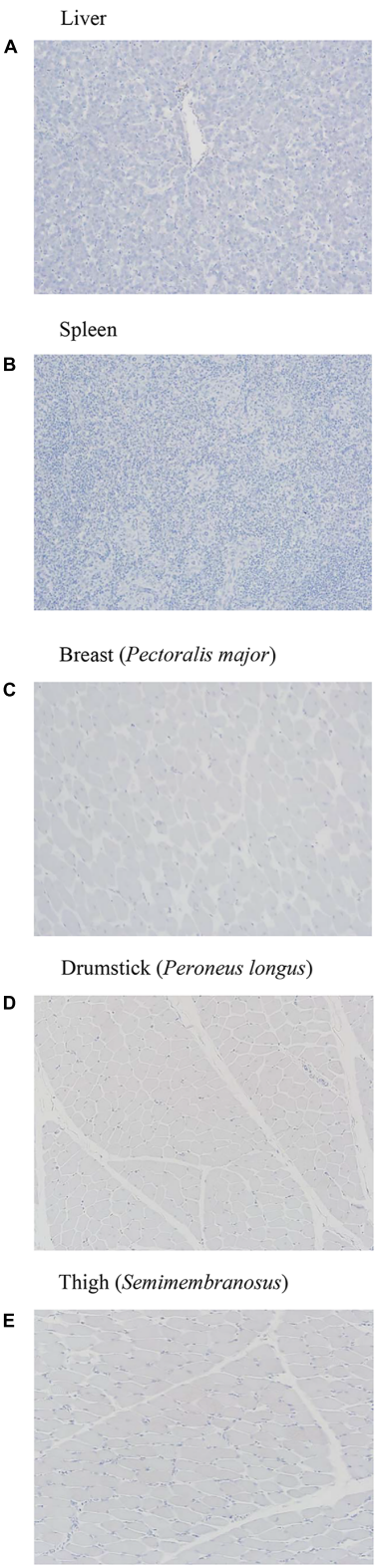


FIGURE 8 | (A–E) Immunohistochemistry of liver, spleen, and muscle samples after 14 days of MDR *S.* Heidelberg challenge in poults did not reveal any pathological changes signifying an infection [only the representative samples from the highest inoculum (8 $\log_{10}$ CFU) are included].

TABLE 1 | Effect of various inoculum levels of MDR *S.* Heidelberg on cecal colonization, dissemination to internal organs and deposition in skeletal muscles of adult turkey hens 16 days post inoculation (4 birds/group/study; 2 total studies).

Description	Experimental groups				
	0 CFU	10^2 CFU	10^4 CFU	10^6 CFU	10^8 CFU
Cecum*	0/8 (0%)	4/8 (50%)	3/8 (37.5%)	4/8 (50%)	6/8 (62.5%)
Liver	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
Spleen	0/8 (0%)	0/8 (0%)	0/8 (0%)	1/8 (12.5%)	2/8 (25%)
Semimembranosus	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
Peroneus longus	0/8 (0%)	1/8 (12.5%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
Pectoralis major	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)

*P-value for linear dose response < 0.05.

TABLE 2 | Excretion level of MDR *S.* Heidelberg after challenging the adult turkey hens with different inoculum levels (by fecal enrichment; 2 samples/day/isolation room/study; total 2 studies).

	Experimental groups				
Days	0 CFU	10^2 CFU	10^4 CFU	10^6 CFU	10^8 CFU
1–11	0/4 (0%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
12	0/4 (0%)	2/4 (50%)	2/4 (50%)	4/4 (100%)	4/4 (100%)
13	0/4 (0%)	4/4 (100%)	0/4 (0%)	4/4 (100%)	4/4 (100%)
14	0/4 (0%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
15	0/4 (0%)	3/4 (75%)	2/4 (50%)	4/4 (100%)	3/4 (75%)
16*	0/4 (0%)	1/4 (25%)	2/4 (50%)	4/4 (100%)	4/4 (100%)

*P-value for linear dose response < 0.05.

inoculum levels of *S.* Heidelberg, the pathogen recovery was 0.4–1.0 (**Figure 4**), 0.2–0.7- (**Figure 5**) and 0.1–0.3- (**Figure 6**) $\log_{10}$ CFU/g *S.* Heidelberg from thigh (49 positive samples /77 total), drumstick (41 positive samples /77 total), and breast muscles (18 positive samples /77 total), respectively.

This study revealed that even with a lower inoculum of 2-log *S.* Heidelberg, the colonization could reach ∼4 $\log_{10}$ CFU/g in the cecum of poults. This means that the ingestion of ∼100 *Salmonella* cells (2 $\log_{10}$ CFU/ml) could cause effective pathogen colonization in poults after 14 days of challenge. The results showed a significant dissemination of MDR *S.* Heidelberg to the internal organs, and resulted in muscle tissue deposition in poults. Both colonization and dissemination of *S.* Heidelberg in poults underscore the potential role of infected poults as sources of farm and product contamination. The situation warrants adoption of effective *Salmonella* intervention strategies starting at day 0. Appropriate hygienic measures have to be adopted to reduce the colonization between the flocks.

Previous studies have indicated *S.* Heidelberg as a colonizer in poultry similar to other predominant serovars such as *S.* Enteritidis. A survey carried out by Borsoi et al. (2011) found that *S.* Heidelberg colonized in the cecum of broiler chicks and appeared in the cecum after 6 h post-infection. After 12 h postinfection, *S.* Heidelberg counts were higher in the cecum compared to *S.* Enteritidis. To follow, after 72 h of infection, both the serovars showed similar colonization in the cecum. In the same study, *S.* Heidelberg showed similar invasion potential to the liver as that of *S.* Enteritidis. Similarly, Menconi et al. (2011) reported high colonization of *S.* Heidelberg in poults where 5 $\log_{10}$ CFU *S.* Heidelberg challenge resulted in ∼7.0 $\log_{10}$ CFU/g colonization in the cecum of poults. However, the same inoculum level resulted only in ∼3.0 $\log_{10}$ CFU/g colonization in the cecum of broiler chicks, indicating comparative propensity of the serovar for colonization in poults.

The high colonization potential and survival and multiplication of MDR *S.* Heidelberg in poultry could be attributed to its virulence mechanisms. *S.* Heidelberg possesses different transmissible plasmids that contain genes encoding antimicrobial resistance, virulence, and a VirB4/D4 type-IV secretion system. The plasmids having VirB4/D4 type-IV secretion system is unique to *S.* Heidelberg that promotes the invasion and prolonged survival in the intestinal epithelial cells and macrophages. In addition, the presence of VirB4/D4 type-IV secretion system increases the virulence of *S.* Heidelberg and enables the pathogen to down-regulate host immune system (Gokulan et al., 2013). The plasmids that encode resistance genes such as *bla*TEM-1, *aac (3)-IIa*, *aadA1*, *ant(3_)-Ia*, and *tetA* are poultry related plasmids which also accounts for increased virulence and survival of *S.* Heidelberg in poultry (Folster et al., 2012).

In this study, the H&E and immunohistochemistry staining did not show pathological changes despite dissemination to liver, spleen or muscles (**Figures 7A–E**, **8A–E**). This could be because of the lower number of samples used for the analyses.

Adult Turkey Hen Study

The recovery of *S.* Heidelberg from the cecum, internal organs (liver and spleen) and muscles (thigh, breast, and drumstick)

of adult turkey hens 16 days after inoculation was determined. *S.* Heidelberg could be recovered from all *Salmonella* challenge groups in the cecum by surface plating method. In addition, recovery of MDR *S.* Heidelberg was assessed by enriching the samples for presence or absence the pathogen. The pathogen recovery rates from the cecum of adult turkey hens were 0, 50, 37.5, 50, and 62.5% respectively, for 0-, 2-, 4-, 6-, and 8-log inoculum levels (**Table 1**; $P < 0.05$ for linear dose-response). There was no recovery of *S.* Heidelberg from the liver samples from any of the groups (**Table 1**). Low recovery of MDR *S.* Heidelberg could be obtained from the spleen samples of adult turkey hens. No muscle deposition of MDR *S.* Heidelberg was noticed except in one drumstick sample in the 2-log inoculum group by the enrichment method. However, similar to the turkey poult study, the histology results did not reveal the potential presence of the pathogen (pictures not included) even though it was recovered from spleen, and muscle tissues by enrichment method at the higher inoculum level. In the present study, adult turkey hens seemed to be less susceptible to MDR *S.* Heidelberg infection at lower doses, as determined by surface plating. However, it should be noted that the birds were challenged at week 12. By this time, the turkeys would have developed a strong immune system and the protective natural microbiota (Smith and Tucker, 1980; Corrier et al., 1991).

Enrichment of the fecal samples from the isolation rooms was conducted daily for 16 days following inoculation of the adult turkey hens (**Table 2**). The fecal shedding of MDR *S.* Heidelberg was consistent in the challenge groups from days 1 to 11 post-inoculation, and all samples had MDR *S.* Heidelberg, indicating the constant shedding of the pathogen at all inoculum levels until day 11. This situation continued with the higher inoculum groups (10^6, 10^8) where almost all samples continued to be positive until the end of the study. The results indicated that when the level of inoculum is high, the possibility of shedding of the pathogen through the feces is high. However, for the lower inoculum levels also the excretion of the pathogen through the feces was detected throughout the study, although with differences noticed in the number of positive samples.

CONCLUSION

We demonstrated that MDR *S.* Heidelberg from the 2011 ground turkey outbreak was highly effective in colonizing poults, resulting in the dissemination of pathogen to liver and spleen, and showed potential for deposition in skeletal muscles of poults. The recovery rate of *S.* Heidelberg was highest in cecum followed by spleen, liver, thigh, drumstick, and breast. This is an important finding since most studies have not focused on the potential deposition of this pathogen in skeletal muscle tissues. The adult turkey hens, although positive for the pathogen in the cecum with all inoculum levels, responded well against the dissemination of *S.* Heidelberg in the liver and spleen, and potential deposition in the muscles. The results indicate that MDR *S.* Heidelberg has the capability for becoming a threat to the microbiological safety of turkeys and turkey products, warranting the producers to invest in targeted intervention methods to control it at the farm level. However, longer-duration challenge studies are warranted to determine if the pathogen inoculated at an earlier stage could potentially result in persisting colonization in the cecum, dissemination to the liver and spleen, and deposition in the muscles, posing a significant food safety threat to the industry.

AUTHOR CONTRIBUTIONS

DN, JV, and AK performed the turkey studies. RP read the histopathology and immunohistochemistry slides. DN and AK wrote the manuscript. JV, SN, and RP reviewed and corrected the manuscript before submission. SN helped AK to procure adult turkey hens from the commercial grower as a donation. AK conceived the idea, designed the study, and conducted the statistical analysis of the data.

ACKNOWLEDGMENTS

The authors would like to thank the Grant-in-Aid of Research, Artistry, and Scholarship Program funds administered through the Office of the Vice President of Research, University of Minnesota (Project# 22847; 2014–2015) and the FY16/17 Rapid Agricultural Response Fund Project# RR-212, awarded to the PI, AK. They also thank the USDA NIFA Hatch funds through the Minnesota Agricultural Experiment Station Project# MIN-16-102. They would also like to thank Ms. Jan Shivers, the VDL Section Head of Histology and Immunohistochemistry, and Dr. Marc Schwabenlander DVM, the VDL BSL-2 Necropsy Section Head for their assistance during the experiments. Veterinarian, Dr. Angela Craig, DVM, and the Research Animal Resources team members are also gratefully acknowledged for their help during the experiments. They would also like to thank Dr. Ben Wileman, DVM, Ph.D., Director of Global Technical Services, Life-Science Innovations, and Ziggy Kotze, General Manager – Willmar Poultry Farms, for donating the turkeys for the adult turkey hen study.

REFERENCES

Bearson, B. L., Bearson, S. M. D., Looft, T., Cai, G., and Shippy, D. C. (2017). Characterization of a multidrug-resistant *Salmonella enterica* serovar Heidelberg outbreak strain in commercial turkeys: colonization, transmission, and host transcriptional response. *Front. Vet. Sci.* 4:156. doi: 10.3389/fvets.2017.00156

Borsoi, A., Do Santos, L., Rodrigues, L. B., Luiz De Souza Moraes, H., Tadeu, C., Salle, P., et al. (2011). Behavior of *Salmonella* Heidelberg and *Salmonella* Enteritidis strains following broiler chick inoculation: evaluation of cecal morphometry, liver and cecum bacterial counts and fecal excretion patterns. *Braz. J. Microbiol.* 42, 266–273. doi: 10.1590/S1517-838220110001 00034

Campero, C. M., Odeón, A. C., Cipolla, A. L., Moore, D. P., Poso, M. A., and Odriozola, E. (2002). Demonstration of *Listeria monocytogenes* by immunohistochemistry in formalin-fixed brain tissues from natural cases of ovine and bovine encephalitis. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 49, 379–383. doi: 10.1046/j.1439-0450.2002.00586.x

CDC (2013). *Morbidity and Mortality Weekly Report: Surveillance for Foodborne Disease Outbreaks – United States, 1998–2008*. Available at: https://www.cdc.gov/mmwr/preview/mmwrhtml/ss6202a1.htm [accessed May 22, 2017].

CDC (2014a). *Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Foster Farms Brand Chicken*. Available at: https://www.cdc.gov/salmonella/heidelberg-10-13/ [accessed January 15, 2017].

CDC (2014b). *Outbreak of Salmonella Heidelberg Infections Linked to Tyson Brand Mechanically Separated Chicken at a Correctional Facility*. Available at: https://www.cdc.gov/salmonella/heidelberg-01-14/ [accessed January 15, 2017].

CDC (2016). *Multistate Outbreak of Salmonella Oranienburg Infections Linked to Good Earth Egg Company Shell Eggs*. Available from: https://www.cdc.gov/salmonella/oranienburg-10-16/ [accessed September 17, 2017].

Corrier, D. E., Elissalde, M. H., Ziprin, R. L., and Deloach, J. R. (1991). Effect of immunosuppression with cyclophosphamide, cyclosporin, or dexamethasone on *Salmonella* colonization of broiler chicks. *Avian Dis.* 35, 40–45. doi: 10.2307/1591292

Finstad, S., O'Bryan, C. A., Marcy, J. A., Crandall, P. G., and Ricke, S. C. (2012). *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Res. Int.* 45, 789–794. doi: 10.1016/j.foodres.2011.03.057

Foley, S. L., Lynne, A. M., and Nayak, R. (2008). *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J. Anim. Sci.* 86(Suppl. 14), E149–E162. doi: 10.2527/jas.2007-0464

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., and Ricke, S. C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Appl. Environ. Microbiol.* 77, 4273–4279. doi: 10.1128/AEM.00598-11

Folster, J. P., Pecic, G., Rickert, R., Taylor, J., Zhao, S., Fedorka-Cray, P. J., et al. (2012). Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg from a ground turkey-associated outbreak in the United States in 2011. *Antimicrob. Agents Chemother.* 56, 3465–3466. doi: 10.1128/AAC.00201-12

FSIS (2010). *Serotypes Profile of Salmonella Isolates from Meat and Poultry Products, 1998-2009*. Available at: https://www.fsis.usda.gov/shared/PDF/Serotypes_Profile_Salmonella_2009.pdf?redirecthttp=true [accessed September 18, 2017].

Gokulan, K., Khare, S., Rooney, A. W., Han, J., Lynne, A. M., and Foley, S. L. (2013). Impact of plasmids, including those encodingVirB4/D4 Type IV secretion systems, on *Salmonella enterica* serovar Heidelberg virulence in macrophages and epithelial cells. *PLOS ONE* 8:e77866. doi: 10.1371/journal.pone.0077866

Gonzalez-Escobedo, G., La Perle, K. M. D., and Gunn, J. S. (2013). Histopathological analysis of *Salmonella* chronic carriage in the mouse hepatopancreatobiliary system. *PLOS ONE* 8:e84058. doi: 10.1371/journal.pone.0084058

Gu, Y., Liang, X., Huang, Z., and Yang, Y. (2015). Outbreak of *Listeria monocytogenes* in pheasants. *Poult. Sci.* 94, 2905–2908. doi: 10.3382/ps/pev264

Hammack, T. S., Amagua, R. M., June, G. A., Sherrod, P. S., and Andrews, W. H. (1999). Relative effectiveness of selenite cystine broth, tetrathionate broth, and rappaport-vassiliadis medium for the recovery of *Salmonella* spp. from foods with a low microbial load. *J. Food Prot.* 62, 16–21. doi: 10.4315/0362-028X-62.1.16

Hindermann, D., Gopinath, G., Chase, H., Negrete, F., Althaus, D., Zurfluh, K., et al. (2017). *Salmonella enterica* serovar Infantis from food and human infections, Switzerland, 2010-2015: poultry-related multidrug resistant clones and an emerging ESBL producing clonal lineage. *Front. Microbiol.* 8:1322. doi: 10.3389/fmicb.2017.01322

Hoffmann, M., Zhao, S., Luo, Y., Li, C., Folster, J. P., Whichard, J., et al. (2012). Genome sequences of five *Salmonella enterica* serovar Heidelberg isolates associated with a 2011 multistate outbreak in the United States. *J. Bacteriol.* 194, 3274–3275. doi: 10.1128/JB.00419-12

Hoffmann, M., Zhao, S., Pettengill, J., Luo, Y., Monday, S. R., Abbott, J., et al. (2014). Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. *Genome Biol. Evol.* 6, 1046–1068. doi: 10.1093/gbe/evu079

Hoover, N. J., Kenney, P. B., Amick, J. D., and Hypes, W. A. (1997). Preharvest sources of *Salmonella* colonization in turkey production. *Poult. Sci.* 76, 1232–1238. doi: 10.1093/ps/76.9.1232

Hopper, S. A., and Mawer, S. (1988). *Salmonella* Enteritidis in a commercial layer flock. *Vet. Rec.* 123:351. doi: 10.1136/VR.123.13.351

Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., and Chai, S. J. (2013). Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998-2008. *Emerg. Infect. Dis.* 19, 1239–1244. doi: 10.3201/eid1908.121511

Jones, F., Axtell, R., and Rives, D. (1991). A survey of *Salmonella* contamination in modern broiler production. *J. Food Prot.* 54, 502–507. doi: 10.4315/0362-028X-54.7.502

Kennedy, M., Villar, R., Vugia, D. J., Rabatsky-Ehr, T., Farley, M. M., Pass, M., et al. (2004). Hospitalizations and deaths due to *Salmonella* infections, FoodNet, 1996-1999. *Clin. Infect. Dis.* 38(Suppl. 3), S142–S148. doi: 10.1086/381580

Kollanoor Johny, A., and Kumar, V. (2016). "Preharvest food safety – potential use of plant-derived compounds in layer chickens," in *Producing Safe Eggs: Microbial Ecology of Salmonella*, eds S. C. Ricke and R. K. Gast (Cambridge, MA: Academic Press), 347–364.

Kollanoor-Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A., Babapoor, S., March, B., et al. (2012a). Reduction of *Salmonella enterica* serovar enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds *trans*-cinnamaldehyde and eugenol. *Appl. Environ. Microbiol.* 78, 2981–2987. doi: 10.1128/AEM.07643-11

Kollanoor-Johny, A., Upadhyay, A., Baskaran, S. A., Upadhyaya, I., Mooyottu, S., Mishra, N., et al. (2012b). Effect of therapeutic supplementation of the plant compounds *trans*-cinnamaldehyde and eugenol on *Salmonella enterica* serovar enteritidis colonization in market-age broiler chickens. *J. Appl. Poult. Res.* 21, 816–822. doi: 10.3382/japr.2012-00540

Marder, E. P., Cieslak, P. R., Cronquist, A. B., Dunn, J., Lathrop, S., Rabatsky-Ehr, T., et al. (2017). Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance — foodborne diseases active surveillance network, 10 U.S. sites, 2013–2016. *MMWR Morb. Mortal. Wkly. Rep.* 66, 397–403. doi: 10.15585/mmwr.mm6615a1

Mead, G., Lammerding, A. M., Cox, N., Doyle, M. P., Humbert, F., Kulikovskiy, A., et al. (2010). Scientific and technical factors affecting the setting of *Salmonella* criteria for raw poultry: a global perspective. *J. Food Prot.* 73, 1566–1590. doi: 10.4315/0362-028X-73.8.1566

Menconi, A., Wolfenden, A. D., Shivaramaiah, S., Terraes, J. C., Urbano, T., Kuttel, J., et al. (2011). Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella enterica* serovar Heidelberg in neonatal broiler chickens and turkey poults. *Poult. Sci.* 90, 561–565. doi: 10.3382/ps.2010-01220

Nair, D. V. T., and Kollanoor Johny, A. (2017). Food grade Pimenta leaf essential oil reduces the attachment of *Salmonella enterica* Heidelberg (2011 ground turkey outbreak isolate) on to turkey skin. *Front. Microbiol.* 8:2328. doi: 10.3389/fmicb.2017.02328

NCC (2017). *Per Capita Consumption of Poultry and Livestock, 1965 to Estimated 2018, in Pounds. The National Chicken Council*. Available at: http://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/ [accessed September 6, 2017].

Rothrock, M. J. Jr., Ingram, K. D., Gamble, J., Guard, J., Cicconi-Hogan, K. M., Hinton, A. Jr., et al. (2015). The characterization of *Salmonella enterica* serotypes isolated from the scalder tank water of a commercial poultry processing plant: recovery of a multidrug-resistant Heidelberg strain. *Poult. Sci.* 94, 467–472. doi: 10.3382/ps/peu060

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17, 7–15. doi: 10.3201/eid1701.P11101

Scharff, R. L. (2012). *Economic Burden from Health Losses Due to Foodborne Illness in the United States - ProQuest*. Available at: https://search.proquest.com/docview/913142449?pq-origsite=gscholar [accessed September 5, 2017].

Smith, W., and Tucker, J. F. (1980). The virulence of *Salmonella* strains for chickens: their excretion by infected chickens. *J. Hyg.* 84, 479–488. doi: 10.1017/S0022172400027017

Suzuki, S. (1994). Pathogenicity of *Salmonella* enteritidis in poultry. *Int. J. Food Microbiol.* 21, 89–105. doi: 10.1016/0168-1605(94)90203-8

Tavakkoli, H., Rahmani, M., Ghanbarpoor, R., Kheirandish, R., and Kheirandish, A. R. (2015). Induced systemic listeriosis in *Alectoris chukar* chicks: clinical, histopathological and microbiological findings. *Br. Poult. Sci.* 56, 651–657. doi: 10.1080/00071668.2015.1113505

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Nair, Vazhakkattu Thomas, Noll, Porter and Kollanoor Johny. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 February 2018
doi: 10.3389/fmicb.2018.00152

Determination of Lipophilic Marine Biotoxins in Mussels Harvested from the Adriatic Sea by LC-MS/MS

Maria Schirone[1†], Miriam Berti[2†], Pierina Visciano[1], Francesco Chiumiento[3], Giacomo Migliorati[3], Rosanna Tofalo[1], Giovanna Suzzi[1], Federica Di Giacinto[2] and Nicola Ferri[2]*

[1] Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy, [2] Biologia delle Acque Interne, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy, [3] Bromatologia e Residui negli Alimenti per l'Uomo e gli Animali, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy

Lipophilic marine biotoxins include okadaic acid, pectenotoxin, yessotoxin and azaspiracid groups. The consumption of contaminated molluscs can lead to acute food poisoning syndromes depending on the exposure level. Regulatory limits have been set by Regulation (European Community, 2004a) No 853/2004 and LC-MS/MS is used as the official analytical method according to Regulation (European Community, 2011) No 15/2011. In this study specimens of mussels (*Mytilus galloprovincialis*) were collected along the coasts of the central Adriatic Sea during the years 2015–2017 and analyzed by the European harmonized Standard Operating Procedure. The method was validated for linearity, specificity, repeatability and reproducibility and it revealed able to be used for the detection of the lipophilic marine biotoxins. Levels of okadaic acid, pectenotoxin, yessotoxin and its analogs were detected at different concentrations in 148 (37%) out of a total of 400 samples, always below the maximum limits, except for 11 (4.3%) of them that were non-compliant because they exceeded the regulatory limit. Moreover, some samples were exposed to a multi-toxin mixture with regards to okadaic acid, yessotoxin and 1-Homo yessotoxin. Following these results, the aquaculture farms from which the non-compliant samples derived were closed until the analytical data of two consecutive samplings returned favorable. Besides the potential risk of consumption of mussels contaminated by lipophilic marine biotoxins, these marine organisms can be considered as bio-indicators of the contamination status of the marine ecosystem.

Keywords: marine biotoxins, okadaic acid, dinophysistoxin, azaspiracid, yessotoxin, *Mytilus galloprovincialis*, LC-MS/MS

OPEN ACCESS

Edited by:
Rosalba Lanciotti, Università di Bologna, Italy

Reviewed by:
Fatih Ozogul, Çukurova University, Turkey
Jorge Reinheimer, National University of the Littoral, Argentina

***Correspondence:**
Maria Schirone
mschirone@unite.it

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 December 2017*
Accepted: *23 January 2018*
Published: *12 February 2018*

Citation:
Schirone M, Berti M, Visciano P, Chiumiento F, Migliorati G, Tofalo R, Suzzi G, Di Giacinto F and Ferri N (2018) Determination of Lipophilic Marine Biotoxins in Mussels Harvested from the Adriatic Sea by LC-MS/MS. Front. Microbiol. 9:152. doi: 10.3389/fmicb.2018.00152

INTRODUCTION

Lipophilic marine biotoxins (LMB) are toxic metabolites produced by some species of unicellular algae developing during natural phenomena known as harmful algal blooms. They are grouped in different classes, i.e., okadaic acid (OA), azaspiracids (AZA), yessotoxins (YTX), pectenotoxins (PTX), and spirolides (Ferron et al., 2016).

The OA group consists of OA and its isomers, the dinophysistoxins1 and 2 (DTX1, DTX2) and in addition the fatty acid ester derivatives of OA or DTX1 and DTX2 named DTX3 (Braga et al., 2017). These marine biotoxins are responsible of the human diarrhetic shellfish poisoning (DSP) characterized by gastrointestinal disorders such as nausea, vomiting, severe diarrhea and abdominal

cramps (García et al., 2016). The mechanism of action of OA and DTX is linked to the inhibition of serine/threonine protein phosphatases (Ferreiro et al., 2015).

Besides gastrointestinal symptoms, AZA can be carcinogenic in mice and teratogenic to the developing fish (Twiner et al., 2012). Moreover, azaspiracid1 (AZA1) was demonstrated to be toxic to some human cell lines (B lymphocyte, monocyte, lung epithelial, T lymphocyte); azaspiracid2 (AZA2) has been shown to have a similar cytotoxicity with cytoskeleton alterations (Kim et al., 2017). Ferreiro et al. (2014a,b) reported that AZA2 could have acute arrhythmogenic potential *in vivo* and chronic effects on a specific cardiac potassium channel *in vitro*. Also YTX and its analogs can present cardiotoxicity with mitochondrial damage in cardiomyocytes after repeated exposure and marked bradycardia and hypotension in rats (Ferreiro et al., 2016), while some PTX are hepatotoxic to mice by intraperitoneal injection (Trainer et al., 2013). The European Legislation set maximum levels in Regulations (European Community, 2004a) No 853/2004 and (European Community, 2013) No 786/2013, corresponding to 160 μg of OA equivalent kg^{-1} for OA, DTX and PTX together, 160 μg of AZA equivalent kg^{-1} for AZA and 3.75 mg YTX equivalent kg^{-1} for YTX-group.

The Regulation (European Community, 2005) No 2074/2005 established the official analytical methods to be used for the detection of LMB, which were represented by the mouse bioassay (MBA) and the rat bioassay (RBA). However, these methods showed some disadvantages other than ethical, such as the high variability in results, the insufficient detection capability and the limited specificity. Therefore, developed alternatives to the biological methods were successfully tested and a liquid chromatography-mass spectrometry (LC-MS/MS) method was validated and recognized as the official method by the Regulation (European Community, 2011) No 15/2011 since 31 December 2014. The aim of this study was the application of the EU Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS (2015) in specimens of *Mytilus galloprovincialis* coming from different aquaculture farms located along the central Adriatic coasts. The samples were collected according to the multi-annual regional control plan 2015–2018, which requires the samplings twice a month. Another objective of this study was the validation of the method for the criteria established by Regulation (European Community, 2004c) No 882/2004.

MATERIALS AND METHODS

Collection of Specimens and Sample Preparation

Specimens of *M. galloprovincialis* were collected from 12 aquaculture farms located along the coasts of Abruzzo and Molise regions, in coastal areas belonging to the following 4 provinces: Teramo, Pescara, Chieti (Abruzzo) and Campobasso (Molise).

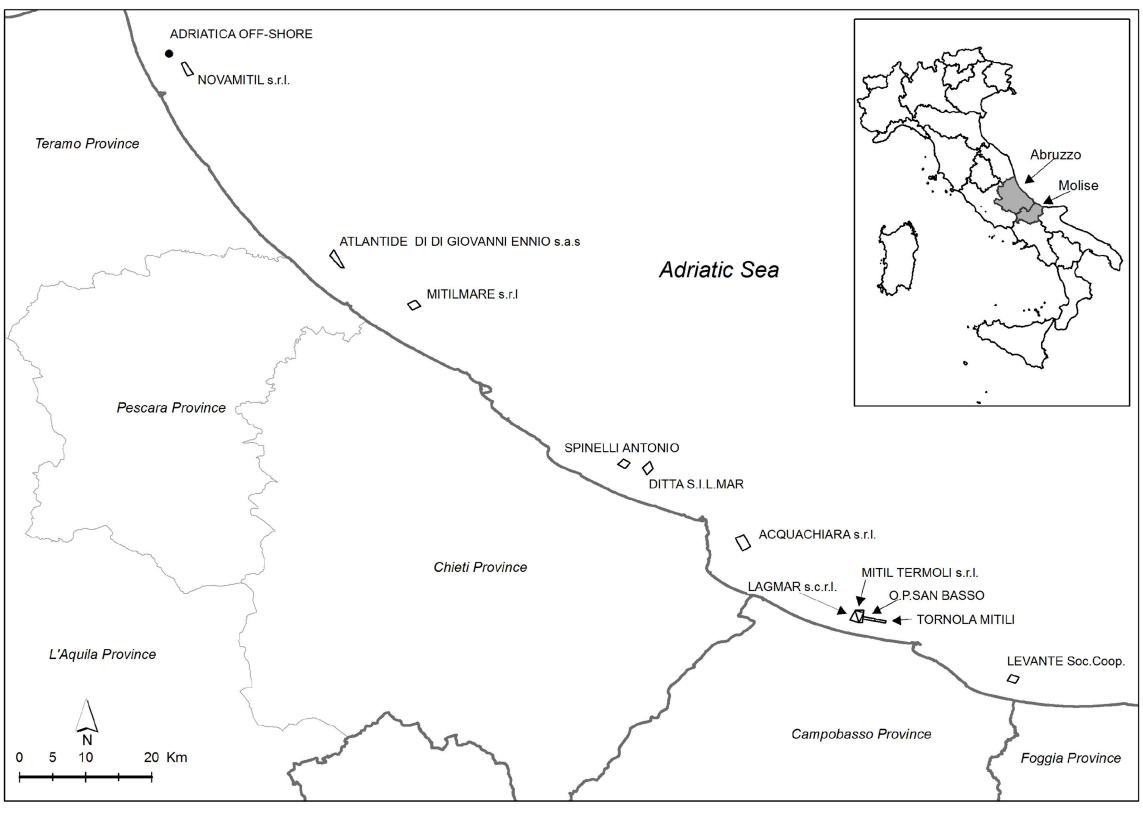


FIGURE 1 | Map of the sampling sites along the coasts of the central Adriatic Sea.

TABLE 1 | Longitude and latitude of the sampling points.

Region	Aquaculture Farm	Points	Longitude (East)	Latitude (Nord)
Abruzzo	Adriatica Off-Shore	A	13,985277	42,808611
	Novamitil S.R.L.	A-D	14,001666/14,006667/14,018334/14,009999	42,794999/42,797499/42,780833/42,778331
	Atlantide Di Giovanni	A-D	14,204166/14,209722/14,223611/14,218055	42,533333/42,541666/42,516666/42,518055
	Mitilmare S.R.L.	A-D	14,310097/14,321057/14,327828/14,316638	42,467663/42,472476/42,464385/42,459421
	Spinelli Antonio	A-D	14,596670/14,604170/14,613330/14,606670	42,248670/42,256170/42,251000/42,243500
	Ditta S.I.L. Mar	A-D	14,630555/14,640277/14,644444/14,636111	42,244444/42,252777/42,243055/42,234722
	Acquachiara S.R.L.	A-D	14,756833/14,768333/14,777130/14,766667	42,148167/42,152667/42,137642/42,131167
Molise	Lagmar S.C.R.L.	A-D	14,920000/14,931667/14,925000/14,913333	42,050000/42,046670/42,033330/42,036670
	Mitil Termoli S.R.L.	A-D	14,920000/14,931944/14,930000/14,925000	42,050000/42,050000/42,033330/42,033330
	O.P. San Basso	A-D	14,931110/14,947500/14,946944/14,930000	42,041390/42,038610/42,035000/42,038060
	Tornola Mitili	A-D	14,949157/14,962088/14,961113/14,948183	42,037970/42,035570/42,032620/42,034900
	Levante Soc. Coop.	A-D	15,132375/15,143406/15,138489/15,127458	41,962340/41,958650/41,950450/41,954140

TABLE 2 | LC-MS/MS parameters for detection of marine biotoxins in MRM mode.

Biotoxin	ESI	Precursor ion ⇒ product ion (*m/z*)	DP[a]	FP[b]	EP[c]	CE[d]	CXP[e]
OA	N[1]	803.6 ⇒ 255.3	−146	−350	−10	−65	−4
		803.6 ⇒ 113.3				−78	
DTX1	N	817.6 ⇒ 255.5	−130	−340	−13	−90	−10
		817.6 ⇒ 112.8				−70	
DTX2	N	803.6 ⇒ 255.3	−80	−315	−13	−65	−1
		803.6 ⇒ 113.3				−88	−6
YTX	N	1,141.6 ⇒ 1,061.7	−110	−350	−14	−45	−16
		1,141.6 ⇒ 855.6				−96	−10
1-Homo YTX	N	1,155.6 ⇒ 1,075.6	−100	−350	−14	−46	−18
		1,155.6 ⇒ 869.6				−100	−10
45 OH YTX	N	1,157.5 ⇒ 1,077.7	−110	−350	−14	−45	−16
		1,157.5 ⇒ 871.5				−96	−10
45 OH-Homo YTX	N	1,171.5 ⇒ 1,091.5	−100	−350	−14	−46	−18
		1,171.5 ⇒ 869.9				−100	−10
AZA1	P[2]	842.4 ⇒ 824.4	85	385	10	40	10
		842.6 ⇒ 806.7				55	11
AZA2	P	856.6 ⇒ 838.5	81	360	10	43	12
		856.6 ⇒ 820.6				53	
AZA3	P	828.6 ⇒ 810.6	72	360	8	43	18
		828.6 ⇒ 792.4				53	11
PTX2	P	876.6 ⇒ 823.5	74	375	9	33	11
		876.6 ⇒ 212.9				36	
PTX1	P	892.5 ⇒ 821.5	74	375	9	33	11
		892.5 ⇒ 213.2				36	

[1]N, Negative; [2]P, Positive; [a]DP, Declustering Potential; [b]FP, Focusing Potential; [c]EP, Entrance Potential; [d]CE, Collision Energy; [e]CXP, Collision Cell Exit Potential.

The samples were taken from 4 different sampling points (from A to D), except for a farm in which just only one sampling point was considered (**Figure 1**). In **Table 1**, longitude and latitude for each sampling point were reported.

The sampling was made according to the multi-annual regional control plan 2015–2018. The frequency for the determination of marine biotoxins was established twice in a month or more frequently when the contamination in mussels increased.

A total of 400 global samples, each formed by 150 specimens of *M. galloprovincialis*, were collected during the investigated years, and more in detail 126 samples in 2015, 173 samples in 2016, and 101 samples from January to July 2017.

The mussels were opened, removed from the shell, washed with running water to remove any residues and pooled according to their origin, to form the global samples. Then, each global sample was homogenized with a blender and stored at −20°C until the analysis. The extraction procedure was carried out for 2 g of the homogenate.

Chemicals and Standards Preparation

All chemicals were of analytical reagent grade: methanol (LC-MS grade), acetonitrile (LC-MS grade) and ammonium formate (99% purity), formic acid (98%), hydrochloric acid (37%), and sodium hydroxide (99%) and were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was prepared with high-purity water obtained from a Milli-Q® system (Merck Millipore, Darmstadt, Germany).

The certified reference standard solutions of OA (CRM-OA-c), DTX1 (CRM-DTX1) and DTX2 (CRM-DTX2), PTX2 (CRM-PTX2), AZA1 (CRM-AZA1), AZA2 (CRM-AZA2), AZA3 (CRM-AZA3), YTX (CRM-YTX), 1-Homo YTX (CRM-Homo YTX), certified reference material with OA, and DTX (CRM-DSP-MUS-b), homogenate of digestive gland of mussel (*Mytilus edulis*) with OA and DTX were all purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council Canada (Ottawa, Ontario, Canada). The calibration curve for all biotoxins was prepared in methanol following the EU-harmonized SOP.

Instrumental Analysis

The instrumental analysis was performed by a Perkin Elmer HPLC system (Perkin Elmer, Waltham, MA, USA) constituted of a model 200 microbinary pump and a model 200 auto-sampler. A reversed-phase HPLC column X-Bridge C18 (50 × 2.1 mm, 2.5 μm) with relative guard column X-Bridge C18 (10 × 2.1 mm, 2.5 μm) both from Waters (Milford, MA, USA) were used. The flow rate was set to 0.3 ml min^{-1} and the injection volume was 20 μl. The mobile phase was used in gradient mode as follows: 90% of eluent A (100% water containing 2 mM ammonium formate and 50 mM formic acid) and 10% of eluent B (95% acetonitrile: 5% water containing 2 mM ammonium formate and 50 mM

TABLE 3 | Simultaneous presence of okadaic acid (OA), yessotoxin (YTX), and 1-Homo YTX in samples distinguished by provenance.

Sample	Province	Region	OA (μg kg^{-1} ± MU)	YTX (mg/kg)	1-Homo YTX (mg/kg)	PTX2 (μg kg^{-1} ± MU)
2015						
TM1	Campobasso	Molise	51.2		0.072	
C1	Chieti	Abruzzo	46.7		0.088	
V1	Chieti	Abruzzo	143	0.172	0.165	
C2	Chieti	Abruzzo	179 ± 42	0.330	0.247	
O1	Chieti	Abruzzo	172 ± 40	0.490	0.434	
TM2	Campobasso	Molise	141	0.121	0.107	
V2	Chieti	Abruzzo	132	0.220	0.196	
P1	Pescara	Abruzzo	120	0.093	0.075	
V3	Chieti	Abruzzo	157	0.183	0.224	
O2	Chieti	Abruzzo	122	0.134	0.153	
O3	Chieti	Abruzzo	111	0.076		
C3	Chieti	Abruzzo	83.7		0.109	
O4	Chieti	Abruzzo	316 ± 74*	0.178	0.160	53.2 ± 18.0
C4	Chieti	Abruzzo	758 ± 177*	0.199	0.170	93.5 ± 31.6
G1	Teramo	Abruzzo	45.9	0.133	0.107	
TM3	Campobasso	Molise	146	0.167	0.175	
TM4	Campobasso	Molise	409 ± 96*	0.179	0.151	75.1 ± 25.4
TM5	Campobasso	Molise	369 ± 86*	0.226	0.168	61.7 ± 20.9
P2	Pescara	Abruzzo		0.193	0.203	
C5	Chieti	Abruzzo	179 ± 42	0.244	0.230	
V4	Chieti	Abruzzo	342 ± 80*	0.187	0.193	72.6 ± 17.4
F1	Chieti	Abruzzo	523 ± 122*	0.144	0.140	
2016						
TM6	Campobasso	Molise	265 ± 62*	0.215	0.172	
TM7	Campobasso	Molise	139	0.124	0.092	
P3	Pescara	Abruzzo		0.158	0.203	
TM8	Campobasso	Molise	227 ± 53*	0.150	0.071	
V5	Chieti	Abruzzo	168 ± 39	0.226	0.153	
O5	Chieti	Abruzzo	202 ± 47	0.188	0.130	
C6	Chieti	Abruzzo	472 ± 110*	0.251	0.214	59.3 ± 14.2
TM9	Campobasso	Molise	55.8	0.104	0.063	
O6	Chieti	Abruzzo	128	0.121	0.113	
C7	Chieti	Abruzzo	132	0.272	0.239	
C8	Chieti	Abruzzo	206 ± 48	0.243	0.145	
TM10	Campobasso	Molise	229 ± 53*	0.217	0.124	
C9	Chieti	Abruzzo	214 ± 51*	0.091		
TM11	Campobasso	Molise	57.2		0.196	

**Samples exceeding the maximum limit and non-compliant based on the Measurement Uncertainty (MU).*

formic acid) at 0–10 min, then eluent B increased up to 90% at time 10–13 min and decreased again to 10% at 13–16 min up to 20 min.

The LC-MS/MS analysis was carried out by a mass spectrometer API 3000 PE SCIEX (Applied Biosystems, Toronto, ON, Canada) equipped with an electrospray interface set in the positive ionization mode (ESI+) for PTX and AZA-groups, and the negative ionization mode (ESI−) for OA, DTX- and YTX-groups. The mass spectrometer was set in multiple reaction monitoring (MRM) mode, with specific transition parameters as reported in **Table 2**. The capillary voltage was set at 5.5 kV for ESI+, −4.5 kV for ESI−, and the ion source temperature at 550°C.

RESULTS AND DISCUSSION

Concentrations of Lipophilic Marine Biotoxins

In this study, the monitoring for LMB occurrence in specimens of *M. galloprovincialis* collected during the years 2015–2017 showed a good trend with regards to the compliance with the regulatory limits. To express the results by each toxin group according

TABLE 4 | Concentration of okadaic acid (OA) in samples distinguished by provenance.

Sample	Province	Region	OA (μg kg^{-1} ± MU)
2015			
O7	Chieti	Abruzzo	57.4
T1	Teramo	Abruzzo	95.9
TM12	Campobasso	Molise	86.7
V6	Chieti	Abruzzo	202 ± 47
2016			
TM13	Campobasso	Molise	84.2
TM14	Campobasso	Molise	91.3
TM15	Campobasso	Molise	118
TM16	Campobasso	Molise	72.5
TM17	Campobasso	Molise	103
C10	Chieti	Abruzzo	114
TM18	Campobasso	Molise	42.5
TM19	Campobasso	Molise	44.6
O8	Chieti	Abruzzo	65.6
O9	Chieti	Abruzzo	55.1
C11	Chieti	Abruzzo	45.3
O10	Chieti	Abruzzo	60.0
TM20	Campobasso	Molise	57.2

to the European legislation (i.e., as μg equivalents kg^{-1} or mg equivalents kg^{-1}) the use of the Toxicity Equivalent Factors (TEFs) was required. Therefore, the individual content of each detected biotoxin/analog was multiplied with the corresponding TEF before summarizing the total equivalents for the respective group toxins (European Food Safety Authority, 2009).

Among the investigated LMB, concentrations above the LOQ were found only for OA, PTX, YTX, and its analogs. Moreover, mussels were often exposed to a multi-toxin mixture because some samples contained more than one LMB. The simultaneous presence of OA, YTX, and 1-Homo YTX were detected in 17 and 11 samples collected in 2015 and 2016 years, respectively. Moreover, 6 out of these 28 samples had also PTX2 concentrations. A total of 11 samples exceeded the regulatory maximum limit of 160 μg of OA equivalents kg^{-1} (**Table 3**). The samples showing only OA levels ranging from 42.5 to 114 μg of OA equivalents kg^{-1} were reported in **Table 4**. The sample named V6, collected from Chieti province, had a value of 202 ± 47 μg of OA equivalents kg^{-1}, but it was considered compliant based on the measurement uncertainty. **Table 5** showed samples containing only YTX and its analogs, even if none of them exceeded the maximum limit for this group. The remaining investigated LMB, i.e., AZA- and DTX- groups, and 45 OH-Homo YTX, were never detected.

The occurrence of marine harmful algae is increasing worldwide and therefore, the accumulation of LMB from harmful phytoplankton represents a food safety threat in the shellfish industry. In the present study, LMB belonging to the OA-group were the only compounds exceeding the regulatory limits of 160 μg of OA equivalent kg^{-1}. In particular, the samples coming from Chieti and Campobasso provinces resulted non-compliant and therefore their production areas were closed according the multi-annual regional control plan 2015–2018 for Abruzzo[1] and Molise regions. Moreover, this plan established that in case of non-compliant samples, the rapid alert system should be activated and harvesting molluscs from the contaminated areas should be suspended until the results of two consecutive samplings are compliant. In addition, Regulation (European Community, 2004b) No 854/2004 affirms that in such circumstance the production area of bivalve molluscs should be closed by the competent authority and it could be re-opened when at least two consecutive results of biotoxin levels meet with the legislative criteria.

Also other authors reported frequent closures of bivalve fisheries of the west coast of Ireland due to AZA presence in the blue edible mussel *M. edulis* (Murray et al., 2017).

Our previous studies carried out in mussels coming from the central Adriatic Sea showed a similar trend with regards to YTX content, while no presence of the other investigated LMB was observed. The monitoring plans for the determination of LMB and domoic acid (DA) in samples of *M. galloprovincialis* during the years 2006–2009 revealed no presence of these compounds, neither by MBA used for LMB, nor by a chromatographic analysis for DA detection (Schirone et al., 2011). These results demonstrated a good condition of the monitored marine zones for the occurrence of marine biotoxins, even if a high intensity of algal blooms in Adriatic Sea have been frequently reported in the last years. However, the DSP outbreak occurrence remains still very difficult to predict due to the high variability of biotoxin content in phytoplankton cells (Leonardo et al., 2017). In another study (Schirone et al., 2013), YTX levels were found in *M. galloprovincialis* specimens taken from three Italian regions (i.e., Abruzzo, Molise, Emilia Romagna) along the coasts of the Adriatic Sea, at concentrations ranging from 0.2 to 1.8 mg of YTX equivalent kg^{-1}. Some samples coming from Emilia Romagna region exceeded the maximum limit (1 mg of YTX equivalent kg^{-1}) that was in force in the period of the investigation, instead of the new limit of 3.75 mg of YTX equivalent kg^{-1} fixed by Regulation (European Community, 2013) No 786/2013. These analyses were carried out by a functional method as alternative to MBA for the *in vitro* quantitative detection of YTX. The positive samples were also confirmed by MBA causing the death of two out three mice within 24 h of inoculation with the extract of the mollusc, even if this method showed low specificity and sensitivity because it did not provide the identification and quantification of the biotoxin causing the death of mice. A comparison among the two cited assays and a LC-MS/MS method was studied by analyzing other samples of *M. galloprovincialis* collected from the Adriatic Sea (Visciano et al., 2013). The results showed the presence of YTX at concentrations up to 1.63 and 1.97 mg of YTX equivalent kg^{-1} by the functional assay and LC-MS/MS, respectively. Moreover, the last method allowed the detection also of YTX analogs, i.e., homo YTX and carboxy homo YTX. The authors supposed that the influx of

[1] *Piano Pluriennale Regionale Integrato dei Controlli della Sanità Pubblica veterinaria e Sicurezza Alimentare della Regione Abruzzo 2015–2018. 3rd Edn.* 1–892.

TABLE 5 | Concentrations (mg/kg) of yessotoxin (YTX) and its analogs distinguished for provenance.

N° of samples	Province	Region	YTX	45 OH-YTX	1-Homo YTX
2015					
19	Teramo	Abruzzo	0.095 (T2), 0.081 (R1), 0.106 (G3), 0.112 (R2), 0.068 (R3), 0.080 (G9), 0.265 (T3), 0.208 (G10), 0.070 (R4), 1.400 (G11), 0.189 (G12), 0.172 (G13), 0.078 (G14)	0.095 (T2), 0.077 (G3), 0.099 (G5), 0.080 (G6), 0.088 (R2)	0.183 (T2), 0.073 (R1), 0.122 (G2), 0.150 (G3), 0.088 (G4), 0.135 (G6), 0.116 (G7), 0.083 (G8), 0.898 (R2), 0.088 (G9), 0.224 (T3), 0.245 (G10), 1.290 (G11), 0.204 (G12), 0.168 (G13)
8	Pescara	Abruzzo	0.099 (P4), 0.093 (P6), 0.134 (P7), 0.096 (P9), 0.213 (P11)	0.085 (P7)	0.160 (P4), 0.128 (P5), 0.078 (P6), 0.143 (P7), 0.078 (P8), 0.069 (P10), 0.204 (P11)
16	Chieti	Abruzzo	0.067 (V7), 0.088 (V8), 0.099 (F2), 0.110 (V9), 0.084 (F3), 0.132 (C13), 0.210 (V11), 0.092 (F4), 0.121 (O11), 0.183 (V12), 0.172 (O12), 0.165 (C14), 0.126 (FS1), 1.080 (O13)	0.074 (V8), 0.082 (F2), 0.091 (V9)	0.091 (V8), 0.106 (F2), 0.100 (C12), 0.143 (V9), 0.112 (V10), 0.124 (C13), 0.173 (V11), 0.077 (F4), 0.118 (O11), 0.154 (V12), 0.160 (O12), 0.104 (C14), 0.084 (FS1), 1.02 (O13)
14	Campobasso	Molise	0.103 (TM22), 0.093 (TM23), 0.101 (TM25), 0.092 (TM26), 0.200 (TM27), 0.087 (TM28), 0.136 (TM29), 0.176 (TM30), 0.111 (TM31), 0.118 (TM32), 0.105 (TM33)	0.091 (TM23)	0.110 (TM21), 0.131 (TM22), 0.076 (CB1), 0.116 (TM24), 0.095 (TM25), 0.145 (TM27), 0.093 (TM28), 0.115 (TM29), 0.107 (TM30), 0.062 (TM31), 0.089 (TM32), 0.072 (TM33)
2016					
1	Teramo	Abruzzo	0.096 (G15)		
1	Pescara	Abruzzo	0.073 (P12)		0.072 (P12)
2	Chieti	Abruzzo	0.109 (C15), 0.094 (V13)		
2	Campobasso	Molise	0.107 (TM34), 0.105 (TM35)		
2017					
6	Teramo	Abruzzo	0.133 (T4), 0.108 (T5), 0.090 (T6), 0.167 (T7), 0.186 (T8), 0.232 (T9)		
17	Chieti	Abruzzo	0.229 (O14), 0.397 (V14), 0.286 (O15), 0.156 (V15), 0.257 (C16), 0.245 (C17), 0.167 (C18), 0.137 (V16), 0.522 (C19), 0.215 (C20), 0.145 (O16), 0.282 (O17), 0.153 (C21), 0.184 (V17), 0.125 (O18), 0.162 (C22), 0.173 (C23)		
10	Campobasso	Molise	0.344 (TM36), 0.172 (TM37), 0.238 (TM38), 0.267 (TM39), 0.192 (TM40), 0.142 (TM41), 0.135 (TM42), 0.155 (TM43), 0.144 (TM44), 0.130 (TM45)		

TABLE 6 | Correlation coefficient for the investigated lipophilic marine biotoxins.

Biotoxin	R^2
OA	0.9993
DTX1	0.9991
DTX2	0.9944
PTX2	0.9996
AZA1	0.9998
AZA2	0.9990
AZA3	0.9998
YTX	0.9992
1-Homo YTX	0.9997

the waters from the Po, the most important river of Italy, on phytoplankton bloom dynamics, as well as the clear seasonal variability in the circulation and ecosystem of the Adriatic Sea due to strong forcing functions could affect the presence of this compound.

In the present study the concentrations of YTX and its analogs were lower than the above reported values (Schirone et al., 2013; Visciano et al., 2013) while levels of LMB belonging to OA-group were detected for the first time. These results confirmed that OA is gaining a high critical interest because it represents the most predominant DSP biotoxin in the European coasts (González-Romero et al., 2012). Bacchiocchi et al. (2015) found concentrations ranging between 5 and 25 μg of OA equivalent kg^{-1} in samples of *M. galloprovincialis* collected along the coast of Marche region, Adriatic Sea (Italy) during the years 2012–2013, whereas DTX were never detected. Levels of YTX and its analogs were also reported, reaching values near or slightly above the regulatory limit. However, Pistocchi et al. (2012) described that the Northern Adriatic Sea has been characterized by the presence of toxic algae producing OA and DTX until 1997 and YTX in the past decades. Moreover, Orellana et al. (2017) found similar results to those obtained in the present study, showing that OA/DTX2 and YTX were the most abundantly accumulated LMB in the analyzed mussels (*M. edulis*, *Crassostrea gigas* and *Patella* sp.) coming from the Belgian Part of the North Sea and reaching values of 25.4 and 169.2 μg equivalent kg^{-1} wet, respectively. Gerssen et al. (2010) found concentrations ranging from 18.2 to 67.5 μg of OA equivalent kg^{-1} in mussels (*M. edulis*) collected from the Dutch shellfish harvesting areas.

TABLE 7 | Levels of validation for the determination of Toxicity Equivalent Quantitation for OA-group.

Biotoxin	TEF	Level 1 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 1 ($\mu g\ kg^{-1}$)	Level 2 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 2 ($\mu g\ kg^{-1}$)	Level 3 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 3 ($\mu g\ kg^{-1}$)
OA	1	45	45	67.5	67.5	90	90
DTX1	1	45	45	67.5	67.5	90	90
DTX2	0.6	45	27	67.5	40.5	90	54
PTX2	1	45	45	67.5	67.5	90	90
Sum (as μg of OA equivalent kg^{-1})			162		243		324

TABLE 8 | Levels of validation for the determination of Toxicity Equivalent Quantitation for AZA-group.

Biotoxin	TEF	Level 1 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 1 ($\mu g\ kg^{-1}$)	Level 2 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 2 ($\mu g\ kg^{-1}$)	Level 3 ($\mu g\ kg^{-1}$) ($n = 6$)	TEQ level 3 ($\mu g\ kg^{-1}$)
AZA1	1	40	40	60	60	80	80
AZA2	1.8	40	72	60	108	80	144
AZA3	1.4	40	56	60	84	80	112
Sum (as μg of AZA equivalent kg^{-1})			168		252		336

TABLE 9 | Levels of validation for the determination of Toxicity Equivalent Quantitation for YTX group.

Biotoxin	TEF	Level 1 (mg kg^{-1}) ($n = 6$)	TEQ level 1 (mg kg^{-1})	Level 2 (mg kg^{-1}) ($n = 6$)	TEQ level 2 (mg kg^{-1})	Level 3 (mg kg^{-1}) ($n = 6$)	TEQ level 3 (mg kg^{-1})
YTX	1	0.94	0.94	1.88	1.88	2.82	2.82
1-Homo YTX	1	0.94	0.94	1.88	1.88	2.82	2.82
Sum (as mg of YTX equivalent kg^{-1})			1.88		3.75		5.64

TABLE 10 | Repeatability data.

Biotoxin	First level ($n = 6$)			Second level ($n = 6$)			Third level ($n = 6$)		
	R%	SD%	CV%	R%	SD%	CV%	R%	SD%	CV%
OA	92.4	10.0	10.8	94.5	6.7	7.1	86.1	6.9	8.0
DTX1	96.6	7.4	7.7	102.0	10.0	9.8	90.0	9.2	10.2
DTX2	101.6	8.9	8.8	104.6	4.9	4.7	94.9	10.0	10.5
PTX2	93.3	7.0	7.5	100.4	6.2	6.2	93.4	5.5	5.9
AZA1	89.5	7.1	7.9	91.5	4.5	5.2	87.4	3.2	3.6
AZA2	100.4	5.5	5.5	102.1	4.4	4.3	97.7	3.1	3.2
AZA3	89.5	4.7	5.3	84.5	3.5	4.2	88.4	7.4	8.4
YTX	84.7	9.5	11.2	90.6	7.5	8.3	92.6	5.1	5.5
1-Homo YTX	87.4	7.5	8.6	91.4	6.3	6.9	95.3	4.1	4.3

R, Recovery; SD, Standard Deviation; CV, Coefficient Variation.

Levels of OA, ranging from 40 to 611 μg of OA equivalent kg^{-1} were also detected by Garibo et al. (2014) in samples of *M. galloprovincialis* obtained from the shellfish monitoring program of the Catalan littoral (NW Mediterranean) during a DSP event in 2012. Also in that circumstance, the closure of the production area due to OA levels above the regulatory limit was observed. On the contrary, other studies (Blanco et al., 2017) reported AZA concentrations up to a maximum of 5.4 mg of AZA1 equivalent kg^{-1} in mussels (*M. galloprovincialis*) collected during the official monitoring programs of production areas located along the Atlantic and Cantabrian coasts of Spain. The authors supposed that this contamination was linked to the downwelling or upwelling relaxation in the outer (more oceanic) part of the sampling zones, and the molluscs became affected when the plankton populations were advected to the shore.

Validation Study

The method was validated for the criteria established in Regulation (European Community, 2004c) No 882/2004. The linearity was tested by five points calibration curves in the range 1.5–50 $\mu g\ l^{-1}$ for OA-group, 1.5–40 $\mu g\ l^{-1}$ for AZA- and PTX-group, and 3.75–250 $\mu g\ l^{-1}$ for YTX-group. The correlation

TABLE 11 | Reproducibility data.

Biotoxi n	First level ($n = 12$)			Second level ($n = 12$)			Third level ($n = 12$)		
	R%	SD%	CV%	R%	SD%	CV%	R%	SD%	CV%
OA	91.0	9.7	10.7	91.6	10.0	10.9	89.0	8.6	9.6
DTX1	96.3	7.1	7.4	102.8	8.1	7.8	96.2	10.1	10.5
DTX2	101.6	10.1	9.9	102.3	7.7	7.6	92.0	4.7	5.1
PTX2	93.7	5.6	6.0	96.5	6.9	7.2	93.4	5.5	5.9
AZA1	89.3	5.8	6.5	90.6	4.4	4.8	88.1	3.1	3.5
AZA2	100.0	6.6	6.6	103.7	4.6	4.4	101.3	5.8	5.7
AZA3	89.0	5.2	5.9	85.7	3.7	4.4	86.3	5.9	6.8
YTX	89.7	9.3	10.4	91.4	6.3	6.9	90.7	5.0	5.5
1-Homo YTX	89.9	6.8	7.5	91.7	7.0	7.6	92.6	4.7	5.1

R, Recovery; SD, Standard Deviation; CV, Coefficient Variation.

TABLE 12 | Measurement uncertainty (MU).

Biotoxin	MU (%)
OA	22.8
DTX1	20.7
DTX2	13.0
PTX2	8.4
AZA1	10.8
AZA2	12.7
AZA3	16.1
YTX	5.5
1-Homo YTX	8.4

coefficient indicated a good fit for all the analytes as reported in **Table 6**.

The specificity was tested by analyzing 20 blank samples of different mollusc species (mussels, clams and oysters). All blank samples showed no interfering peaks in the retention time of interest for all the analytes. These blank samples were used to evaluate the limit of detection (LOD) and the limit of quantitation (LOQ) of the method, that resulted below the target concentration established by the EU harmonized SOP (2015). The LOD corresponded to 8 $\mu g\ kg^{-1}$ for OA-, PTX-, DTX-, and AZA-group, and to 0.013 $mg\ kg^{-1}$ for YTX-group, while the LOQ was 40 $\mu g\ kg^{-1}$ for OA-, PTX-, DTX-, and AZA-group, and 0.060 $mg\ kg^{-1}$ for YTX-group.

A negative sample spiked with all biotoxins for six samples at three levels in two different days considering the maximum limits and the TEF to calculate the TEQ (Toxicity Equivalent Quantitation) was used to calculate the recovery. In **Tables 7–9** the three selected levels for validation were shown. Repeatability and reproducibility data were reported for each level in **Tables 10**, **11**. The measurement uncertainty (MU) was calculated according to EURACHEM/CITAC Guide CG4 (2012) and shown in **Table 12**.

CONCLUSION

The legislation of the European Union is particularly careful about the protection of consumers from biological and chemical hazards such as the presence of contaminants in food. With regards to marine biotoxins, it requires that the sampling is carried out weekly during the period at which harvesting is allowed. The small quantities of LMB found in mussels analyzed in the present study demonstrated the good condition of the investigated marine areas as well as the security of bivalve molluscs for public health. However, when these values exceeded the maximum limits, the closure of the examined aquaculture farms should have a negative impact on the seafood industry. The method applied in this study was able to define both presence and concentrations of LMB, that must be routinely monitored in order to avoid the risk of a chronic exposure in regular consumers.

AUTHOR CONTRIBUTIONS

MB and GM conceived and designed the experiments; FC and FD performed the experiments; MS, PV, RT, and GS analyzed the data; NF contributed reagents, materials, analysis tools; MS and PV wrote the paper.

REFERENCES

Bacchiocchi, S., Siracusa, M., Ruzzi, A., Gorbi, S., Ercolessi, M., Cosentino, M. A., et al. (2015). Two-year study of lipophilic marine toxin profile in mussels of the North-central Adriatic Sea: first report of azaspiracids in Mediterranean seafood. *Toxicon* 108, 115–125. doi: 10.1016/j.toxicon.2015.10.002

Blanco, J., Arévalo, F., Moroño, Á., Correa, J., Muñíz, S., Mariño, C., et al. (2017). Presence of azaspiracids in bivalve molluscs from Northern Spain. *Toxicon* 137, 135–143. doi: 10.1016/j.toxicon.2017.07.025

Braga, A. C., Lage, S., Pacheco, M., Rydberg, S., and Costa, P. R. (2017). Native (*Ruditapes decussatus*) and non-indigenous (*R. philippinarum*) shellfish species living in sympatry: Comparison of regulated and

non-regulated biotoxins accumulation. *Mar. Environ. Res.* 129, 147–155. doi: 10.1016/j.marenvres.2017.05.002

EU Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS (2015). *Version* 5, 1–33.

European Community (2004a). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Off. J. Eur. Union L*226, 22–82.

EURACHEM/CITAC Guide CG4 (2012). *Quantifying Uncertainty in Analytical Measurement. 3rd Edn.*, eds S. L. R. Ellison and A. Williams (London, UK).

European Community (2004b). Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Off. J. Eur. Union* L226, 83–127.

European Community (2004c). Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. *Off. J. Eur. Union* L191, 1–64.

European Community (2005). Commission Regulation (EC) No 2074/2005 of 5 December of 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off. J. Eur. Union* L338, 27–59.

European Community (2011). Commission Regulation (EU) No 15/2011 of 10 January of 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Union* L6, 3–6.

European Community (2013). Commission Regulation (EU) No 786/2013 of 16 August of 2013 amending Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as regards the permitted limits of yessotoxins in live bivalve molluscs. *Off. J. Eur. Union* L220:14.

European Food Safety Authority (2009). Scientific opinion of the panel on contaminants in the food chain on a request from the european commission on marine biotoxins in shellfish–summary on regulated marine biotoxins. *EFSA J.* 1306, 1–23.

Ferreiro, S. F., Carrera, C., Vilariño, N., Louzao, M. C., Santamarina, G., Cantalapiedra, A. G., et al. (2015). Acute cardiotoxicity evaluation of the marine biotoxins OA, DTX-1 and YTX. *Toxins* 7, 1030–1047. doi: 10.3390/toxins7041030

Ferreiro, S. F., Vilariño, N., Carrera, C., Louzao, M. C., Cantalapiedra, A. G., Santamarina, G., et al. (2016). Subacute cardiotoxicity of yessotoxin: *in vitro* and *in vivo* studies. *Chem. Res. Toxicol.* 29, 981–990. doi: 10.1021/acs.chemrestox.6b00012

Ferreiro, S. F., Vilariño, N., Carrera, C., Louzao, M. C., Santamarina, G., Cantalapiedra, A. G., et al. (2014a). *In vivo* arrythmogenicity of the marine biotoxin azaspiracid-2 in rats. *Arch. Toxicol.* 88, 425–434. doi: 10.1007/s00204-013-1115-4

Ferreiro, S. F., Vilariño, N., Louzao, M. C., Nicolau, K. C., Frederick, M. O., and Botana, L. M. (2014b). *In vitro* chronic effects on hERG channel caused by the marine biotoxin azaspiracid-2. *Toxicon* 91, 69–75. doi: 10.1016/j.toxicon.2014.09.012

Ferron, P. J., Hogeveen, K., De Sousa, G., Rahmani, R., Dubreil, E., Fessard, V., et al. (2016). Modulation of CYP3A4 activity alters the cytotoxicity of lipophilic phycotoxins in human hepatic HepaRG cells. *Toxicol. In Vitro* 33, 136–146. doi: 10.1016/j.tiv.2016.02.021

García, C., Oyaneder-Terrazas, J., Contreras, C., del Campo, M., Torres, R., and Contreras, H. R. (2016). Determination of the toxic variability of lipophilic biotoxins in marine bivalve and gastropod tissues treated with an industrial canning process. *Food Addit. Contam. A Chem.* 33, 1711–1727. doi: 10.1080/19440049.2016.1239032

Garibo, D., Campbell, K., Casanova, A., de la Iglesia, P., Fernández-Tejedor, M., Diogène, J., et al. (2014). SPR immunosensor for the detection of okadaic acid in mussels using magnetic particles as antibody carries. *Sensors Actuat. B Chem.* 190, 822–828. doi: 10.1016/j.snb.2013.09.037

Gerssen, A., van Olst, E. H. W., Mulder, P. P. J., and de Boer, J. (2010). In-house validation of a liquid chromatography tandem mass spectrometry method for the analysis of lipophilic marine toxins in shellfish using matrix-matched calibration. *Anal. Bioanal. Chem.* 397, 3079–3088. doi: 10.1007/s00216-010-3886-2

González-Romero, R., Rivera-Casas, C., Fernández-Tajes, J., Ausió, J., Méndez, J. (2012). Chromatin specialization in bivalve molluscs: a leap forward for the evaluation of okadaic acid genotoxicity in the marine environment. *Comp. Biochem. Phys. C* 155, 175–181. doi: 10.1016/j.cbpc.2011.09.003

Kim, J. H., Tillmann, U., Adams, N. G., Krock, B., Stutts, W. L., Deeds, J. R., et al. (2017). Identification of *Azadinium species* and a new azaspiracid from *Azadinium poporum* in Puget sound, Washington State, U.S.A. *Harmful Algae* 68, 152–167. doi: 10.1016/j.hal.2017.08.004

Leonardo, S., Toldrà, A., Rambla-Alegre, M., Fernández-Tejedor, M., Andree, K. B., Ferreres, L., et al. (2017). Self-assembled monolayer-based immunoassays for okadaic acid detection in seawater as monitoring tools. *Mar. Environ. Res.* 133, 6–14. doi: 10.1016/j.marenvres.2017.11.004

Murray, I. M. T., Rowan, N. J., McNamee, S., Campbell, K., and Fogarty, A. M. (2017). Pulsed light reduces the toxicity of the algal toxin okadaic acid to freshwater crustacean *Daphnia pulex*. *Environ. Sci. Pollut. Res. Int.* 25, 607–614. doi: 10.1007/s11356-017-0472-6

Orellana, G., Van Meulebroek, L., De Rijcke, M., Janssen, C. R., and Vanhaecke, L. (2017). High resolution mass spectrometry-based screening reveals lipophilic toxins in multiple trophic levels from the North Sea. *Harmful Algae* 64, 30–41. doi: 10.1016/j.hal.2017.03.005

Pistocchi, R., Guerrini, F., Pezzolesi, L., Riccardi, M., Vanucci, S., Ciminiello, P., et al. (2012). Toxin levels and profiles in microalgae from the North-Western Adriatic Sea-15 years of studies on cultured species. *Mar. Drugs* 10, 140–162. doi: 10.3390/md10010140

Schirone, M., Berti, M., Zitti, G., Ferri, N., Tofalo, R., Suzzi, G., et al. (2011). Monitoring of marine biotoxins in *Mytilus galloprovincialis* of Central Adriatic Sea (2006–2009). *Ital. J. Food Sci.* 23, 431–435.

Schirone, M., Visciano, P., Luciani, M., Ciarelli, A., Berti, M., Tofalo, R., et al. (2013). Yessotoxin determination in *Mytilus galloprovincialis* revealed by an *in vitro* functional assay. *Environ. Sci. Pollut. Res.* 20, 1189–1192. doi: 10.1007/s11356-012-1216-2

Trainer, V. L., Moore, L., Bill, B. D., Adams, N. G., Harrington, N., Borchert, J., et al. (2013). Diarrhetic shellfish toxins and other lipophilic toxins of human health concern in Washington State. *Mar. Drugs* 11, 1815–1835. doi: 10.3390/md11061815

Twiner, M. J., Hanagrif, J. C., Butler, S., Madhkoor, A. K., and Doucette, G. J. (2012). Induction of apoptosis pathways in several cell lines following exposure to the marine algal toxin azaspiracid. *Chem. Res. Toxicol.* 25, 1493–1501. doi: 10.1021/tx3001785

Visciano, P., Schirone, M., Tofalo, R., Berti, M., Luciani, M., Ferri, N., et al. (2013). Detection of yessotoxin by three different methods in *Mytilus galloprovincialis* of Adriatic Sea. *Chemosphere* 90, 1077–1082. doi: 10.1016/j.chemosphere.2012.09.012

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Schirone, Berti, Visciano, Chiumiento, Migliorati, Tofalo, Suzzi, Di Giacinto and Ferri. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 March 2018
doi: 10.3389/fmicb.2018.00289

Phenotypic and Genotypic Characterization of *Klebsiella pneumoniae* Isolated From Retail Foods in China

Shuhong Zhang[1,2], *Guangzhu Yang*[1], *Qinghua Ye*[1], *Qingping Wu*[1]*, *Jumei Zhang*[1] and *Yuanbin Huang*[1]

[1] State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou, China, [2] College of Natural Resources and Environment, South China Agricultural University, Guangzhou, China

OPEN ACCESS

Edited by:
Pierina Visciano,
Università di Teramo, Italy

Reviewed by:
Stella Maris Reginensi Rivera,
University of the Republic, Uruguay
Christine Elizabeth Ruth Dodd,
University of Nottingham,
United Kingdom
Vesna Milanović,
Università Politecnica delle Marche,
Italy

***Correspondence:**
Qingping Wu
wuqp203@163.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 03 July 2017
Accepted: 07 February 2018
Published: 01 March 2018

Citation:
Zhang S, Yang G, Ye Q, Wu Q, Zhang J and Huang Y (2018) Phenotypic and Genotypic Characterization of Klebsiella pneumoniae Isolated From Retail Foods in China. Front. Microbiol. 9:289. doi: 10.3389/fmicb.2018.00289

Klebsiella pneumoniae is not only a major hospital-acquired pathogen but also an important food-borne pathogen that can cause septicaemia, liver abscesses, and diarrhea in humans. The phenotypic and genotypic characteristics of *K. pneumoniae* in retail foods have not been thoroughly investigated in China. The objective of this study was to characterize *K. pneumoniae* isolates through biotyping, serotyping, determination of virulence factors, antibiotic resistance testing, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), and $(GTG)_5$-PCR molecular typing. From May 2013 to April 2014, a total of 61 *K. pneumoniae* isolates were collected from retail foods in China. Using API 20E test strips, five different biotype profiles were identified among these isolates. The majority of isolates belonged to biochemical profile "5215773" (50 isolates, 80.6%). The capsular serotypes of the 61 *K. pneumoniae* isolates and one reference strain were determined by PCR. Of the seven capsular serotypes tested, four different capsular serotypes were identified. Serotypes K1, K20, K57, and K2 were detected in two, three, two, and one isolates, respectively. Serotypes K3, K5, and K54 were not detected. The presence of 11 virulence genes was assessed by PCR. The most common virulence genes were *fimH* (85.5%), *ureA* (79.0%), *wabG* (77.4%), *uge* (56.5%), and *kfuBC* (29.0%). ERIC-PCR and $(GTG)_5$-PCR molecular typing indicated high genetic diversity among *K. pneumoniae* isolates. We identified 60 different ERIC patterns and 56 distinct $(GTG)_5$ patterns. Genotypic results indicated that isolates carrying similar virulence factors were generally genetically related. Some isolates from the same geographic area have a closer relationship. The isolates showed high levels of resistance to ampicillin (51/62, 82.2%). Resistance to streptomycin (11/62, 17.7%) and piperacillin (10/62, 16.1%) was also common. The presence of virulent and antibiotic-resistant *K. pneumoniae* in foods poses a potential health hazard for consumers. Our findings highlight the importance of surveillance of *K. pneumoniae* in foods.

Keywords: food, *Klebsiella pneumoniae*, biotypes, serotypes, virulence genes, antibiotic resistance, (ERIC-PCR), $(GTG)_5$-PCR

INTRODUCTION

Klebsiella pneumoniae is an important opportunistic pathogen that causes a variety of infectious diseases in humans, including septicaemia, liver abscesses, diarrhea, and pneumonia (Bi and Xu, 2013; Cao et al., 2014; Guo et al., 2017). It is a well-known hospital-acquired pathogen and associated with increased patient morbidity and mortality (Brisse et al., 2009; Cabral et al., 2012). In addition to the clinical environment, *K. pneumoniae* is frequently found in foods including raw vegetables, powdered infant formula, meat, fish, and street foods, and has been considered as an important food-borne pathogen (Haryani et al., 2007; Sun et al., 2010; Puspanadan et al., 2012; Overdevest et al., 2014; Kim et al., 2015; Davis and Price, 2016). In powdered infant formula, *K. pneumoniae* is included in the hazard identification category "B" according to the FAO and WHO guidelines on microorganisms (FAO-WHO, 2004). In recent years, an increasing number of food-borne outbreaks caused by *K. pneumoniae* have been reported in different countries (Calbo et al., 2011; Tambekar et al., 2011; Zhou et al., 2011; Bi and Xu, 2013; Yu and Zhou, 2013).

K. pneumoniae can express a variety of virulence factors including capsules, endotoxins, siderophores, iron-scavenging systems, and adhesins, which have been shown to play important roles in its pathogenesis. Capsule is an important virulence factor, which is involved in at least two pathogenic mechanisms: (1) protection of the bacteria from phagocytosis, and (2) direct inhibition of the host immune response (Kang et al., 2015). Some capsular (K) types, particularly K1, K2, K54, K57, K20, and K5, are often associated with community-acquired invasive pyogenic liver abscess syndrome, septicemia, and pneumonia (Fang et al., 2007; Siri et al., 2011; He, 2012). K1, K2, K20, K54, and K57 are highly virulent in experimental infections in mice and are often associated with severe infections in humans and animals (Turton et al., 2008; Yu et al., 2008; Cheng et al., 2015; Wei et al., 2016). K2 and K5 are frequent causes of metritis in mares and are associated with community-acquired pneumonia (Brisse et al., 2009). K3 is generally associated with rhinoscleroma (He, 2012). Capsule typing is currently the most widely used technique for typing *K. pneumoniae* isolates and exhibits good reproducibility in differentiating clinical isolates (Siu et al., 2011). Several PCRs targeting the *wzy* genes have been developed for capsule typing of *K. pneumoniae* (Turton, 2010; Cheng et al., 2015). Other virulence factors such as the *rmpA* gene (regulator of mucoid phenotype A); *allS* gene (encoding the activator of the allantoin regulon, associated with allantoin metabolism); endotoxin-related genes *wabG*, *uge*, and *wcaG*; iron acquisition system-related genes *iucB*, *iroNB*, *ybtA*, and *kfuBC*; adhesin gene *fimH* (type I fimbriae); and *ureA* gene (α-subunit of the urease, invasin related) are also believed to be involved in virulence processes (Brisse et al., 2009; Turton, 2010; El et al., 2013; Calhau et al., 2014). The detection of such virulence factors is important in understanding the pathogenic characteristics of *K. pneumoniae* isolates and enhancing our knowledge of the health risks posed by this pathogen.

The emergence of antimicrobial resistance in *Klebsiella* spp. isolates is of great concern worldwide in human medicine (Hu et al., 2013). Multidrug-resistant *K. pneumoniae* strains have been isolated from different samples (Nawaz et al., 2012; Falomir et al., 2013; Guo et al., 2016; Yaici et al., 2017). Dietary intake is one of the primary routes for the introduction of antibotic-resistant bacteria and their genes into the human digestive tract. Consumption of specific food categories might influence gut antibiotic resistance gene diversity (Milanović et al., 2017). Moreover, such bacteria may transfer antibiotic resistance determinants to other pathogenic bacteria (Machado et al., 2008). Therefore, surveillance and monitoring of drug-resistant bacteria in foods is important for implementing targeted control strategies and selecting effective drugs for treatment.

Molecular typing is a useful tool for determining the genetic relationships of food-borne bacteria and identifying probable sources of infections. This is particularly important in endemic and epidemic nosocomial outbreaks of *K. pneumoniae* infections to improve the management of such outbreaks. A variety of methods have been used for *K. pneumoniae* typing, including pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), randomly amplified polymorphic DNA (RAPD), and $(GTG)_5$ oligonucleotide PCR (Haryani et al., 2007; Ryberg et al., 2011; Barus et al., 2013; Sachse et al., 2014). The ERIC, RAPD, and $(GTG)_5$-PCR assays are relatively simple and cost-effective methods that have been successfully used for genotyping *K. pneumoniae* isolates from various sources (Ryberg et al., 2011; Barus et al., 2013).

In recent years, the number of food-borne illness outbreaks caused by *K. pneumoniae* has increased. However, until now, limited information has been available on the characteristics of *K. pneumoniae* isolated from foods. The purpose of the present study was to determine the biotypes, serotypes, virulence genes, and antimicrobial resistance patterns of food isolates and to further analyse their genetic diversity using ERIC-PCR and $(GTG)_5$-PCR molecular typing.

MATERIALS AND METHODS

Bacterial Isolation and Biochemical Identification

From May 2013 to April 2014, a total of 1,200 retail foods, including 312 ready-to-eat foods (roasted meat, cooked meat, and meatballs), 336 raw meat (poultry, pork, and beef), 192 edible mushrooms (Flammulina velutipes), 240 aquatic products (fish, shrimp, and oysters), and 120 vegetables (cucumber and lettuce), were purchased randomly from supermarkets and farmer's markets in 24 cities of China. The sampling sites covered most of the provincial capitals of China. In each city, 50 samples were randomly collected from two supermarkets and two farmers' markets. The samples were placed in separate sterile plastic bags and then immediately transported to the laboratory in a cooler with ice packs (below 4°C) and processed within 4–6 h.

About 25 g of each food sample was enriched in 225 mL nutrient broth (Huankai Ltd., Guangzhou, China) for 24 h at 37°C. Thereafter, the enrichment was streaked onto MacConkey agar (Huankai Ltd., Guangzhou, China), followed by incubation

at 37°C for 24 h. From MacConkey agar, three pink, mucoid colonies were picked up and ubcultured onto nutrient agar at 37°C for 24 h, followed by biochemical identification using API 20 E (BioMe′rieux, Marcy I′Etoile, France). Finally, 61 *K. pneumoniae* isolates were recovered from retail foods. Among these isolates, 12 were from ready-to-eat foods, six were from vegetables, six were from edible mushrooms, 16 were from raw meat, and 21 were from aquatic products. Confirmed cultures were preserved in Luria-Bertani broth containing 20% glycerol and stored at −80°C for further study.

PCR Confirmation of *K. pneumoniae*

All confirmed *K. pneumoniae* isolates were grown overnight in lactose broth at 37°C. Genomic DNA was extracted using a commercial Universal DNA Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Confirmation of *K. pneumoniae* isolates was performed by PCR as previously described (Neuberger et al., 2008). The primer sequences and amplicon size are shown in **Table 1**. All oligonucleotide primers were synthesized by Sangon Biotech. The PCR mixture (total volume 25 μL) contained 1× DreamTaqTM Green PCR Master Mix (Fermentas, Waltham, MA, USA), 4 μL primer mixture, and 2 μL DNA template. PCR was conducted in a Bio-Rad PTC-200 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reference strain *K. pneumoniae* GIM 46117 (khe +) was used as a positive control. The amplified products were analyzed by electrophoresis on 1.5% agarose gels containing Gold View (0.005% v/v) (SBS Genetech, Beijing, China) in 1×TAE buffer (40 mM Tris–HCl, 1.18 mL acetic acid, 2 mM EDTA, pH 8.0), and the bands were visualized using an ImageQuant 350 Capture system (GE Healthcare, Waukesha, WI, USA).

Serotyping by PCR

The serotypes of 61 *K. pneumoniae* isolates and one reference strain were determined using the PCR-based capsular antigen as previously described (Turton, 2010; He, 2012; Lin et al., 2014; Yu et al., 2015). The primers used are shown in **Table 1**. These assays differentiate isolates into seven major serovars: K1, K2, K5, K20, K54, K57, and K3.

Detection of Virulence Genes of *K. pneumoniae*

Eleven individual PCRs were performed to detect the presence of virulence genes (*rmpA, allS, kfuBC, ybtA, iucB, iroNB, fimH, ureA, uge, wabG,* and *wcaG*) in *K. pneumoniae* isolates as in previous studies (Brisse et al., 2009; Turton, 2010). Primer sequences, amplification conditions, and amplicon sizes are shown in **Table 1**. Amplified PCR products were analyzed by gel electrophoresis on 1.5% agarose gels containing Gold View (0.005% v/v) in 1×TAE buffer (40 mM Tris–HCl, 1.18 mL acetic acid, 2 mM EDTA, pH 8.0), and imaged using the ImageQuant 350 Capture system.

ERIC-PCR

For ERIC-PCR, the primers ERIC1 (5′-ATGTAAGCTCCTG GGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGG TGAGCG-3′) were used (Versalovic et al., 1991). PCR was performed in a 25 μL solution containing 1.0 U of Taq DNA polymerase (Dongsheng Biotech, Guangdong, China), 1.0 μM of each primer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 40 ng of template genomic DNA. Amplifications were performed with a Bio-Rad PTC-200 Thermal Cycler (Bio-Rad) under the following conditions: an initial denaturation at 94°C for 5 min; 35 cycles of 1 min at 94°C, 1 min at 49°C, and 3 min at 72°C; and a final extension at 72°C for 10 min. ERIC-PCR products were detected by a 2.0% agarose gel electrophoresis

TABLE 1 | PCR primers used in this study.

Target virulence genes	Primer Sequence(5′-3′)	Size (bp)	References
khe	F: TGATTGCATTCGCCACTGG R: GGTCAACCCAACGATCCTG	428	Neuberger et al., 2008
wzyK1 (magA)	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	1283	Turton et al., 2008
wzyK2	F: GACCCGATATTCATACTTG ACAGAG R: CCTGAAGTAAAATCGTAAATA GATGGC	641	Turton et al., 2008
zxK5	F: TGGTAGTGATGCTCGCGA R: CCTGAACCCACCCCAATC	280	Turton et al., 2008
wzyK20	F: CGGTGCTACAGTGCATCATT R: GTTATACGATGCTCAGTCGC	741	Fang et al., 2007
wzxK54	F: CATTAGCTCAGTGGTTGGCT R: GCTTGACAAACACCATAGCAG	881	Fang et al., 2007
wzyK57	F: CTCAGGGCTAGAAGTGTCAT R: CACTAACCCAGAAAGTCGAG	1037	Pan et al., 2008
wzyK3	F: TAGGCAATTGACTTTAGGTG R: AGTGAATCAGCCTTCACCT	549	Fevre et al., 2011
rmpA	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	535	Brisse et al., 2009
allS	F: CCGTTAGGCAATCCAGAC R: TCTGATTTA(A/T)CCCACATT	1090	Brisse et al., 2009
kfuBC	F: GAAGTGACGCTGTTTCTGGC R: TTTCGTGTGGCCAGTGACTC	797	Brisse et al., 2009
ybtA	F: ATGACGGAGTCACCGCAAAC R: TTACATCACGCGTTTAAAGG	960	He, 2012
iucB	F: ATGTCTAAGGCAAACATCGT R: TTACAGACCGACCTCCGTGA	948	He, 2012
iroNB	F: GGCTACTGATACTTGACTATTC R: CAGGATACAATAGCCCATAG	992	He, 2012
fimH	F: GCTCTGGCCGATAC(C/T)AC (C/G)ACGG R: GC(G/A)(A/T)A(G/A)TAACG(T/C) GCCTGGAACGG	423	Brisse et al., 2009
ureA	F: GCTGACTTAAGAGAACGTTATG R: GATCATGGCGCTACCT(C/T)A	337	Brisse et al., 2009
uge	F: GATCATCCGGTCTCCCTGTA R: TCTTCACGCCTTCCTTCACT	534	Brisse et al., 2009
wabG	F: CGGACTGGCAGATCCATATC R: ACCATCGGCCATTTGATAGA	683	Brisse et al., 2009
wcaG	F: GGTTGGKTCAGCAATCGTA R: ACTATTCCGCCAACTTTTGC	169	Turton, 2010

with Gold View (0.005% v/v) in 1×TAE buffer (40 mM Tris–HCl, 1.18 mL acetic acid, 2 mM EDTA, pH 8.0), and the gel was photographed using a UV Imaging System (GE Healthcare). The images were captured in TIFF file format for further analysis with BioNumerics software version 6.0 (Applied Maths, Kortrijk, Belgium).

$(GTG)_5$-PCR

$(GTG)_5$ oligonucleotide typing was performed as previously described (Ryberg et al., 2011) with minor modifications. The amplification reaction was carried out in a total volume of 25 μL consisting of 1.0 U of Taq DNA polymerase (Dongsheng Biotech,Guangzhou, China), 1.0 μM of primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 40 ng of template genomic DNA. PCR conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 30 s, and extension at 72°C for 3 min; followed by a final extension at 72°C for 10 min. Genetic relationships among *K. pneumoniae* isolates were analyzed using BioNumerics software version 6.0.

Antimicrobial Susceptibility Testing

The strains were tested for antimicrobial susceptibility to 21 antibiotics using the agar disc diffusion method on Mueller–Hinton agar (Oxoid Ltd., Basingstoke, UK) following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI (Clinical and Laboratory Standards Institute), 2011). The 21 antibiotics (Oxoid) tested were as follows: ampicillin (10 μg), amoxicillin-clavulanic acid (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefoperazone/sulbactam (105 μg), cephalothin (30 μg), piperacillin (30 μg), piperacillin/tazobactam (110 μg), imipenem (10 μg), cefoxitin (30 μg), kanamycin (30 μg), gentamicin (10 μg), amikacin (10 μg), streptomycin (10 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (25 μg), and tetracycline (30 μg). These antibiotics are representative of the major classes of antimicrobial drugs important to both veterinary and human medicine. Isolates were classified as susceptible, moderately resistant, and resistant using breakpoints specified by the CLSI, and *Escherichia coli* ATCC 25922 was used as the quality control strain. Moderately resistant isolates were considered resistant.

RESULTS

Biochemical and Molecular Identification of *K. pneumoniae*

A total of five different biochemical profiles were identified among the isolates using API 20E test strips (Table S1). The majority of isolates belonged to biochemical profile "5215773" (97.6% $T = 1.0$; 50 isolates). Other biochemical profiles included "1215773" (LDC-, 98.0%, $T = 0.93$; six isolates), "7215773" (ADH+, 97.5%, $T = 0.5$; three isolates), "5215373" (SOR-, 93.0% $T = 0.72$; two isolates), and "5214773" (VP-, 95.0% $T = 0.85$; one isolate). All isolates were identified as *K. pneumoniae* by PCR.

Serotypes of *K. pneumoniae*

Capsular serotyping results of the 61 *K. pneumoniae* isolates and one reference strain showed that eight isolates were typeable serotypes and 53 were untypeable serotypes. A total of four different capsular serotypes were identified, with serotype K1 detected in two isolates from fish samples, K20 detected in three isolates (two from chicken and one from mushroom), K57 detected in two isolates (one from shrimp and one from pork), and K2 detected in the reference strain. The other serotypes (K3, K5, and K54) were not detected.

Distribution of Virulence Genes

The distributions of the 11 virulence genes are shown in **Figure 1**. Of the 62 strains, 56 carried at least one virulence gene. The virulence genes *fimH*, *ureA*, *wabG*, and *uge* were commonly presented in the strains from different sources and detected in 85.5% (53/62), 79.0% (49/62), 77.4% (48/62), and 56.5% (35/62) of the 62 strains, respectively. Virulence genes *kfuBC*, *allS*, *wcaG*, *rmpA*, and *ybtA* were detected in 29.0% (18/62), 6.5% (4/62), 6.5% (4/62), 1.6% (1/62), and 1.6% (1/62) of the strains, respectively, while *iucB* and *iroNB* were not detected.

Antimicrobial Susceptibility Testing

The results of antimicrobial susceptibility testing of the 62 strains are shown in **Table 2**. A high prevalence of resistance to ampicillin (51/62, 82.2%) was observed. Resistance to streptomycin (11/62, 17.7%), piperacillin (10/62, 16.1%), and tetracycline (8/62, 12.9%) was also common. Most of the isolates were susceptible to quinolone and fluoroquinolone antimicrobials including ciprofloxacin, nalidixic acid, and norfloxacin. Some isolates were resistant to β-lactamase antibiotics such as cefotaxime (3/62, 4.8%), cefoxitin (2/62, 3.2%), and amoxycillin-clavulanic acid (2/62, 3.2%). All strains were susceptible to piperacillin/tazobactam, ceftazidime, and imipenem.

With regards to multidrug resistance, 17.7% (11/62) of isolates were resistant to three or more of the tested agents. Some of these multidrug-resistant isolates were resistant to 10–15 of these antibiotics (**Table 2**).

ERIC-PCR and $(GTG)_5$-PCR Molecular Typing

ERIC-PCR classified the 62 strains into 60 different patterns (**Figure 2**). Two isolates (numbers 53 and 54) obtained from frozen chicken wings in Xiamen exhibited identical patterns and carried the same virulence gene, *fimH*. Likewise, two isolates (numbers 29 and 30) obtained from a beef sample in Guangzhou also showed identical genetic profiles. All other isolates belonged to distinct genetic types. Two isolates (numbers 11 and 21) obtained from Fuzhou but different food samples exhibited similar genetic profiles and virulent gene profiles. Similar findings were observed for two isolates (numbers 3 and 5) obtained from Shaoguan and the other two isolates (numbers 50 and 2) obtained from Shaoguan.

$(GTG)_5$-PCR analysis revealed that the food isolates and reference strain could be divided into 56 different genetic patterns (**Figure 3**). Again isolates 53 and 54 exhibited the same genetic

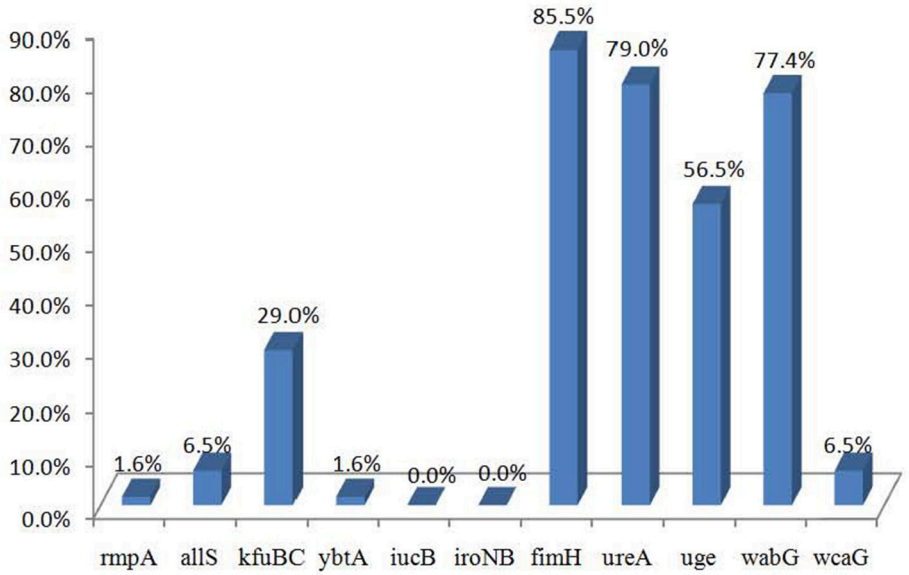


FIGURE 1 | The distribution of virulence genes in *Klebsiella pneumoniae* strains.

profile, as did isolates 29 and 30. Some isolates with similar virulence genes but from different cities (isolates 34 and 56, 19 and 20, and 8 and 9) also yielded the same genetic profiles. One isolate (10) yielded a genetic pattern identical to that of the reference strain. With the exception of these six pairs of isolates with identical patterns, all other isolates showed unique genetic patterns. Some isolates obtained from the same cities (isolates 3 and 5, 17 and 23, 31 and 40) showed similar genetic profiles and virulent gene profiles, respectively.

DISCUSSION

Biotyping, Serotypes, and Virulence Gene Distributions

Biotyping of *K. pneumoniae* is an important method of bacterial characterization for supplementing epidemiological studies. Our results showed that the biochemical profiles of the food isolates were diverse. We identified five different biotypes among the 62 isolates, among which "5215773" was the most prevalent biotype profile. However, no obvious correlations were observed between the biotype and other phenotypic factors, such as serotype and virulence factors. Therefore, the biotyping method should be combined with other methods to accurately study this pathogen.

Capsules are important virulence factors that are associated with the severity of infection. In our study, four capsular types (K1, K2, K20, and K54) were identified among the isolates. Most of the isolates were non-K1/K2 serotypes. This finding is inconsistent with those of previous studies. Wei et al. (2016) reported that capsule serotypes K1, K2, and K57 accounted for 82.1% of clinical high-virulence *K. pneumoniae* strains. In other studies, serotypes K1, K2, and K54 were found to be the most common capsular types among *K. pneumoniae* clinical isolates (Yu et al., 2008; Cheng et al., 2015). The difference in results may be related to the origins and geographic distributions of the isolates examined. Although K1/K2 are considered to be the most virulent serotypes, increasing clinical and epidemiological evidence suggests that some non-K1/K2 serotypes are also virulent and can cause various human infections (Yao et al., 2015; Yu et al., 2015). Therefore, the presence of these serotypes in foods still poses a potential public health risk. In addition, the strains with high virulent capsular types were mainly isolated from meat and aquatic products, indicating the high risk of these food types.

The results of the assessment of virulence factors suggested that the *fimH*, *ureA*, *uge*, and *wabG* genes were commonly distributed among food isolates, which is consistent with the results reported for clinical isolates (Yu et al., 2008; Calhau et al., 2014; Cheng et al., 2015). The presence of these genes in food isolates suggests the pathogenic potential of these isolates and a potential risk to human health. The *rmpA* gene is a putative virulence factor and has been found to be associated with highly virulent *K. pneumoniae* (Yu et al., 2006). Yu et al. (2007) found that *rmpA* is mainly prevalent among *K. pneumoniae* isolates categorized as capsular serotypes K1 and K2, causing internal infections with abscessation in Southeast Asia. Other studies have also reported that capsular serotypes K1 and K2 are often associated with the *rmpA* gene (Turton, 2010; Cheng et al., 2015). However, we did not detect the *rmpA* gene in serotype K1 strains. In addition, Yu et al. (2008) reported that there was a strong association between the *kfuB* and *allS* genes and K1 serotype isolates, with all K1 clinical isolates testing positive for *kfuB* and *allS*. However, in the present study, we found no obvious correlation between capsular serotype and any virulence genes. *kfuB* was not detected in K1 serotype isolates. Furthermore, *kfuB* and *allS* were present in isolates belonging to other serotypes. Our results indicate that the virulence profiles

TABLE 2 | Antibiotic susceptibility of *Klebsiella pneumoniae* strains.

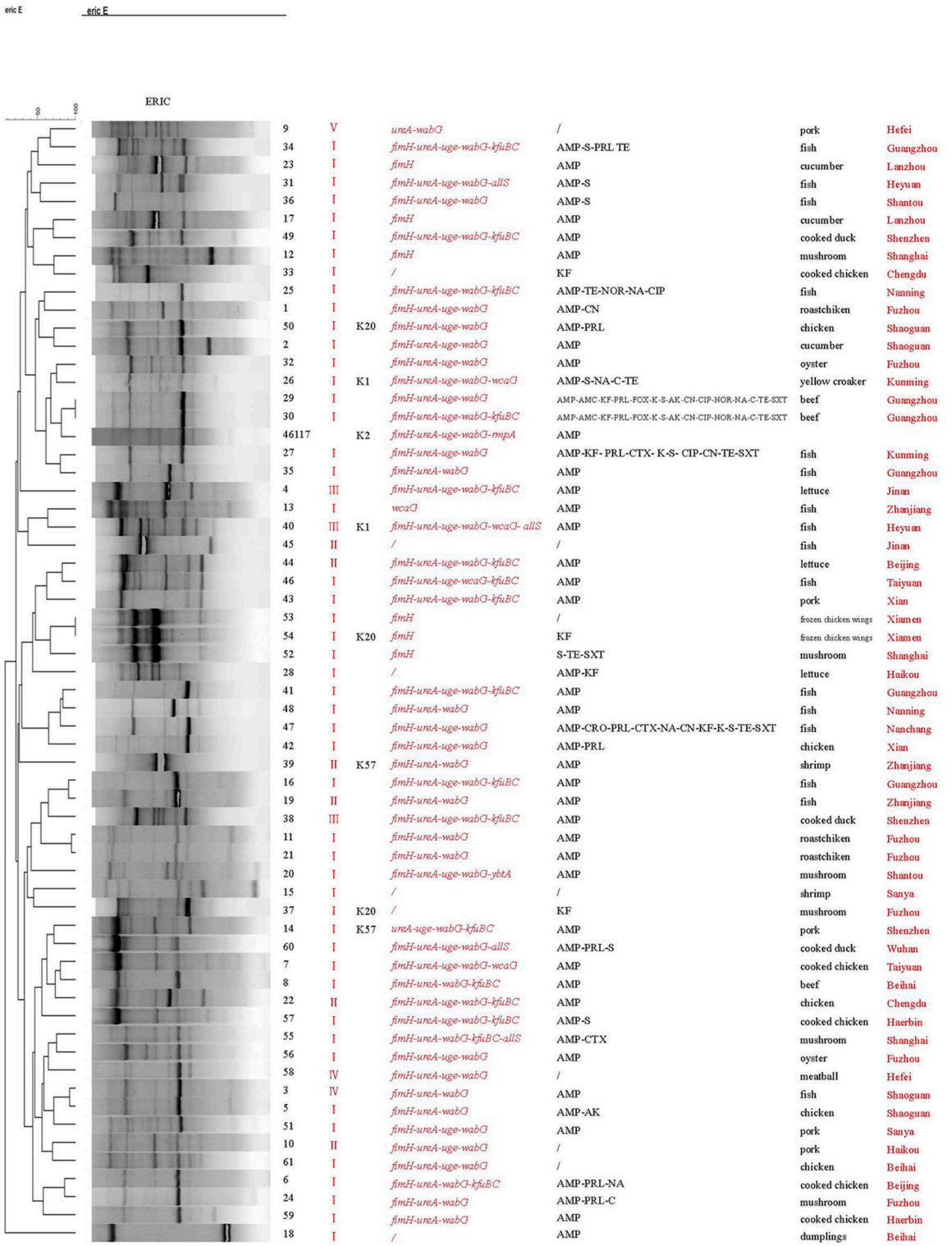
Antibiotics	Antibiotic disc content (μg)	Susceptibility		
		Resistant No. (%)	Intermediate No. (%)	Susceptible No. (%)
β-LACTAMS				
Ampicillin (AMP)	10	51 (82.3)	0	11 (17.7)
Amoxycillin-clavulanicAcid (AMC)	30	2 (3.2)	0	60 (96.8)
Ceftazidime (CAZ)	30	0	0	62 (100)
Cefotaxime (CTX)	30	2 (3.2)	1 (1.6)	59 (95.2)
Ceftriaxone(CRO)	30	1 (1.6)	0	61 (98.4)
Cefoperazone/sulbactam(SCF)	105	0	1 (1.6)	61 (98.4)
Cephalothin(KF)	30	7 (11.3)	1 (1.6)	54 (87.1)
Piperacillin(PRL)	100	2 (3.2)	8 (12.9)	52 (83.9)
Piperacillin/tazobactam(TZP)	110	0	0	62 (100)
Imipenem(IPM)	10	0	0	62 (100)
Cefoxitin (FOX)	30	2 (3.2)	0	60 (96.8)
QUINOLONES AND FLUOROQUINOLONES				
Ciprofloxacin(CIP)	5	3 (4.8)	1 (1.6)	58 (93.6)
Nalidixic acid (NA)	30	4 (6.5)	2 (3.2)	56 (90.3)
Norfloxacin (NOR)	10	2 (3.2)	1 (1.6)	59 (95.2)
AMINOGLYCOSIDES				
Amikacin (AK)	30	0	3 (4.8)	59 (95.2)
Gentamicin (CN)	10	4 (6.5)	1 (1.6)	57 (91.9)
Kanamycin (K)	30	2 (3.2)	2 (3.2)	58 (93.6)
Streptomycin (S)	10	7 (11.3)	4 (6.5)	51 (82.2)
Phenicols				
Chloramphenicol (C)	30	4 (6.5)	0	58 (93.5)
SULFONAMIDES AND SYNERGISTIC AGENTS				
Trimethoprim-sulfamethoxazole (SXT)	25	5 (8.1)	0	57 (91.9)
Tetracyclines				
Tetracycline (TE)	30	7 (11.3)	1 (1.6)	54 (87.1)

of the isolates were diverse and that virulence genes were commonly present in different capsular types. These findings may be related to the limited number of isolates in our study. In future studies, a greater number of isolates should be analyzed to assess the relationship between capsular type and virulence factors.

Antibiotic Resistance of *K. pneumoniae*

The extensive use of antimicrobials has led to a high incidence of resistance in *K. pneumoniae* (Cao et al., 2014; Kim et al., 2015). The food isolates in our study showed a high prevalence of resistance to ampicillin. Resistance to piperacillin, cephalothin, streptomycin, and tetracycline was also common. These results are consistent with previous findings (Haryani et al., 2007; Hassan et al., 2011; Nawaz et al., 2012). Nawaz et al. (2012) reported that 47% of *K. pneumoniae* isolated from shrimp were resistant to trimethoprim/sulfamethaxazole and chloramphenicol. In our study, most isolates were susceptible to these two antibiotics. Quinolones are broad-spectrum antimicrobial agents that have been widely used in clinical medicine and in raising food-producing animals (such as chicken in China). Wu et al. (2016) found that 80.0% of isolates from chicken broilers were resistant to ciprofloxacin. Kim et al. (2015) also found that 26.3% of *K. pneumoniae* isolates from ready-to-eat vegetables were resistant to ciprofloxacin. In contrast, more than 90% of our isolates were susceptible to quinolone antibiotics, including ciprofloxacin, nalidixic acid, and norfloxacin. The third-generation cephalosporins, such as cefotaxime, ceftriaxone, and piperacillin, play an important role in clinical therapeutic use. In this study, most food isolates were susceptible to these antibiotics, indicating that third-generation cephalosporin antibiotics could be effective against food isolates. However, three isolates were found to be resistant to cefotaxime, which merits concern. These strains usually produce extended spectrum β-lactamases and often confer resistance to almost all β-lactam antibiotics, including 3rd and 4th generation cephalosporins and other kinds of antibiotics (quinolones, trimethoprim/sulfamethoxazole, and aminoglycosides). The presence of such strains in food may represent a significant threat to consumers, as these pathogens have been recorded to cause obstinate infections with increased morbidity and mortality (Warjri et al., 2015; Koovapra et al., 2016).

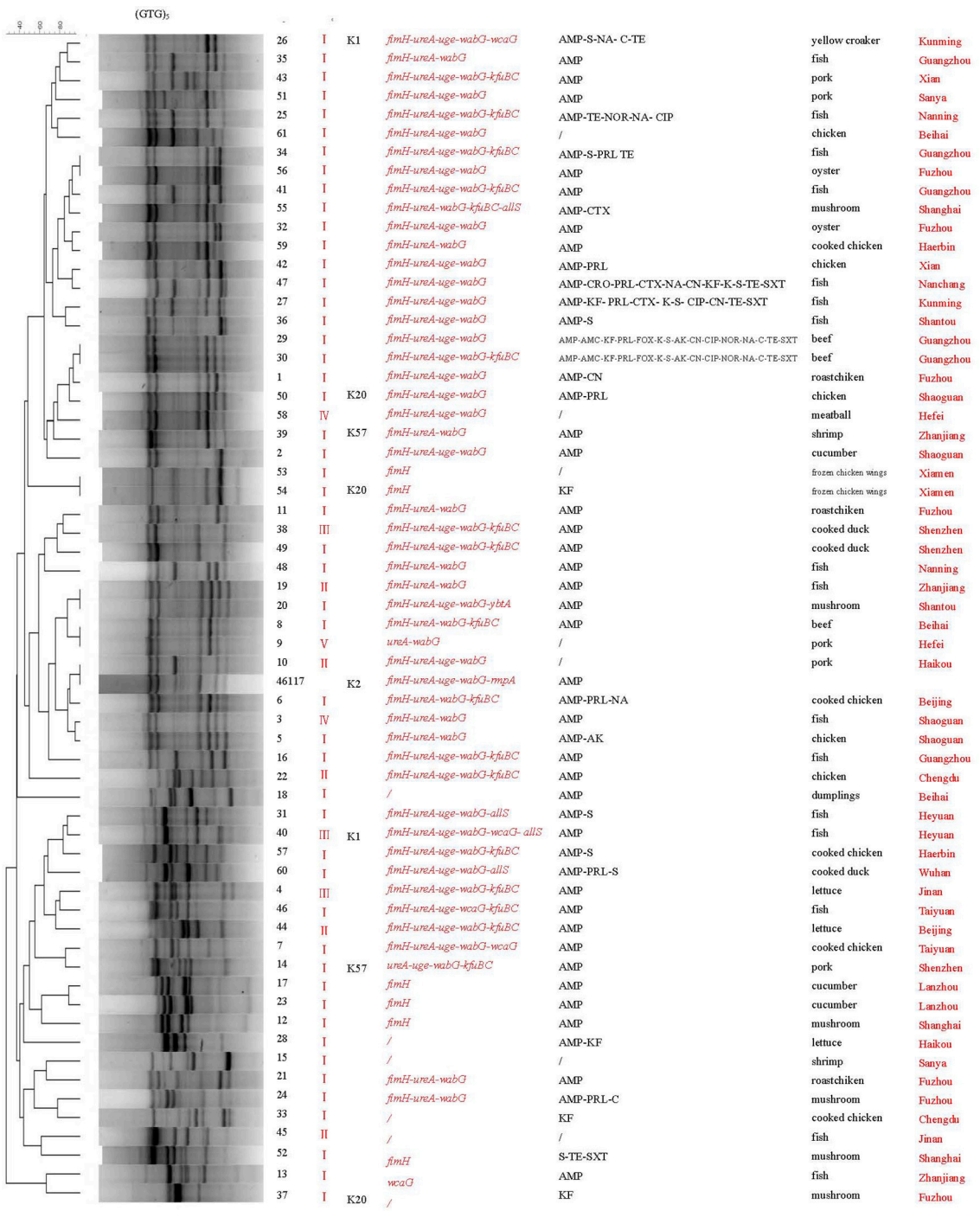
In addition, we also detected 11 multidrug-resistant (≥3 drugs) isolates. Multidrug-resistant strains are of great



9	V		*ureA-wabG*	/	pork	Hefei
34	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-S-PRL TE	fish	Guangzhou
23	I		*fimH*	AMP	cucumber	Lanzhou
31	I		*fimH-ureA-uge-wabG-allS*	AMP-S	fish	Heyuan
36	I		*fimH-ureA-uge-wabG*	AMP-S	fish	Shantou
17	I		*fimH*	AMP	cucumber	Lanzhou
49	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	cooked duck	Shenzhen
12	I		*fimH*	AMP	mushroom	Shanghai
33	I		/	KF	cooked chicken	Chengdu
25	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-TE-NOR-NA-CIP	fish	Nanning
1	I		*fimH-ureA-uge-wabG*	AMP-CN	roastchiken	Fuzhou
50	I	K20	*fimH-ureA-uge-wabG*	AMP-PRL	chicken	Shaoguan
2	I		*fimH-ureA-uge-wabG*	AMP	cucumber	Shaoguan
32	I		*fimH-ureA-uge-wabG*	AMP	oyster	Fuzhou
26	I	K1	*fimH-ureA-uge-wabG-wcaG*	AMP-S-NA-C-TE	yellow croaker	Kunming
29	I		*fimH-ureA-uge-wabG*	AMP-AMC-KF-PRL-FOX-K-S-AK-CN-CIP-NOR-NA-C-TE-SXT	beef	Guangzhou
30	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-AMC-KF-PRL-FOX-K-S-AK-CN-CIP-NOR-NA-C-TE-SXT	beef	Guangzhou
46117		K2	*fimH-ureA-uge-wabG-rmpA*	AMP		
27	I		*fimH-ureA-uge-wabG*	AMP-KF- PRL-CTX- K-S- CIP-CN-TE-SXT	fish	Kunming
35	I		*fimH-ureA-wabG*	AMP	fish	Guangzhou
4	III		*fimH-ureA-uge-wabG-kfuBC*	AMP	lettuce	Jinan
13	I		*wcaG*	AMP	fish	Zhanjiang
40	III	K1	*fimH-ureA-uge-wabG-wcaG- allS*	AMP	fish	Heyuan
45	II		/	/	fish	Jinan
44	II		*fimH-ureA-uge-wabG-kfuBC*	AMP	lettuce	Beijing
46	I		*fimH-ureA-uge-wcaG-kfuBC*	AMP	fish	Taiyuan
43	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	pork	Xian
53	I		*fimH*	/	frozen chicken wings	Xiamen
54	I	K20	*fimH*	KF	frozen chicken wings	Xiamen
52	I		*fimH*	S-TE-SXT	mushroom	Shanghai
28	I		/	AMP-KF	lettuce	Haikou
41	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	fish	Guangzhou
48	I		*fimH-ureA-wabG*	AMP	fish	Nanning
47	I		*fimH-ureA-uge-wabG*	AMP-CRO-PRL-CTX-NA-CN-KF-K-S-TE-SXT	fish	Nanchang
42	I		*fimH-ureA-uge-wabG*	AMP-PRL	chicken	Xian
39	II	K57	*fimH-ureA-wabG*	AMP	shrimp	Zhanjiang
16	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	fish	Guangzhou
19	II		*fimH-ureA-wabG*	AMP	fish	Zhanjiang
38	III		*fimH-ureA-uge-wabG-kfuBC*	AMP	cooked duck	Shenzhen
11	I		*fimH-ureA-wabG*	AMP	roastchiken	Fuzhou
21	I		*fimH-ureA-wabG*	AMP	roastchiken	Fuzhou
20	I		*fimH-ureA-uge-wabG-ybtA*	AMP	mushroom	Shantou
15	I		/	/	shrimp	Sanya
37	I	K20	/	KF	mushroom	Fuzhou
14	I	K57	*ureA-uge-wabG-kfuBC*	AMP	pork	Shenzhen
60	I		*fimH-ureA-uge-wabG-allS*	AMP-PRL-S	cooked duck	Wuhan
7	I		*fimH-ureA-uge-wabG-wcaG*	AMP	cooked chicken	Taiyuan
8	I		*fimH-ureA-wabG-kfuBC*	AMP	beef	Beihai
22	II		*fimH-ureA-uge-wabG-kfuBC*	AMP	chicken	Chengdu
57	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-S	cooked chicken	Haerbin
55	I		*fimH-ureA-wabG-kfuBC-allS*	AMP-CTX	mushroom	Shanghai
56	I		*fimH-ureA-uge-wabG*	AMP	oyster	Fuzhou
58	IV		*fimH-ureA-uge-wabG*	/	meatball	Hefei
3	IV		*fimH-ureA-wabG*	AMP	fish	Shaoguan
5	I		*fimH-ureA-wabG*	AMP-AK	chicken	Shaoguan
51	I		*fimH-ureA-uge-wabG*	AMP	pork	Sanya
10	II		*fimH-ureA-uge-wabG*	/	pork	Haikou
61	I		*fimH-ureA-uge-wabG*	/	chicken	Beihai
6	I		*fimH-ureA-wabG-kfuBC*	AMP-PRL-NA	cooked chicken	Beijing
24	I		*fimH-ureA-wabG*	AMP-PRL-C	mushroom	Fuzhou
59	I		*fimH-ureA-wabG*	AMP	cooked chicken	Haerbin
18	I		/	AMP	dumplings	Beihai

FIGURE 2 | ERIC-PCR molecular fingerprint profiles of *Klebsiella pneumoniae*.

public health concern, as they may further complicate the treatment of human infections caused by *K. pneumoniae*. Multidrug resistance increases the risk of antimicrobial treatment failure in humans. Continuous surveillance of the antibiotic resistance of *K. pneumoniae* in foods is therefore needed.



26	I	K1	*fimH-ureA-uge-wabG-wcaG*	AMP-S-NA- C-TE	yellow croaker	Kunming
35	I		*fimH-ureA-wabG*	AMP	fish	Guangzhou
43	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	pork	Xian
51	I		*fimH-ureA-uge-wabG*	AMP	pork	Sanya
25	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-TE-NOR-NA- CIP	fish	Nanning
61	I		*fimH-ureA-uge-wabG*	/	chicken	Beihai
34	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-S-PRL TE	fish	Guangzhou
56	I		*fimH-ureA-uge-wabG*	AMP	oyster	Fuzhou
41	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	fish	Guangzhou
55	I		*fimH-ureA-wabG-kfuBC-allS*	AMP-CTX	mushroom	Shanghai
32	I		*fimH-ureA-uge-wabG*	AMP	oyster	Fuzhou
59	I		*fimH-ureA-wabG*	AMP	cooked chicken	Haerbin
42	I		*fimH-ureA-uge-wabG*	AMP-PRL	chicken	Xian
47	I		*fimH-ureA-uge-wabG*	AMP-CRO-PRL-CTX-NA-CN-KF-K-S-TE-SXT	fish	Nanchang
27	I		*fimH-ureA-uge-wabG*	AMP-KF- PRL-CTX- K-S- CIP-CN-TE-SXT	fish	Kunming
36	I		*fimH-ureA-uge-wabG*	AMP-S	fish	Shantou
29	I		*fimH-ureA-uge-wabG*	AMP-AMC-KF-PRL-FOX-K-S-AK-CN-CIP-NOR-NA-C-TE-SXT	beef	Guangzhou
30	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-AMC-KF-PRL-FOX-K-S-AK-CN-CIP-NOR-NA-C-TE-SXT	beef	Guangzhou
1	I		*fimH-ureA-uge-wabG*	AMP-CN	roastchiken	Fuzhou
50	I	K20	*fimH-ureA-uge-wabG*	AMP-PRL	chicken	Shaoguan
58	IV		*fimH-ureA-uge-wabG*	/	meatball	Hefei
39	I	K57	*fimH-ureA-wabG*	AMP	shrimp	Zhanjiang
2	I		*fimH-ureA-uge-wabG*	AMP	cucumber	Shaoguan
53	I		*fimH*	/	frozen chicken wings	Xiamen
54	I	K20	*fimH*	KF	frozen chicken wings	Xiamen
11	I		*fimH-ureA-wabG*	AMP	roastchiken	Fuzhou
38	III		*fimH-ureA-uge-wabG-kfuBC*	AMP	cooked duck	Shenzhen
49	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	cooked duck	Shenzhen
48	I		*fimH-ureA-wabG*	AMP	fish	Nanning
19	II		*fimH-ureA-wabG*	AMP	fish	Zhanjiang
20	I		*fimH-ureA-uge-wabG-ybtA*	AMP	mushroom	Shantou
8	I		*fimH-ureA-wabG-kfuBC*	AMP	beef	Beihai
9	V		*ureA-wabG*	/	pork	Hefei
10	II		*fimH-ureA-uge-wabG*	/	pork	Haikou
46117		K2	*fimH-ureA-uge-wabG-rmpA*	AMP		
6	I		*fimH-ureA-wabG-kfuBC*	AMP-PRL-NA	cooked chicken	Beijing
3	IV		*fimH-ureA-wabG*	AMP	fish	Shaoguan
5	I		*fimH-ureA-wabG*	AMP-AK	chicken	Shaoguan
16	I		*fimH-ureA-uge-wabG-kfuBC*	AMP	fish	Guangzhou
22	II		*fimH-ureA-uge-wabG-kfuBC*	AMP	chicken	Chengdu
18	I		/	AMP	dumplings	Beihai
31	I		*fimH-ureA-uge-wabG-allS*	AMP-S	fish	Heyuan
40	III	K1	*fimH-ureA-uge-wabG-wcaG- allS*	AMP	fish	Heyuan
57	I		*fimH-ureA-uge-wabG-kfuBC*	AMP-S	cooked chicken	Haerbin
60	I		*fimH-ureA-uge-wabG-allS*	AMP-PRL-S	cooked duck	Wuhan
4	III		*fimH-ureA-uge-wabG-kfuBC*	AMP	lettuce	Jinan
46	I		*fimH-ureA-uge-wcaG-kfuBC*	AMP	fish	Taiyuan
44	II		*fimH-ureA-uge-wabG-kfuBC*	AMP	lettuce	Beijing
7	I		*fimH-ureA-uge-wabG-wcaG*	AMP	cooked chicken	Taiyuan
14	I	K57	*ureA-uge-wabG-kfuBC*	AMP	pork	Shenzhen
17	I		*fimH*	AMP	cucumber	Lanzhou
23	I		*fimH*	AMP	cucumber	Lanzhou
12	I		*fimH*	AMP	mushroom	Shanghai
28	I		/	AMP-KF	lettuce	Haikou
15	I		/	/	shrimp	Sanya
21	I		*fimH-ureA-wabG*	AMP	roastchiken	Fuzhou
24	I		*fimH-ureA-wabG*	AMP-PRL-C	mushroom	Fuzhou
33	I		/	KF	cooked chicken	Chengdu
45	II		/	/	fish	Jinan
52	I		*fimH*	S-TE-SXT	mushroom	Shanghai
13	I		*wcaG*	AMP	fish	Zhanjiang
37	I	K20	/	KF	mushroom	Fuzhou

FIGURE 3 | $(GTG)_5$-PCR molecular fingerprint profiles of *Klebsiella pneumoniae.* AMP, Antibiotics: ampicillin; AMC, amoxycillin-clavulanicAcid; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; SCF, cefoperazone/sulbactam; KF, cephalothin; PRL, piperacillin; TZP, piperacillin/tazobactam; IPM, imipenem; FOX, cefoxitin; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; AK, amikacin; CN, gentamicin; K, kanamycin; S, streptomycin; C, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline; /, susceptible Biotypes: I"7215773"; II "1215773"; III "7215773"; IV " 5215373"; V "5214773".

ERIC-PCR and $(GTG)_5$-PCR Molecular Typing

Both ERIC-PCR and $(GTG)_5$-PCR molecular typing results revealed a high degree of genetic diversity among the *K. pneumoniae* isolates from retail foods. Interestingly, a good concordance was observed between ERIC and $(GTG)_5$ typing in four isolates. Isolates 53 and 54 from the same sample were 100% identical according to the two genetic typing methods. Likewise,

isolates 29 and 30 from the same sample also showed identical genotyping patterns with both methods. These results reflect the reliability and accuracy of these molecular methods.

Further analysis of the associations between phenotypic types and molecular subtypes indicated that genotyping results generally correlated with virulence gene profiles. In $(GTG)_5$-PCR typing, most isolates with similar or identical virulence gene profiles exhibited close genetic relationships (**Figure 3**). Similarly, in ERIC typing, some isolates (53, 54, and 52; 44, 46, and 43; 11 and 21; 15 and 37; 3 and 5; 10 and 61) carrying similar virulence factors were genetically related.

Based on the isolation sources, we found that some isolates obtained from the same geographic area showed similar genetic profiles and had a closer relationship. Additionally, some isolates from the same city were divided into different clusters in ERIC or $(GTG)_5$ typing, indicating the genetic heterogeneity of these isolates. However, no clear correlation was observed between the genotyping patterns of isolates and food types, consistent with that of a previous study (Barus et al., 2013). The isolates with the same biotypes also exhibited diversity genetic profiles and were distributed in different clusters in ERIC or $(GTG)_5$ typing. Some isolates with different biotypes were clustered together in ERIC and $(GTG)_5$ profiles. Further studies are needed to confirm the relationship between biotypes and genotypes of *K. pneumoniae* with a larger number of food strains.

Compared with $(GTG)_5$-PCR, ERIC-PCR exhibited a higher discriminatory ability to distinguish these isolates, as ERIC-PCR was able to differentiate even the isolates with the same $(GTG)_5$ genetic profiles, including isolates 34 and 56, 19 and 20, and 8 and 9, which originated from different sources. Merging the results from the two fingerprinting methods enhanced detection of polymorphisms. Our analyses suggest that a combination of ERIC-PCR and $(GTG)_5$-PCR is more effective for analysing the epidemiological and virulence characteristics of *K. pneumoniae*.

CONCLUSIONS

Our results indicate that food-borne *K. pneumoniae* exhibit diverse virulence gene profiles, antibiotic resistance profiles, and genotypes. Highly virulent serotypes and multidrug-resistant isolates were present in foods. The potential health risks posed by such isolates should not be underestimated. Our findings highlight the need for increasing the surveillance of *K. pneumoniae* in foods. These data improve our understanding of the epidemiological and public health implications of this pathogen.

AUTHOR CONTRIBUTIONS

SZ, GY, and QW: conceived and designed the experiments. SZ and GY: performed the experiments. SZ, GY, QY, and YH: analyzed the data. SZ, GY, QW, and JZ: contributed reagents, materials and analysis tools.

FUNDING

The authors would like to acknowledge the financial support of National Key Research and Development Program of China (2016YFD0401204) and the Science and Technology Planning Project of Guangdong Province (2016A040403088; 201704020089). We are very grateful to the reviewers for their valuable suggestions and comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00289/full#supplementary-material

REFERENCES

Barus, T., Hanjaya, I., Sadeli, J., Lay, B. W., Suwanto, A., and Yulandi, A. (2013). Genetic diversity of *Klebsiella* spp. isolated from tempe based on enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). *Hayati J. Biosci.* 20, 171–176. doi: 10.4308/hjb.20.4.171

Bi, X., and Xu, W. Y. (2013). An investigation of food poisoning caused by *Klebsiella pneumoniae*. *Chin. Pract. Med.* 8, 275–276. doi: 10.3969/j.issn.1673-7555.2013.26.224

Brisse, S., Fevre, C., Passet, V., Issenhuthjeanjean, S., Tournebize, R., Diancourt, L., et al. (2009). Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS ONE* 4:e4982. doi: 10.1371/journal.pone.0004982

Cabral, A. B., Melo Rde C., Maciel, M. A., and Lopes, A. C. (2012). Multidrug resistance genes, including blaKPC and blaCTX-M-2, among *Klebsiella pneumoniae* isolated in Recife, Brazil. *Rev. Soc. Bras. Med. Trop.* 45, 572–578. doi: 10.1590/S0037-86822012000500007

Calbo, E., Freixas, N., Xercavins, M., Riera, M., Nicolás, C., Monistrol, O., et al. (2011). Foodborne nosocomial outbreak of SHV1 and CTX-M-15-producing *Klebsiella pneumoniae*: epidemiology and control. *Clin. Infect. Dis.* 52, 743–749. doi: 10.1093/cid/ciq238

Calhau, V., Boaventura, L., Ribeiro, G., Mendonça, N., and Da, S. G. (2014). Molecular characterization of *Klebsiella pneumoniae* isolated from renal transplanted patients: virulence markers, extended-spectrum β-lactamases, and genetic relatedness. *Diagn. Microbiol. Infect. Dis.* 79, 393–395. doi: 10.1016/j.diagmicrobio.2013.08.031

Cao, X., Xu, X., Zhang, Z., Han, S., Chen, J., and Zhang, K. (2014). Molecular characterization of clinical multidrug-resistant *Klebsiella pneumoniae* isolates. *Ann. Clin. Microbiol. Antimicrob.* 13, 1–6. doi: 10.1186/1476-07 11-13-16

Cheng, L., Cao, X. L., Shen, H., Zhang, Z. F., Ning, M. Z., Zhou, W. Q., et al. (2015). Investigations on the virulence, serotypes and genotyping of *Klebsiella pneumoniae* producing KPC-2. *Chin. J. Clin. Lab. Sci.* 33, 591–595. doi: 10.13602/j.cnki.jcls.2015.08.08

CLSI (Clinical and Laboratory Standards Institute) (2011). *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-First Informational Supplement 2012,* Vol. 31. Wayne, IL: CLSI.

Davis, G. S., and Price, L. B. (2016). Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Curr. Environ. Health Rep.* 3, 1–8. doi: 10.1007/s40572-016-0089-9

El, F. R., Messai, Y., Alouache, S., and Bakour, R. (2013). Virulence profiles and antibiotic susceptibility patterns of *Klebsiella pneumoniae* strains isolated from different clinical specimens. *Pathol. Biol.* 6, 209–216. doi: 10.1016/j.patbio.2012.10.004

Falomir, M. P., Rico, H., and Gozalbo, D. (2013). Enterobacter and Klebsiella species isolated from fresh vegetables marketed in valencia (spain) and their clinically relevant resistances to chemotherapeutic agents. *Foodborne Pathog. Dis.* 10, 1002–1007. doi: 10.1089/fpd.2013.1552

Fang, C. T., Lai, S. Y., Yi, W. C., Hsueh, P. R., Liu, K. L., and Chang, S. C. (2007). *Klebsiella pneumoniae* genotype k1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. *Clin. Infect. Dis.* 45, 284–293. doi: 10.1086/519262

FAO-WHO. (2004). *Joint FAO/WHO Workshop on Enterobacter sakazakii* and *Other Microorganisms in Powdered Infant Formula*. Geneva. Available online at: http://www.fao.org/docrep/007/y5502e/y5502e00.htm

Fevre, C., Passet, V., Deletoile, A., Barbe, V., Frangeul, L., Almeida, A. S., et al. (2011). PCR-based identification of *Klebsiella pneumoniae* subsp. *rhinoscleromatis*, the agent of rhinoscleroma. *PLoS Negl. Trop. Dis.* 5:e1052. doi: 10.1371/journal.pntd.0001052

Guo, Y., Wang, S., Zhan, L., Jin, Y., Duan, J., Hao, Z., et al. (2017). Microbiological and clinical characteristics of hypermucoviscous *Klebsiella pneumoniae* isolates associated with invasive infections in China. *Front. Cell. Infect. Microbiol.* 7:24. doi: 10.3389/fcimb.2017.00024

Guo, Y., Zhou, H., Qin, L., Pang, Z., Qin, T., Ren, H., et al. (2016). Frequency, antimicrobial resistance and genetic diversity of *Klebsiella pneumoniae* in food samples. *PLoS ONE* 11:e0153561. doi: 10.1371/journal.pone.0153561

Haryani, Y., Noorzaleha, A. S., Fatimah, A. B., Noorjahan, B. A., Patrick, G. B., Shamsinar, A. T., et al. (2007). Incidence of *Klebsiella pneumoniae* in street foods sold in Malaysia and their characterization by antibiotic resistance, plasmid profiling, and RAPD–PCR analysis. *Food Control* 18, 847–853. doi: 10.1016/j.foodcont.2006.04.009

Hassan, S. A., Altalhi, A. D., Gherbawy, Y. A., and El-Deeb, B. A. (2011). Bacterial load of fresh vegetables and their resistance to the currently used antibiotics in Saudi Arabia. *Foodborne Pathog. Dis.* 8, 1011–1018. doi: 10.1089/fpd.2010.0805

He, J. Y. (2012). Study on serotype and distribution on characterization of virulence genes of *Klebsiella pheumoniae [D]*. Chongqing Medical University.

Hu, L., Zhong, Q., Tu, J., Xu, Y., Qin, Z., Parsons, C., et al. (2013). Emergence of blaNDM-1 among *Klebsiella pneumoniae* ST15 and novel ST1031 clinical isolates in China. *Diagn. Microbiol. Infect. Dis.* 75, 373–376. doi: 10.1016/j.diagmicrobio.2013.01.006

Kang, Y. F., Tian, P. F., and Tan, T. W. (2015). Research progress on virulence factors of *Klebsiella pneumoniae*. *Acta Microbiol. Sin.* 55, 1245–1252. doi: 10.13343/j.cnki.wsxb.20140614

Kim, H. S., Chon, J. W., Kim, Y. J., Kim, D. H., Kim, M. S., and Seo, K. H. (2015). Prevalence and characterization of extended-spectrum-β-lactamase-producing *Escherichia coli*, and *Klebsiella pneumoniae*, in ready-to-eat vegetables. *Int. J. Food Microbiol.* 207, 83–86. doi: 10.1016/j.ijfoodmicro.2015.04.049

Koovapra, S., Bandyopadhyay, S., Das, G., Bhattacharya, D., Banerjee, J., Mahanti, A., et al. (2016). Molecular signature of extended spectrum β-lactamase producing *Klebsiella pneumoniae*, isolated from bovine milk in eastern and north-eastern India. *Infect. Genet. Evol.* 44, 395–402. doi: 10.1016/j.meegid.2016.07.032

Lin, J. C., Koh, T. H., Lee, N., Fung, C. P., Chang, F. Y., Tsai, Y. K., et al. (2014). Genotypes and virulence in serotype k2 *Klebsiella pneumoniae* from liver abscess and non-infectious carriers in Hong Kong, Singapore and Taiwan. *Gut Pathog.* 6:21. doi: 10.1186/1757-4749-6-21

Machado, E., Coque, T. M., Canton, R., Sousa, J. C., and Peixe, L. (2008). Antibiotic resistance integrons and extended-spectrum β-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal. *J. Antimicrob. Chem.* 62, 296–302. doi: 10.1093/jac/dkn179

Milanović, V., Osimani, A., Aquilanti, L., Tavoletti, S., Garofalo, C., Polverigiani, S., et al. (2017). Occurrence of antibiotic resistance genes in the faecal DNA of healthy omnivores, Ovo-Lacto vegetarians and vegans. *Mol. Nutr. Food Res.* 61:1601098. doi: 10.1002/mnfr.201601098

Nawaz, M., Khan, S. A., Tran, Q., Sung, K., Khan, A. A., Adamu, I., et al. (2012). Isolation and characterization of multidrug-resistant Klebsiella spp. isolated from shrimp imported from Thailand. *Int. J. Food Microbiol.* 155, 179–184. doi: 10.1016/j.ijfoodmicro.2012.02.002

Neuberger, A., Oren, I., and Sprecher, H. (2008). Clinical impact of a pcr assay for rapid identification of *Klebsiella pneumoniae* in blood cultures. *J. Clin. Microbiol.* 46, 377–379. doi: 10.1128/JCM.00568-07

Overdevest, I. T., Heck, M., van der Zwaluw, K., Huijsdens, X., van Santen, M., Rijnsburger, M., et al. (2014). Extended-spectrum β-lactamase producing Klebsiella spp. in chicken meat and humans: a comparison of typing methods. *Clin. Microbiol. Infect.* 20, 251–255. doi: 10.1111/1469-0691.12277

Pan, Y. J., Fang, H. C., Yang, H. C., Lin, T. L., Hsieh, P. F., Tsai, F. C., et al. (2008). Capsular polysaccharide synthesis regions in *Klebsiella pneumoniae* serotype K57 and a new capsular serotype. *J. Clin. Microbiol.* 46, 2231–2240. doi: 10.1128/JCM.01716-07

Puspanadan, S., Afsahhejri, L., Loo, Y. Y., Nillian, E., Kuan, C. H., Goh, S. G., et al. (2012). Detection of *Klebsiella pneumoniae* in raw vegetables using most probable number-polymerase chain reaction (MPN-PCR). *Int. Food Res. J.* 19, 1757–1762.

Ryberg, A., Olsson, C., Ahrné, S., and Monstein, H. J. (2011). Comparison of (GTG)5-oligonucleotide and ribosomal intergenic transcribed spacer (ITS)-PCR for molecular typing of Klebsiella isolates. *J. Microbiol. Methods* 84, 183–188. doi: 10.1016/j.mimet.2010.11.019

Sachse, S., Bresan, S., Erhard, M., Edel, B., Pfister, W., Saupe, A., et al. (2014). Comparison of multilocus sequence typing, RAPD, and MALDI-TOF mass spectrometry for typing of β-lactam-resistant *Klebsiella pneumoniae* strains. *Diagn. Microbiol. Infect. Dis.* 80, 267–271. doi: 10.1016/j.diagmicrobio.2014.09.005

Siri, G. P., Sithebe, N. P., and Ateba, C. N. (2011). Identification of Klebsiella species isolated from modimola dam (mafikeng) north west province – South Africa. *Afr. J. Microbiol. Res.* 5, 3958–3963. doi: 10.5897/AJMR11.690

Siu, L. K., Fung, C. P., Chang, F. Y., Lee, N., Yeh, K. M., Koh, T. H., et al. (2011). Molecular typing and virulence analysis of serotype k1 *Klebsiella pneumoniae* strains isolated from liver abscess patients and stool samples from noninfectious subjects in Hong Kong, Singapore, and Taiwan. *J. Clin. Microbiol.* 49, 3761–3765. doi: 10.1128/JCM.00977-11

Sun, F., Wu, D., Qiu, Z., Jin, M., Wang, X., and Li, J. (2010). Development of real time PCR systems based on SYBR Green fro specific detection and quantification of *Klebsiella pneumoniae* in infant formula. *Food Control* 21, 487–491. doi: 10.1016/j.foodcont.2009.07.014

Tambekar, D. H., Kulkarni, R. V., Shirsat, S. D., and Bhadange, D. G. (2011). Bacteriological quality of street vended food Pani Puri: a case study of Amravati city (MS) India. *Biosci. Disc.* 2, 350–354.

Turton, J. F. (2010). PCR characterization and typing of *Klebsiella pneumoniae* using capsular type-specific, variable number tandem repeat and virulence gene targets. *J. Med. Microbiol.* 59, 541–547. doi: 10.1099/jmm.0.015198-0

Turton, J. F., Baklan, H., Siu, L. K., Kaufmann, M. E., and Pitt, T. L. (2008). Evaluation of a multiplex PCR for detection of serotypes K1, K2 and K5 in Klebsiella sp. and comparison of isolates within these serotypes. *FEMS Microbiol. Lett.* 284, 247–252. doi: 10.1111/j.1574-6968.2008.01208.x

Versalovic, J., Koeuth, T., and Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 19, 6823–6831. doi: 10.1093/nar/19.24.6823

Warjri, I., Dutta, T. K., Lalzampuia, H., and Chandra, R. (2015). Detection and characterization of extended-spectrum β-lactamases (blaCTX-M-1 and blaSHV) producing *Escherichia coli*, Salmonella spp. and *Klebsiella pneumoniae* isolated from humans in Mizoram. *Vet. World* 8, 599–604. doi: 10.14202/vetworld.2015.599-604

Wei, D. D., Chen, K. Q., and Wang, L. H. (2016). Clinical and molecular characteristics of high virulent *Klebsiella pneumonia* in infection in intensive care unit. *Chin. J. Nosocomiol.* 26, 5056–5059.

Wu, H., Wang, M., Liu, Y., Wang, X., Wang, Y., Lu, J., et al. (2016). Characterization of antimicrobial resistance in Klebsiella species isolated from chicken broilers. *Int. J. Food Microbiol.* 232, 95–102. doi: 10.1016/j.ijfoodmicro.2016.06.001

Yaici, L., Haenni, M., Métayer, V., Saras, E., Mesbah, Z. F., Ayad, M., et al. (2017). Spread of esbl/ampc-producing *Escherichia coli* and *Klebsiella pneumoniae* in the community through ready-to-eat sandwiches in algeria. *Int. J. Food Microbiol.* 245, 66–72. doi: 10.1016/j.ijfoodmicro.2017.01.011

Yao, B., Xiao, X., Wang, F., Zhou, L., Zhang, X., and Zhang, J. (2015). Clinical and molecular characteristics of multi-clone carbapenem-resistant hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* isolates in a tertiary hospital in Beijing, China. *Int. J. Infect. Dis.* 37, 107–112. doi: 10.1016/j.ijid.2015.06.023

Yu, Q., Li, J., Li, Y. C., Tian, B., and Hu, Z. D. (2015). Apsular serotypes and virulence genes of *Klebsiella pneumoniae* in patients with bloodstream infection. *Chin. J. Clin. Lab. Sci.* 33, 785–788. doi: 10.13602/j.cnki.jcls.2015.10.19

Yu, V. L., Hansen, D. S., Chien, K. W., Asia, S., Klugman, K. P., Anne, V. G., et al. (2007). Virulence characteristics of Klebsiella and clinical manifestations of *K. pneumonia* bloodstream infections. *Emerg. Infect. Dis.* 13, 986–993. doi: 10.3201/eid1307.070187

Yu, W. L., Ko, W. C., Cheng, K. C., Lee, C. C., Lai, C. C., and Chuang, Y. C. (2008). Comparison of prevalence of virulence factors for *Klebsiella pneumoniae* liver abscesses between isolates with capsular k1/k2 and non-k1/k2 serotypes. *Diagn. Microbiol. Infect. Dis.* 62, 1–6. doi: 10.1016/j.diagmicrobio.2008.04.007

Yu, W. L., Ko, W. C., Cheng, K. C., Lee, H. C., Ke, D. S., Lee, C. C., et al. (2006). Association between rmpA and magA genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. *Clin. Infect. Dis.* 42, 1351–1358. doi: 10.1086/503420

Yu, Y., and Zhou, Z. Q. (2013). Detection of food-borne diseases caused by *Klebsiella pneumoniae*. *ZheJiang Prevent. Med.* 25, 93–94. doi: 10.3969/j.issn.1007-0931.2013.02.039

Zhou, X., Gao, J., Huang, Y., Fu, S., and Chen, H. (2011). Antibiotic resistance pattern of *Klebsiella pneumoniae* and *Enterobacter sakazakii* isolates from powdered infant formula. *Afr. J. Microbiol. Res.* 5, 3073–3077. doi: 10.5897/AJMR10.867

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Zhang, Yang, Ye, Wu, Zhang and Huang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 March 2018
doi: 10.3389/fmicb.2018.00596

Listeria monocytogenes Sequence Types 121 and 14 Repeatedly Isolated Within One Year of Sampling in a Rabbit Meat Processing Plant: Persistence and Ecophysiology

Frédérique Pasquali[1], Federica Palma[1], Laurent Guillier[2], Alex Lucchi[1], Alessandra De Cesare[1] and Gerardo Manfreda[1]*

[1] *Dipartimento di Scienze e Tecnologie Agro-Alimentari, Alma Mater Studiorum – Università di Bologna, Bologna, Italy,*
[2] *Laboratoire de Sécurité des Aliments, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail, Maisons-Alfort, France*

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Beatrix Stessl, Veterinärmedizinische Universität Wien, Austria
Hana Drahovska, Comenius University, Slovakia

***Correspondence:**
Frédérique Pasquali
frederique.pasquali@unibo.it

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *16 December 2017*
Accepted: *15 March 2018*
Published: *29 March 2018*

Citation:
Pasquali F, Palma F, Guillier L, Lucchi A, De Cesare A and Manfreda G (2018) Listeria monocytogenes Sequence Types 121 and 14 Repeatedly Isolated Within One Year of Sampling in a Rabbit Meat Processing Plant: Persistence and Ecophysiology. Front. Microbiol. 9:596. doi: 10.3389/fmicb.2018.00596

Listeria monocytogenes is a foodborne pathogen adapted to survive and persist in multiple environments. Following two previous studies on prevalence and virulence of *L. monocytogenes* ST121 and ST14 repeatedly collected in a the same rabbit-meat processing plant, the research questions of the present study were to: (1) assess persistence of *L. monocytogenes* isolates from the rabbit-plant; (2) select genes associated to physiological adaptation to the food-processing environment; (3) compare presence/absence/truncation of these genes in newly sequenced and publicly available ST121 and ST14 genomes.A total of 273 draft genomes including ST121 and ST14 newly sequenced and publicly available draft genomes were analyzed. Whole-genome Single Nucleotide Polymorfism (wgSNP) analysis was performed separately on the assemblies of ST121 and ST14 draft genomes. SNPs alignments were used to infer phylogeny. A dataset of *L. monocytogenes* ecophysiology genes was built based on a comprehensive literature review. The 94 selected genes were screened on the assemblies of all ST121 and ST14 draft genomes. Significant gene enrichments were evaluated by statistical analyses.A persistent ST14 clone, including 23 out of 27 newly sequenced genomes, was circulating in the rabbit-meat plant along with two not persistent clones. A significant enrichment was observed in ST121 genomes concerning stress survival islet 2 (SSI-2) (alkaline and oxidative stress), *qacH* gene (resistance to benzalkonium chloride), *cadA1C* gene cassette (resistance to 70 mg/l of cadmium chloride) and a truncated version of *actA* gene (biofilm formation). Conversely, ST14 draft genomes were enriched with a full-length version of *actA* gene along with the *Listeria* Genomic Island 2 (LGI 2) including the *ars* operon (arsenic resistance) and the *cadA4C* gene cassette (resistance to 35 mg/l of cadmium chloride). Phenotypic tests confirmed ST121 as a weak biofilm producer in comparison to ST14.

In conclusion, ST121 carried the *qacH* gene and was phenotypically resistant to quaternary ammonium compounds. This property might contribute to the high prevalence of ST121 in food processing plants. ST14 showed greater ability to form biofilms, which might contribute to the occasional colonization and persistence on harborage sites where sanitizing procedures are difficult to display.

Keywords: *Listeria monocytogenes*, the food processing environment, persistence, stress, adaptation

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen adapted to survive in a variety of environmental locations including soil, groundwater, decaying vegetation (Gray et al., 2006). In food-processing plants, *L. monocytogenes* has been repeatedly isolated from both food and the environment. Based on different molecular typing methods, isolates sharing the same profile have been collected over months or years in fish, meat, dairy and vegetable processing plants (Leong et al., 2014; Stasiewicz et al., 2015; Véghová et al., 2017). Strains repeatedly isolated over time in the same plant are considered as persistent. Unfortunately, there is not yet an agreement on specific issues related to the definition of persistence. In particular, the number of times of re-isolation, the sources as well as the period of isolation are not yet uniquely defined (Ferreira et al., 2014).

Persistent strains of high virulence are of major concern since they commonly colonize harborage sites difficult to clean or to reach by sanitizing procedures. These strains typically contaminate different lots of food during several months of production and have been described as responsible of outbreaks including few to hundreds cases spread in time and geographical areas (Tompkin, 2002).

The reasons why *L. monocytogenes* persists in food processing plants is still on debate. One strain can colonize harborage sites by chance (Carpentier and Cerf, 2011). In this view, the persistence of the strain was supposed as more related to characteristics of the environment rather than of the strain itself (Carpentier and Cerf, 2011). Authors failed to identify associations among persistence and particular genes/features of the strain (Ferreira et al., 2011; Stasiewicz et al., 2015). Nevertheless, a differential distribution of specific subtypes in food environments and clinical samples was observed, suggesting that *L. monocytogenes* strains might harbor unique genotypic and phenotypic features facilitating survival and growth and ultimately spread to humans. In foods and food processing environments, *L. monocytogenes* of lineage II and serotype 1/2a has been more frequently collected than lineage I (Orsi et al., 2011). Within serotype 1/2a, Clonal Complex (CC) 121 was the most prevalent clone (17%) (Maury et al., 2016). In particular within CC 121, food source was overrepresented in comparison to clinical one (92.9% vs. 7.0%) (Maury et al., 2016). Human strains belonging to CC121 showed prevalence of 9.5 and 2.3% among 116 strains of the Institute Pasteur *L. monocytogenes* database (Moura et al., 2016) and 262 sporadic cases collected throughout Europe, respectively (Nielsen et al., 2017). Low frequency of CC121 in clinical samples was associated to an attenuated virulence of this subtype, which often carries Premature Stop Codon Mutations in the virulence gene marker *inlA*. (Olier et al., 2002, 2003; Van Stelten et al., 2010; Beier and Bertilsson, 2013; Palma et al., 2017).

The CC14 is another clonal complex of serotype 1/2a. Compared to CC121, CC14 was seldom isolated in foods in France (1.4%) (Maury et al., 2016). Similarly, low detection values were reported in 19 meat processing plants located in Northern Italy (5.7% over 69 tested isolates) in comparison to CC121 (23%) (Morganti et al., 2016). Besides a low prevalence in foods, CC14 was described to gather a high percentage of isolates of clinical source (29.5%) (Maury et al., 2016). Within CC14, hypervirulent strains were described. In particular, a Multi Locus Sequence Type (ST) 14 strain was associated to a case of invasive listeriosis. Further molecular analyses revealed that this strain belongs to epidemic clone (EC) III (corresponding to Multi-Virulence Locus Type (VT) 1) previously linked to a sporadic case as well as a multi-state outbreak occurred in United States in 1988 and 2000, respectively (Kathariou, 2002; Mammina et al., 2013).

Physiological adaptation (ecophysiology) to environmental stresses including resistance to antimicrobials, heavy metals and quaternary ammonium compounds (QAC) as well as adaptation to cold, salt, acid, oxidative stresses, desiccation and ability of biofilm formation are often described in *L. monocytogenes* isolated from food processing plants (Lebrun et al., 1994; Kathariou, 2002; Srinivasan et al., 2005; Mullapudi et al., 2008; Ryan et al., 2010; Hein et al., 2011; Ratani et al., 2012; Müller et al., 2013; Ortiz et al., 2015; Kovacevic et al., 2016; Xu et al., 2016; Harter et al., 2017; Hingston et al., 2017). Studies on ST121 strains have been recently performed aiming at identifying genes associated to persistence and/or adaptation to food-processing environmental stresses. In particular, deletions of *lmo02774-lmo2776* were described along with no significant association of genes (Holch et al., 2013; Knudsen et al., 2017). In one study, ST121 isolates were found to harbor the *qacH* carrying transposon Tn*1688* (QAC resistance) (Ortiz et al., 2015). No similar studies are available on ST14.

Analyses based on Whole Genome Sequencing (WGS) have recently revealed an unprecedented potential for multiple investigations as a one-serve-all approach. WGS-based typing showed valuable potential in pathogen fingerprinting. In particular, a high discrimination power has been achieved by single nucleotide polymorphisms (SNPs) analysis. This is particularly relevant to discriminate strains showing high genetic similarity as determined by same Pulse Field Gel Electrophoresis (PFGE) or 7-loci MLST, as it is the case during a persistent event (Ferreira et al., 2014).

Based on WGS-data, studies on presence/absence/truncation of a wide number of genes can be performed at once. Genes described as strongly associated to a specific phenotype can be

selected through a literature search, and traced on sequenced genomes for prediction of that specific phenotype. At present, freely available tools unable a quick screening of genomes for thousands of genes selected for their association to virulence or antimicrobial resistance (McArthur et al., 2013; Zankari et al., 2012). Genes, useful to predict phenotypic traits associated to *L. monocytogenes* physiological adaptation to environmental conditions, have not been collected so far in a unique dataset.

In a previous study on prevalence of *L. monocytogenes* in four Italian rabbit meat processing plants, isolates indistinguishable by 7-loci MLST, Multi Locus Variable number tandem repeats Analysis (MLVA) type and *Apa*I-PFGE were repeatedly collected over time. Isolates were sampled from carcasses, meat cuts, meat products and the meat-processing environment (De Cesare et al., 2017). The dataset of this study was used to investigate further the potential virulence of specific subtypes. For this purpose, in a second study a specific focus was addressed on two subtypes gathering isolates of 7-loci MLST ST121 and ST14 collected over 1 year in the same processing plant (Palma et al., 2017). In this latter study, ST14 isolates showed higher virulence potential than ST121 as suggested by *in silico* virulotyping (Palma et al., 2017). In particular, all ST14 isolates belonged to VT107, which differed from ECIII (VT1) for only four nucleotides (Murugesan et al., 2015). Moreover, all ST121 and no ST14 genomes carried a truncated version of the *actA* gene and an *inlA* gene with a premature stop codon of type 6, both described as associated to attenuated virulence (Smith and Portnoy, 1997; Olier et al., 2002, 2003; Van Stelten et al., 2010).

In the present study, the ST121 and ST14 *L. monocytogenes* isolates, collected over 1 year on the same rabbit meat processing plant, were studied with a specific focus on persistence and physiological adaptation to food-processing environmental stresses. In particular, the research questions were: (1) assess persistence of *L. monocytogenes* isolates from the rabbit-plant; (2) select genes associated to physiological adaptation to the food-processing environment; (3) compare presence/absence/truncation of selected genes in newly sequenced and publicly available ST121 and ST14 genomes. A particular focus has been placed on the evaluation of putative gene enrichment in the two subtypes as well as identification of putative markers of ecophysiology associated to survival and growth of rare subtype ST14 of *L. monocytogenes*.

MATERIALS AND METHODS

Listeria monocytogenes Isolates From Italian Meat Processing Plants, Sequencing and *de Novo* Assembly

Listeria monocytogenes isolates included in the present study belong to a subset of isolates collected from November 2005 to November 2006 within a previous study on prevalence of *L. monocytogenes* in rabbit meat-processing plants (De Cesare et al., 2017). This subset included isolates belonging to two subtypes and collected from plant A. Isolates were considered as potentially persistent when they belonged to the same subtype and they were collected more than six times over a period of more than 6 months from different sources (rabbit carcasses, rabbit meat cuts, rabbit meat products, and the food processing environment). Each subtype gathered isolates indistinguishable by serotype, *Apa*I-PFGE, 7-loci MLST, MLVA and automated ribotype (De Cesare et al., 2017). The genomes of this subset of *L. monocytogenes* isolates were sequenced and *de novo* assembled in a previous study (Palma et al., 2017). Briefly, the genomes of 33 *L. monocytogenes* isolates belonging to ST121 (6) and ST14 (27) were previously sequenced by MiSeq (Illumina) platform. Paired-end reads were *de novo* assembled using the INNUca pipeline[1], which consists of several modules (e.g., Trimmomatic, SPAdes, Pilon) and QA/QC steps. Information on quality parameters of *de novo* assemblies were included in the previous study (Palma et al., 2017).

Publicly Available *L. monocytogenes* Genomes Included in the Present Study

A selection of draft genomes from ENA was carried out in order to: (1) explore the genetic distances of newly sequenced *L. monocytogenes* genomes in comparison to publicly available genomes; (2) study ST-related genetic markers associated to ecophysiology. In particular, 196 ST121 and 44 ST14 publicly available draft genomes were included. These genomes were from strains isolated from both food processing environments and humans. Moreover, they were widely distributed over time and geographical locations (**Supplementary Table S2**).

Whole-Genome SNP Analysis

Single nucleotide polymorphisms (SNPs) calling was performed using the Snippy v2.6 pipeline[2]. *De novo* assemblies of ST121 and ST14 genomes were analyzed separately. A newly generated draft genome for each *L. monocytogenes* ST was chosen as reference genome: LSALM51 for ST121 and LSALM1 for ST14. After removal of Illumina Nextera adapters and low-quality sequences (Phred scores of <10), the *de novo* assemblies were mapped to the reference genome with the Burrows-Wheeler Aligner (BWA) v0.7.12 using default parameters (Li and Durbin, 2009). After mapping, average depths were determined with SAMtools v1.3 (Li et al., 2009). Variants were called using Freebayes v0.9.20 (Garrison and Marth, 2012) with the following parameters: minimum base quality of 20, minimum read coverage of 10X, and 90% read concordance at a locus. Snippy was used to pool all SNP positions called in at least one isolate and investigate all isolates. Alignments of all SNPs were produced in Snippy and used to infer a high-resolution phylogeny. A maximum likelihood (ML) tree was constructed using the PhyML v2.4.4 program to analyze the SNP differences between isolates and FigTree v1.4.3 software[3] was used to visualize the tree rooting at midpoint for each ST. Draft genomes were tentatively considered as belonging to the same persistent clone if the following criteria were fulfilled: (1) the difference between draft genomes and the reference genome was equal or lower than 25 SNPs; (2) draft genomes belonged to

[1]https://github.com/B-UMMI/INNUca

[2]https://github.com/tseemann/snippy

[3]https://github.com/rambaut/figtree

isolates collected from different origins (food and the processing environment) for at least six times during a time frame of at least 6 months. The cut-off of 25 SNPs was used as previously suggested (Nielsen et al., 2017).

Dataset of Putative Gene Markers of Ecophysiology in *L. monocytogenes*

An extensive literature review was conducted on PubMed[4] using following keywords: "antimicrobials," "QAC," "heavy metals," "bacteriocins," "cold," "high salt concentration," "low pH," "desiccation," "blue-light," "biofilm," "*L. Monocytogenes,*" and "genes." At the time of analysis (October 2017), the system retrieved overall 664 peer-reviewed published papers. The abstract of each of these papers was read with the purpose to identify genes associated to the response of *L. monocytogenes* to each stress. Around 100 papers were selected based on the abstract. These papers were thoroughly read in order to identify and specifically select genes with a strong association to the related phenotype. For this purpose, only genes confirmed by insertional mutagenesis or mutant selection experiments were included (**Supplementary Table S1**). All GenBank accession numbers and related sequences reported in published papers were checked by nucleotide BLAST[5] and CLUSTAL Omega[6] for alignment. For each gene, the following informations were reported: name of the gene, locus tag, annotation, main function, localization, reference paper, GenBank accession number and direct link to the web page of the sequence in NCBI[7] (**Supplementary Table S1**).

Screening of Genes of Ecophysiology

A multifasta file was generated with the sequences of all selected genes (**Supplementary Table S1**). Nucleotide BLAST was run locally using ABRricate pipeline[8] on 202 ST121 as well as 71 ST14 draft genomes (**Supplementary Table S2**) with the mutifasta file as gene database. Based on the output matrix of gene presence/absence, a heatmap was built using morpheus software[9]. Morever, results were analyzed for statistically significant differences at 95% confidence by *t*-test.

Antimicrobial Susceptibility

For phenotypic confirmation, newly sequenced strains carrying antimicrobial resistant genes (*ampC, tetA*) were tested for susceptibility against ampicillin and tetracycline by disk diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2010; Jamali et al., 2015). The assay was performed on Mueller Hinton Agar plates supplemented with 5% defibrinated sheep blood (Thermo Scientific, Milan, Italy). Ampicillin (10 μg) and tetracycline (30 μg), were applied as antibiotic agents following Clinical and Laboratory Standards Institute [CLSI] recommendations for fastidious organisms (document M45-A2).

Cadmium Chloride and Benzalkonium Chloride Susceptibility

Determination of cadmium chloride and benzalkonium chloride resistance was performed as previously described (Mullapudi et al., 2008). Briefly, a single colony from a blood agar plate culture was suspended in 100 μl of tryptic soy broth (Thermo Fisher Scientific, Milan, Italy). Three microliters of the suspension were spotted in duplicate onto: (1) Mueller Hinton Agar Cation adjusted (MHBII) containing 2% defibrinated sheep blood (Thermo Fisher Scientific) (control); (2) MHBII (Thermo Fisher Scientific) containing 2% defibrinated sheep blood (Thermo Fisher Scientific) and 70 mg/l of cadmium chloride anhydrous (Sigma, Milan, Italy); (3) MHBII (Thermo Fisher Scientific) containing 2% defibrinated sheep blood (Thermo Fisher Scientific) and 35 mg/l of cadmium chloride anhydrous (Sigma, Milan, Italy); (4) Mueller Hinton Agar (MHB) (Thermo Fisher Scientific) containing 2% defibrinated sheep blood (Thermo Fisher Scientific) and 10 mg/l benzalkonium chloride (Sigma). Positive and negative control strains were included. All plates were incubated at 37°C for 48 h.

Crystal Violet Staining Assay

In order to test the ability of biofilm formation, representative isolates of identified clones of ST121 and ST14 isolates of *L. monocytogenes* were submitted to the crystal violet staining assay as previously described (Stepanović et al., 2004). Briefly, 20 μl of an overnight bacterial culture were added to 230 μl of Brain Heart Infusion broth (BHI, Thermo Scientific, Milan, Italy) into each well of a sterile 96-well not tissue treated polystyrene microplate (Sarstedt, Milan Italy). Plates were incubated at 35°C for 24 h. The content of each plate was discarded and 300 μl of sterile distilled water were added to each well. This washing step was repeated three times. Adherent bacteria were fixed with 250 μl of methanol per well. After 15 min, methanol was discarded and plates air-dried overnight. Biofilms were stained with 250 μl per well of Crystal violet (Gram-color staining set for microscopy; Merck) for 5 min. Excess stain was rinsed off and microplates air-dried. Then, attached bacteria were solubilized with 250 μl of 33% (v/v) glacial acetic acid per well. Finally, the optical density (OD) of each well was measured at 570 nm using Infinite® F50 Absorbance Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland). According to Stepanović et al. (2004), based on the OD produced by bacterial films, strains were classified as no, weak, moderate or strong biofilm producers. All isolates were tested in triplicate.

Nucleotide Sequence Accession Numbers

De novo assembled genomes of the 33 *L. monocytogenes* isolates included in this study were submitted to GenBank under BioProject no. PRJNA396103 with individual BioSample identification (ID) numbers SAMN07420940 to SAMN07420973.

[4]https://www.ncbi.nlm.nih.gov/pubmed/
[5]https://blast.ncbi.nlm.nih.gov/Blast.cgi
[6]https://www.ebi.ac.uk/Tools/msa/clustalo/
[7]https://www.ncbi.nlm.nih.gov/nuccore/
[8]https://github.com/tseemann/abricate
[9]https://software.broadinstitute.org/morpheus/

RESULTS

Whole-Genome SNP Analysis

In order to increase the resolution of whole-genome SNP analysis, assemblies of ST121 and ST14 sequenced isolates were mapped separately against *de novo* assemblies of the reference genomes (i.e., LSALM51, LSALM1 strains). The resulting ML trees are reported in **Figures 1**, **2**. Within ST14, 23 out of 27 isolates collected from the rabbit meat-processing environment as well as from rabbit meat carcasses, meat cuts and products from November 2005 to November 2006, shared SNPs counts ranging from 0 to 25 SNPs in comparison to the reference genome LSALM1 and were considered as belonging to the same persistent clone. Three isolates (LSALM8, LSALM9, LSALM10), collected from rabbit carcasses from June to August 2006, showed 29–33 SNPs whereas one isolate (LSALM22), collected in November 2006 showed 359 SNPs (**Figure 1**).

ST121 isolates were clearly differentiated in two distinct clusters (**Figure 2**). One cluster included isolates LSALM50 and LSALM53, collected from rabbit meat cuts in May 2006 and sharing 204 and 214 SNPs in comparison to the reference genome LSALM51. The second cluster included the reference along with LSALM27, LSALM29, LSALM31. These isolates, sharing from 0 to 17 SNPs, were collected from the rabbit meat-processing environment as well as from meat cuts from December 2005 to May 2006 (**Figure 2**).

Public ST121 draft genomes showed genetic distances ranging from 45 SNPs (processing environment, Danmark) to 1584 SNPs (fish product, China) in comparison to the internal reference sequence LSALM51 (**Figure 1**). Public ST14 draft genomes displayed SNPs differences ranging from 60 SNPs (environmental swab, United States) to 1183 SNPs (RTE product, United States). These results confirm the high clonality of *L. monocytogenes* especially within the same ST.

Dataset of Putative Gene Markers of Ecophysiology in *L. monocytogenes*

Overall 94 genes, described in 41 published papers, were included in the dataset (**Supplementary Table S1**). All selected genes were identified as strongly associated to specific phenotypes related to

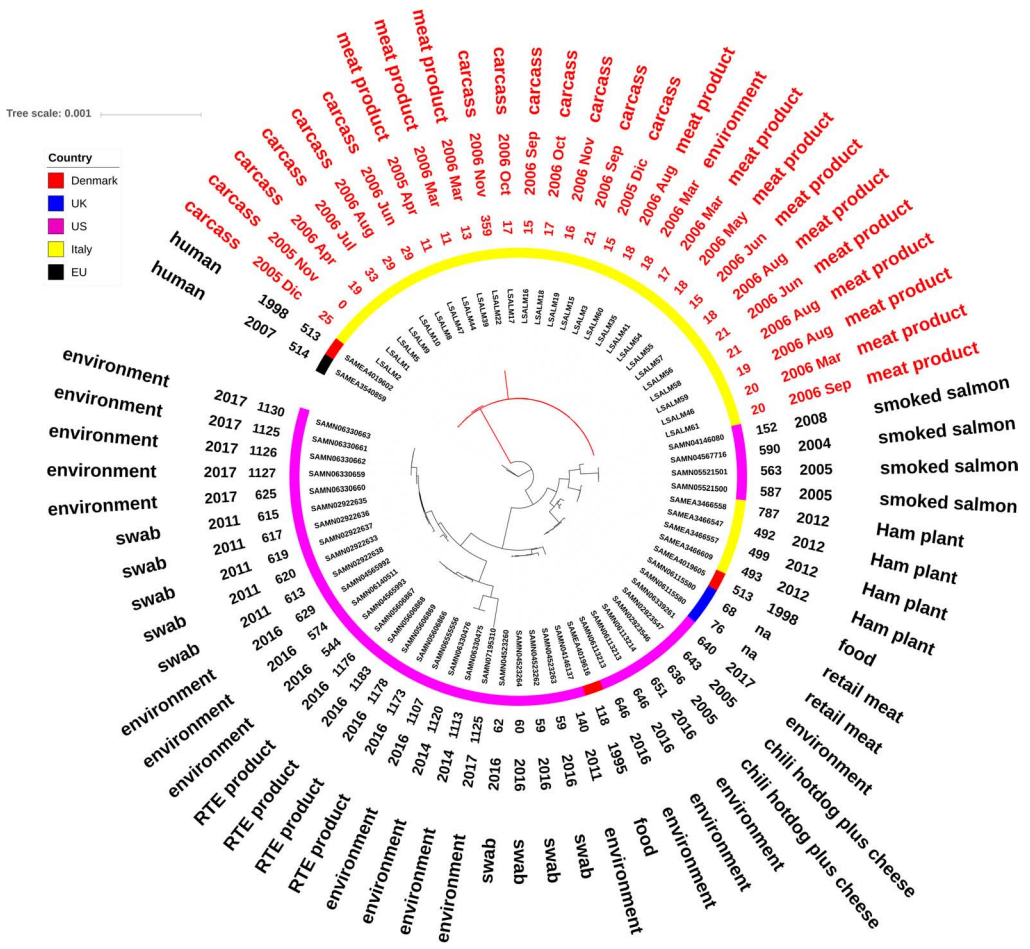


FIGURE 1 | Maximum likelihood tree showing genetic distances between ST14 *L. monocytogenes* draft genomes based on whole-genome core SNPs. The tree tips list study ID of the isolate, date of isolation, origin (rabbit carcass, rabbit meat cut, rabbit meat product, the food processing environment) and the SNP count of the ID genome in comparison to the reference (LSALM1). The scale bar refers to the branch length representing the number of nucleotide substitutions per site.

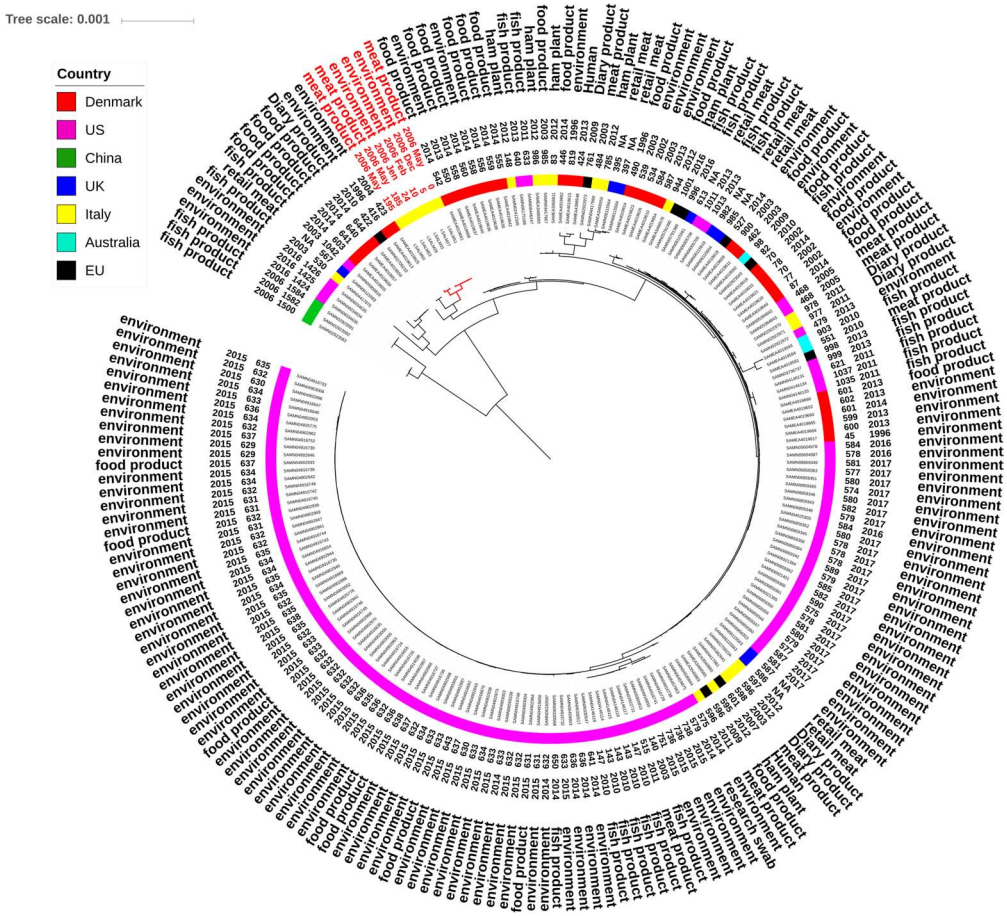


FIGURE 2 | Maximum likelihood tree showing genetic distances between ST121 *L. monocytogenes* draft genomes based on whole-genome core SNPs. The tree tips list study ID of the isolate, date of isolation, origin (rabbit meat cut, the food processing environment) and the SNP count of the ID genome in comparison to the reference (LSALM51). The scale bar refers to the branch length representing the number of nucleotide substitutions per site.

physiological adaptation of *L. monocytogenes* to environmental stresses encountered in food processing plants. In particular the genes were associated to: resistance to antimicrobials, QAC, heavy metals and bacteriocins; adaptation to cold, high salt concentration, low pH, desiccation and biofilm formation (**Supplementary Table S1**).

Fourteen genes were included related to resistance to different antimicrobial classes such as tetracycline, ampicillin, vancomycin, streptomycin, chloramphenicol/florfenicol, sulphonamides, erythromycin, and fluoroquinolones (Poyart-Salmeron et al., 1992; Charpentier et al., 1993; Roberts et al., 1996; Godreuil et al., 2003; Srinivasan et al., 2005; Lungu et al., 2011; Jamali et al., 2015).

Seven genes associated to resistance to benzalkonium chloride were included (*qacH, qacA, qacC, bcrA, bcrB, bcrC,* and *emrE*). These genes are associated to the active efflux pump of QAC (Elhanafi et al., 2010; Müller et al., 2013; Xu et al., 2014; Kovacevic et al., 2016; Nielsen et al., 2017).

Sixteen genes associated to resistance to cadmium and arsenic were included. In particular, three gene cassettes, *cadA1C, cadA2C,* and *cadA3C* were associated to resistance to 70 mg/l of cadmium chloride, whereas *cadA4C* was associated to resistance to 35 mg/l (Mullapudi et al., 2010; Parsons et al., 2017). Regarding resistance to arsenic, *ars* genes were associated to resistance to 500 mg/l of sodium (meta) arsenite (Lee et al., 2013).

Regarding bacteriocins resistance, 5 genes associated to the response of *L. monocytogenes* to cell-envelope stress were included: *virR*, *virS*, *mprF*, *lia*R, *anrB* (Thedieck et al., 2006; Collins et al., 2010; Bergholz et al., 2013; Kang et al., 2015).

As far as stress adaptation is considered, different mutant selection experiments demonstrated the important role of the *sigB* gene (sigma factor B) in the regulation of expression of several genes associated to adaptation to environmental stresses. In particular the knockout of *sigB* was directly associated to adaptation to desiccation in *L. monocytogenes* (Huang et al., 2015).

As far as blue-light is considered, the gene *lmo0799*, coding for a blue-light receptor, was strongly associated to the adaptation of *L. monocytogenes* to this particular stress (Ondrusch and Kreft, 2011; O'Donoghue et al., 2016).

Listeria monocytogenes can adapt to cold following different pathways. One pathway includes cold shock proteins cspB and

cspD (Schmid et al., 2009). Another pathway includes the glycine/betaine transporter system which mediates the uptake of osmolytes, such as glycine, betaine and carnitine, important for adaptation to both cold and high salt concentration (Angelidis and Smith, 2003). Overall, 13 genes related to adaptation of *L. monocytogenes* to cold and/or high salt concentration were included in the dataset (Angelidis and Smith, 2003; Schmid et al., 2009; Markkula et al., 2012; Pöntinen et al., 2015).

The Stress Survival Islet 1, corresponding to a cassette of five genes (lmo0444-lmo0448), was associated to the survival and growth of *L. monocytogenes* under suboptimal conditions. In particular, the knockout of the entire SSI-1 was associated to an impaired ability of this food-borne pathogen to grow at low pH and high salt concentrations (Ryan et al., 2010). Within this gene cassette, *gadD1* (lmo0447) and *gadT1* (lmo0448) encode for a glutamate decarboxylase and an amino acid transporter both described as specifically involved in the adaptation to low pH (Feehily et al., 2014).

Along the GAD System, the ADI system might be activated in response to low pH. The *arc* gene is involved in transformation of arginine into ornithine with ammonia as by product, which increases the pH. The ADI system has been described in response to mild acid pH (Feehily et al., 2014). Overall, 16 genes associated to low pH adaptation were included in the dataset (Abram et al., 2008; Ryan et al., 2009; Ryan et al., 2010; Feehily et al., 2014).

The Stress Survival Islet 2 (SSI-2) was firstly described as a cassette of two genes of *Listeria innocua* often present in place of SSI-1 in *L. monocytogenes* ST121 (Hein et al., 2011). SSI-2 was more recently associated to alkaline and oxidative stress in *L. monocytogenes* (Harter et al., 2017).

Regarding adaptation to desiccation, seven genes related to motility of *L. monocytogenes*, were recently associated to this specific phenotype (Hingston et al., 2015). In particular, these genes were found to be downregulated in desiccation tolerant *L. monocytogenes*.

As far as the ability of biofilm is concerned, 8 genes associated to biofilm formation were included in the dataset. Biofilm formation is essential for survival of *L. monocytogenes* and further contributes to bacterial persistence in the food processing environment (Popowska et al., 2017).

Screening of Putative Gene Markers of Ecophysiology and Related Phenotypic Tests

The 94 putative gene markers of ecophysiology were screened on 202 and 71 genomes of *L. monocytogenes* ST121 and ST14, respectively (**Figure 3**). Twenty-one genes were not found in any of the tested draft genomes and were not included into the heatmap (**Figure 3**).

As far as antimicrobial resistance gene markers are concerned, all genomes were positive for *ampC* and *tetA* associated to penicillin and tetracycline resistance, respectively. However, phenotypic tests did not confirm genetic results. Although disk diffusion breakpoints are not available for *Listeria*, all isolates showed zone diameters equal or higher than 30 mm to tetracycline and ampicillin, suggesting susceptibility (data not



FIGURE 3 | Heatmap of the 202 ST121 and 71 ST 14 genomes function to the ecophysiology gene dataset. The rows represent the genomes included while columns represent each gene of the dataset. The colors represent the percentage of identity and length of genes. Red represents lowest percentage while yellow highest percentage. Genes not found in any of the tested genomes were not included.

shown). Further analysis should be performed in order to assess the reason behind these discordant results.

Regarding resistance to QAC, 25 ST14 genomes (35.2%) and 184 (91.2%) ST121 genomes were positive for the *bcrABC* locus and the *qacH* gene, respectively. All ST14 genomes but 8 (88.7%) and none of the ST121 genomes were positive for the *ars* operon (arsenic resistance) and for the *cadA4C* gene cassette (cadmium resistance). Further analyses on the localization of these two gene clusters within the genome, revealed that both the *ars* operon and the *cadA4C* gene cassette are located in a genomic region already identified as *Listeria* genomic Island 2 (LGI-2) (Lee et al., 2013; Parsons et al., 2017) (data not shown). In ST14 isolates included in the present study, LGI-2 was found in 24 out of 27 isolates and it was inserted within the *gtfA2* gene (data not shown). This gene codes for a sucrose phosphorylase involved in *O*-glycosylation of proteins. Glycosylation of flagellins is essential for bacterial flagellar assembly, motility, virulence, and host specificity (Merino and Tomás, 2014). The three isolates lacking LGI-2 (LSALM16, LSALM17, LSALM18) belonged to the persistent clone and were collected in September and November 2006 at the end of the sampling period. Further analyses should be performed in order to confirm whether these three isolates are natural mutants, which lost the accessory genome sequence of SGI-2.

Twenty *cadA4C* positive ST14 genomes additionally carried *cadA2C* gene cassette. The *cadA1C* gene cassette was found only in ST121 and in particular in 177 out of the 202 tested genomes (87.6%). Phenotypic tests conducted on the 33 *L. monocytogenes* strains from the rabbit meat-plant, confirmed that all *qacH* positive ST121 isolates were resistant to benzalkonium chloride and that all *cadA1C* positive ST121 isolates were resistant to 70 mg/l of cadmium chloride, whereas all *cadA4C* positive ST14 isolates were resistant to 35 mg/l of cadmium chloride.

As for environmental stress adaptation, no genomes were positive for the Stress Survival Islet 1 (SSI-1) and all ST121 but no ST14 were positive for the Stress Survival Islet 2 (SSI-2). All tested genomes carried a full-length version of genes associated to: cell-envelope stress response linked to bacteriocins resistance, adaptation to cold and/or high salt concentration, low pH, blue-light and desiccation.

Regarding biofilm formation, the *actA* gene was truncated in all ST121 and in none of the ST14 genomes. Phenotypic tests confirmed the differential biofilm forming ability of ST121 in comparison to ST14. In particular, tested ST121 isolates were classified as weak biofilm producers with ODC (OD Control) median value of 0.15 and OD values ranging from 0.17 to 0.24 OD, whereas all ST14 isolates but three (LSALM8, LSALM9, LSALM10) were categorized as moderate biofilm producers, with OD values ranging from 0.30 to 0.52 OD at 570 nm. OD values of ST121 isolates were statistically significant different in comparison to ST14 ones ($P = 0{,}00238$).

DISCUSSION

In the present study, the persistence of *L. monocytogenes* ST121 and ST14 repeatedly isolated within 1 year of sampling in a rabbit meat processing plant was studied by a genomic approach. Moreover, 94 putative gene markers of *L. monocytogenes* physiological adaptation to the food processing environment were investigated in newly sequenced as well as publicly available genomes of ST121 and ST14 strains. The aim was to evaluate the significant enrichment of these genes in the two subtypes, with a particular focus on those genes associated to ecophysiology in ST14, a subtype rarely isolated in food processing plants.

Based on PFGE-Typing, persistence of *L. monocytogenes* in dairy, meat, fish and vegetable sectors was extensively observed (Leong et al., 2014; Ortiz et al., 2015; Véghová et al., 2017). However, more recently, whole genome SNPs analysis revealed a superior discriminatory power. This is particularly relevant in studies in which highly similar strains have to be differentiated in order to distinguish true persistent from sporadic strains (Ferreira et al., 2014). SNPs calling revealed that the majority but not all of the ST14 isolates sharing the same PFGE-typing, belonged to the same clone. This clone, gathering 23 out of 27 isolates, was re-isolated more than six times within 1 year from rabbit carcasses, meat cuts, meat products and the processing environment in the same rabbit meat processing plant. Although an agreement has not been achieved on the definition of persistence, for the purpose of the present study, the ST14 clone was considered as persistent (Ferreira et al., 2014). This persistent clone included ST14 genomes sharing a maximum of 25 SNPs differences. A difference of 25 SNPs has been already proposed as cut off for definition of genetically related strains belonging to a single persistent clone of *L. monocytogenes* (Nielsen et al., 2017). The persistence of a *L. monocytogenes* strain for long periods confirms a higher risk of food contamination and human exposure to specific pathogen strains (Tompkin, 2002). The concern is even higher when the persistent clone belongs to subtype ST 14, which was described as including hypervirulent strains (e.g., ST14) (Mammina et al., 2013; Voronina et al., 2015; Maury et al., 2016; Palma et al., 2017).

Single nucleotide polymorphisms (SNPs) analysis gathered ST121 isolates in two clusters of 2 and 4 isolates each. SNPs analyses showed a higher discriminatory power in comparison to other molecular typing methods (*Apa*I-PFGE, 7-loci MLST, MLVA, ribotyping), all of which identified all ST121 isolates as belonging to the same clone (De Cesare et al., 2017). The analyzed dataset suggests that one ST121 clone gathering four isolates, survived over 5 months in the same rabbit meat processing plant. However, since those strains were repeatedly isolated in a relative short time frame, this clone cannot be considered as persistent.

In order to gain more insights on the genomic bases behind the differential frequency of ST121 and ST14 in food processing plants, a comprehensive literature review was performed in order to identify genes associated to physiological adaptation of *L. monocytogenes* to specific food-processing environmental stresses. In the literature, different dataset of genes associated to particular phenotypes, such as antimicrobial resistance and virulence of *L. monocytogenes,* have been described (Zankari et al., 2012; McArthur et al., 2013). However, to the best of author's knowledges, no dataset on genes associated to ecophysiology of *L. monocytogenes* is available.

In the present study, 94 genes associated to ecophysiology were included in a dataset available for the public. Genes were included in the dataset only if their association to the specific phenotype was confirmed by insertional mutagenesis or deletion mutants experiments (**Supplementary Table S2**). In particular the dataset includes genes associated to resistance to antimicrobials, QAC, heavy metals and bacteriocins as well as associated to adaptation to cold, high salt concentration, low pH, desiccation and biofilm formation.

The results on 202 ST121 and 71 ST14 genomes outlined interesting findings with particular reference to genes significantly enriched in one or the other subtype (**Figure 3**). In particular genes *qacH*, *cadA1C*, Stress Survival Islet -2 and a truncated version of the *actA* gene were significantly enriched within the ST121 genomes ($P < 0{,}000001$), whereas the *ars* operon and *cadA4C* gene cassettes included in the *Listeria* Genomic Islands 2, the *bcrABC* locus and a full-length version of the *actA* gene were significantly enriched in ST14 genomes ($P < 0{,}000001$). The presence of a QAC associated genetic determinant (either the *qacH* gene or the *bcrABC* locus) was significantly enriched in ST121 genomes in comparison to ST14 ($P < 0{,}000001$). This observation underlines that subtypes ST121 and ST14 have different patterns of genes associated to ecophysiology. Further studies should confirm whether other subtypes with high and low frequency show the same pattern of genes as identified in ST121 and ST14 subtypes, respectively. Comparing the two patterns it appears that ST121 showed high adaptation to sanitizing procedures (resistance to QAC and adaptation to alkaline stress) (*qacH;* SSI-2) along with adaptation to high cadmium concentration (*cadA1C*) (Mullapudi et al., 2010; Müller et al., 2013; Harter et al., 2017). Moreover, ST121 harbored a truncated version of the *actA* gene whereas ST14 harbored a full-length version of the same gene. The *actA* gene is involved in the polymerization of actin which is important for motility of *L. monocytogenes* within the host cell as well as in the first steps of biofilm formation and in particular in cell-to-cell aggregation (Smith and Portnoy, 1997; Travier et al., 2013). Deletion mutants ΔactA showed attenuated virulence and inability to form biofilms (Travier et al., 2013). The higher biofilm forming ability of ST14 in comparison to ST121, was phenotypically confirmed.

All tested genomes carried a full-length version of genes associated to: cell-envelope stress response linked to bacteriocins resistance; adaptation to cold and/or high salt concentration, low pH and desiccation. These genes common to all *L. monocytogenes* genomes were not informative for the differentiation of the two ST121 and ST14 subtypes. For those genes, mostly located in the core genome, specific allele types can be identified. Genome wide association studies can be performed to associate specific allele types to specific phenotypes. Allele types with a strong association to the phenotype could be then included in the dataset. The usefulness of this approach was already demonstrated. For example a genome-wide association study (GWAS) on *Campylobacter jejuni*, revealed that, based on overrepresented genetic elements, different *C. jejuni* subtypes show distinct genotypes associated with survival from farm to fork (Yahara et al., 2017).

CONCLUSION

The different gene enrichment found in ST121 and ST14 supported by phenotypic confirmations, suggest that ST121 mainly include strains resistant to sanitizers. This feature might in part explain the high frequency of detection of this subtype in food-processing plants. ST14 include biofilm producer strains. This feature suggest that ST14 might occasionally contaminate harborage sites where sanitizing procedures are difficult to be performed. If confirmed, this route of contamination might be of concern since ST14 could potentially spread from harborage sites to different food lots scattered in months or years. The concern is even higher if a hypervirulent strain is the driver of this repeated contamination.

AUTHOR CONTRIBUTIONS

FrP designed the study, performed the literature review of genes related to ecophysiology, and draft the manuscript. FeP performed data analysis and contributed in manuscript writing. LG contributed to the setup of the dataset. AL performed the phenotypic tests. ADC contributed to the literature review. GM collected the samples, revised the manuscript, and coordinated the study. All authors have contributed to data interpretation, have critically reviewed the manuscript, and approved the final version as submitted.

FUNDING

This study was funded by European Union Horizon 2020 Research and Innovation Program: COMPARE (collaborative management platform for detection and analyses of [re-]emerging and foodborne outbreaks in Europe: grant number 643476).

ACKNOWLEDGMENTS

The authors gratefully acknowledge Prof. Mirko Rossi of University of Helsinki for supportive, critical and helpful discussions. Moreover, the authors wish to thank Dr. Jani Halkilahti for support in collecting public *Listeria* genomes.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00596/full#supplementary-material

TABLE S1 | Dataset of putative gene markers of ecophysiology in *L. monocytogenes*.

TABLE S2 | Public genomes of *L. monocytogenes* ST121 and ST14 included in the present study.

REFERENCES

Abram, F., Starr, E., Karatzas, K. A., Matlawska-Wasowska, K., Boyd, A., Wiedmann, M., et al. (2008). Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance. *Appl. Environ. Microbiol.* 74, 6848–6858. doi: 10.1128/AEM.00442-08

Angelidis, A. S., and Smith, G. M. (2003). Three transporters mediate uptake of glycine betaine and carnitine by *Listeria monocytogenes* in response to hyperosmotic stress. *Appl. Environ. Microbiol.* 69, 1013–1022. doi: 10.1128/AEM.69.2.1013-1022.2003

Beier, S., and Bertilsson, S. (2013). Bacterial chitin degradation-mechanisms and ecophysiological strategies. *Front. Microbiol.* 4:149. doi: 10.3389/fmicb.2013.00149

Bergholz, T. M., Tang, S., Wiedmann, M., and Boor, K. J. (2013). Nisin resistance of *Listeria monocytogenes* is increased by exposure to salt stress and is mediated via LiaR. *Appl. Environ. Microbiol.* 79, 5682–5688. doi: 10.1128/AEM.01797-13

Carpentier, B., and Cerf, O. (2011). Review — Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol.* 145, 1–8. doi: 10.1016/j.ijfoodmicro.2011.01.005

Charpentier, E., Gerbaud, G., and Courvalin, P. (1993). Characterization of a new class of tetracycline-resistance gene tet(S) in *Listeria monocytogenes* BM4210. *Gene* 131, 27–34. doi: 10.1016/0378-1119(93)90665-P

Clinical and Laboratory Standards Institute [CLSI] (2010). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*, 2nd Edn. Wayne, PA: CLSI.

Collins, B., Curtis, N., Cotter, P. D., Hill, C., and Ross, R. P. (2010). The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various beta-lactam antibiotics. *Antimicrob. Agents Chemother.* 54, 4416–4423. doi: 10.1128/AAC.00503-10

De Cesare, A., Parisi, A., Mioni, R., Comin, D., Lucchi, A., and Manfreda, G. (2017). *Listeria monocytogenes* circulating in rabbit meat products and slaughterhouses in Italy: prevalence data and comparison among typing results. *Foodborne Pathog. Dis.* 14, 167–176. doi: 10.1089/fpd.2016.2211

Elhanafi, D., Dutta, V., and Kathariou, S. (2010). Genetic characterization of plasmid-associated benzalkonium chloride resistance determinants in a *Listeria monocytogenes* strain from the 1998-1999 outbreak. *Appl. Environ. Microbiol.* 76, 8231–8238. doi: 10.1128/AEM.02056-10

Feehily, C., Finnerty, A., Casey, P. G., Hill, C., Gahan, C. G., O'Byrne, C. P., et al. (2014). Divergent evolution of the activity and regulation of the glutamate decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: roles in virulence and acid tolerance. *PLoS One* 9:e112649. doi: 10.1371/journal.pone.0112649

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Moreno Switt, A., Hogg, T., et al. (2011). Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Appl. Environ. Microbiol.* 77, 2701–2715. doi: 10.1128/AEM.02553-10

Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J. Food Prot.* 77, 150–170. doi: 10.4315/0362-028X.JFP-13-150

Garrison, E., and Marth, G. (2012). *Haplotype-Based Variant Detection from Short-Read Sequencing*. Available at: http://arxiv.org/abs/1207.3907 [accessed August 6, 2017].

Godreuil, S., Galimand, M., Gerbaud, G., Jacquet, C., and Courvalin, P. (2003). Efflux pump Lde is associated with fluoroquinolone resistance in *Listeria monocytogenes*. *Antimicrob. Agents Chemother.* 47, 704–708. doi: 10.1128/AAC.47.2.704-708.2003

Gray, M. J., Freitag, N. E., and Boor, K. J. (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to Pathogenic Mr. Hyde. *Infect. Immun.* 74, 2505–2512. doi: 10.1128/IAI.74.5.2505-2512.2006

Harter, E., Wagner, E. M., Zaiser, A., Halecker, S., Wagner, M., and Rychli, K. (2017). Stress survival islet 2, predominantly present in *Listeria monocytogenes* strains of sequence type 121, is involved in the alkaline and oxidative stress responses. *Appl. Environ. Microbiol.* 83:e00827-17. doi: 10.1128/AEM.00827-17

Hein, I., Klinger, S., Dooms, M., Flekna, G., Stessl, B., Leclercq, A., et al. (2011). Stress survival islet 1 (SSI-1) survey in *Listeria monocytogenes* reveals an insert common to *Listeria innocua* in sequence type 121 L. monocytogenes strains. *Appl. Environ. Microbiol.* 77, 2169–2173. doi: 10.1128/AEM.02159-10

Hingston, P., Chen, J., Dhillon, B. K., Laing, C., Bertelli, C., Gannon, V., et al. (2017). Genotypes associated with *Listeria monocytogenes* isolates displaying impaired or enhanced tolerances to cold, salt, acid, or desiccation stress. *Front. Microbiol.* 8:369. doi: 10.3389/fmicb.2017.00369

Hingston, P. A., Piercey, M. J., and Truelstrup Hansen, L. (2015). Genes associated with desiccation and osmotic stress in *Listeria monocytogenes* as revealed by insertional mutagenesis. *Appl. Environ. Microbiol.* 81, 5350–5362. doi: 10.1128/AEM.01134-15

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M., and Gram, L. (2013). Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Appl. Environ. Microbiol.* 79, 2944–2951. doi: 10.1128/AEM.03715-12

Huang, Y., Ells, T. C., and Truelstrup Hansen, L. (2015). Role of sigB and osmolytes in desiccation survival of *Listeria monocytogenes* in simulated food soils on the surface of food grade stainless steel. *Food Microbiol.* 46, 443–451. doi: 10.1016/j.fm.2014.09.007

Jamali, H., Paydar, M., Ismail, S., Looi, C. Y., Wong, W. F., Radmehr, B., et al. (2015). Prevalence, antimicrobial susceptibility and virulotyping of *Listeria* species and *Listeria monocytogenes* isolated from open-air fish markets. *BMC Microbiol.* 15:144. doi: 10.1186/s12866-015-0476-7

Kang, J., Wiedmann, M., Boor, K. J., and Bergholz, T. M. (2015). VirR-Mediated resistance of *Listeria monocytogenes* against food antimicrobials and cross-protection induced by exposure to organic acid salts. *Appl. Environ. Microbiol.* 81, 4553–4562. doi: 10.1128/AEM.00648-15

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* 65, 1811–1829. doi: 10.4315/0362-028X-65.11.1811

Knudsen, G. M., Nielsen, J. B., Marvig, R. L., Ng, Y., Worning, P., Westh, H., et al. (2017). Genome-wide-analyses of *Listeria monocytogenes* from food-processing plants reveal clonal diversity and date the emergence of persisting sequence types. *Environ. Microbiol. Rep.* 9, 428–440. doi: 10.1111/1758-2229.12552

Kovacevic, J., Ziegler, J., Wałecka-Zacharska, E., Reimer, A., Kitts, D. D., and Gilmour, M. W. (2016). Tolerance of *Listeria monocytogenes* to quaternary ammonium sanitizers is mediated by a novel efflux pump encoded by emrE. *Appl. Environ. Microbiol.* 82, 939–953. doi: 10.1128/AEM.03741-15

Lebrun, M., Audurier, A., and Cossart, P. (1994). Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to cadA and cadC of *Staphylococcus aureus* and are induced by cadmium. *J. Bacteriol.* 176, 3040–3048. doi: 10.1128/jb.176.10.3040-3048.1994

Lee, S., Rakic-Martinez, M., Graves, L. M., Ward, T. J., Siletzky, R. M., and Kathariou, S. (2013). Genetic determinants for cadmium and arsenic resistance among *Listeria monocytogenes* serotype 4b isolates from sporadic human listeriosis patients. *Appl. Environ. Microbiol.* 79, 2471–2476. doi: 10.1128/AEM.03551-12

Leong, D., Alvarez-Ordóñ A., and Jordan, K. (2014). Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. *Front. Microbiol.* 5:436. doi: 10.3389/fmicb.2014.00436

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Lungu, B., O'Bryan, C. A., Muthaiyan, A., Milillo, S. R., Johnson, M. G., Crandall, P. G., et al. (2011). *Listeria monocytogenes*: antibiotic resistance in food production. *Foodborne Pathog. Dis.* 8, 569–578. doi: 10.1089/fpd.2010.0718

Mammina, C., Parisi, A., Guaita, A., Aleo, A., Bonura, C., Nastasi, A., et al. (2013). Enhanced surveillance of invasive listeriosis in the Lombardy region, Italy, in the years 2006-2010 reveals major clones and an increase in serotype 1/2a. *BMC Infect. Dis.* 13:152. doi: 10.1186/1471-2334-13-152

Markkula, A., Mattila, M., Lindström, M., and Korkeala, H. (2012). Genes encoding putative DEAD-box RNA helicases in *Listeria monocytogenes* EGD-e are needed for growth and motility at 3°C. *Environ. Microbiol.* 14, 2223–2232. doi: 10.1111/j.1462-2920.2012.02761.x

Maury, M. M., Tsai, Y.-H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., et al. (2016). Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nat. Genet.* 48, 308–313. doi: 10.1038/ng.3501

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., et al. (2013). The comprehensive antibiotic resistance database. *Antimicrob. Agents Chemother.* 57, 3348–3357. doi: 10.1128/AAC.00419-13

Merino, S., and Tomás, J. M. (2014). Gram-Negative Flagella Glycosylation. *Int. J. Mol. Sci.* 15, 2840–2857. doi: 10.3390/ijms15022840

Morganti, M., Scaltriti, E., Cozzolino, P., Bolzoni, L., Casadei, G., Pierantoni, M., et al. (2016). Processing-dependent and clonal contamination patterns of *Listeria monocytogenes* in the cured ham food chain revealed by genetic analysis. *Appl. Environ. Microbiol.* 82, 822–831. doi: 10.1128/AEM.03103-15

Moura, A., Criscuolo, A., Pouseele, H., Maury, M. M., Leclercq, A., Tarr, C., et al. (2016). Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nat. Microbiol.* 2:16185. doi: 10.1038/nmicrobiol.2016.185

Mullapudi, S., Siletzky, R. M., and Kathariou, S. (2008). Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. *Appl. Environ. Microbiol.* 74, 1464–1468. doi: 10.1128/AEM.02426-07

Mullapudi, S., Siletzky, R. M., and Kathariou, S. (2010). Diverse cadmium resistance determinants in *Listeria monocytogenes* isolates from the turkey processing plant environment. *Appl. Environ. Microbiol.* 76, 627–630. doi: 10.1128/AEM.01751-09

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stessl, B., Guinane, C. M., et al. (2013). Tn6188 - a novel transposon in *Listeria monocytogenes* responsible for tolerance to benzalkonium chloride. *PLoS One* 8:e76835. doi: 10.1371/journal.pone.0076835

Murugesan, L., Kucerova, Z., Knabel, S. J., and LaBorde, L. F. (2015). Predominance and distribution of a persistent *Listeria monocytogenes* clone in a commercial fresh mushroom processing environment. *J. Food Prot.* 78, 1988–1998. doi: 10.4315/0362-028X.JFP-15-195

Nielsen, E. M., Björkman, J. T., Kiil, K., Grant, K., Dallman, T., Painset, A., et al. (2017). *Closing Gaps for Performing a Risk Assessment on Listeria monocytogenes in Ready-to-Eat (RTE) Foods: Activity 3, the Comparison of Isolates from Different Compartments Along the Food Chain, and from Humans using Whole Genome Sequencing (WGS) Analysis*. Parma: EFSA. doi: 10.2903/sp.efsa.2017.EN-1151

O'Donoghue, B., NicAogáin, K., Bennett, C., Conneely, A., Tiensuu, T., and Johansson, J. (2016). Blue-light inhibition of *Listeria monocytogenes* growth is mediated by reactive oxygen species and is influenced by σB and the blue-light sensor Lmo0799. *Appl. Environ. Microbiol.* 82, 4017–4027. doi: 10.1128/AEM.00685-16

Olier, M., Pierre, F., Lemaître, J.-P., Divies, C., Rousset, A., and Guzzo, J. (2002). Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. *Microbiology* 148, 1855–1862. doi: 10.1099/00221287-148-6-1855

Olier, M., Pierre, F., Rousseaux, S., Lemaître, J.-P., Rousset, A., Piveteau, P., et al. (2003). Expression of truncated Internalin A is involved in impaired internalization of some *Listeria monocytogenes* isolates carried asymptomatically by humans. *Infect. Immun.* 71, 1217–1224. doi: 10.1128/IAI.71.3.1217-1224.2003

Ondrusch, N., and Kreft, J. (2011). Blue and red light modulates SigB-dependent gene transcription, swimming motility and invasiveness in *Listeria monocytogenes*. *PLoS One* 6:e16151. doi: 10.1371/journal.pone.0016151

Orsi, R. H., den Bakker, H. C., and Wiedmann, M. (2011). *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol.* 301, 79–96. doi: 10.1016/j.ijmm.2010.05.002

Ortiz, S., López-Alonso, V., Rodríguez, P., and Martínez-Suárez, J. V. (2015). The connection between persistent, disinfectant-resistant *Listeria monocytogenes* strains from two geographically separate Iberian pork processing plants: evidence from comparative genome analysis. *Appl. Environ. Microbiol.* 82, 308–317. doi: 10.1128/AEM.02824-15

Palma, F., Pasquali, F., Lucchi, A., Cesare, A. D., and Manfreda, G. (2017). Whole genome sequencing for typing and characterisation of *Listeria monocytogenes* isolated in a rabbit meat processing plant. *Ital. J. Food Saf.* 6:6879. doi: 10.4081/ijfs.2017.6879

Parsons, C., Lee, S., Jayeola, V., and Kathariou, S. (2017). Novel cadmium resistance determinant in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 83:e2580-e16. doi: 10.1128/AEM.02580-16

Pöntinen, A., Markkula, A., Lindström, M., and Korkeala, H. (2015). Two-component-system histidine kinases involved in growth of *Listeria monocytogenes* EGD-e at low temperatures. *Appl. Environ. Microbiol.* 81, 3994–4004. doi: 10.1128/AEM.00626-15

Popowska, M., Krawczyk-Balska, A., Ostrowski, R., and Desvaux, M. (2017). InlL from *Listeria monocytogenes* is involved in biofilm formation and Adhesion to Mucin. *Front. Microbiol.* 8:660. doi: 10.3389/fmicb.2017.00660

Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C., MacGowan, A., McLauchlin, J., and Courvalin, P. (1992). Genetic basis of tetracycline resistance in clinical isolates of *Listeria monocytogenes*. *Antimicrob. Agents Chemother.* 36, 463–466. doi: 10.1128/AAC.36.2.463

Ratani, S. S., Siletzky, R. M., Dutta, V., Yildirim, S., Osborne, J. A., Lin, W., et al. (2012). Heavy metal and disinfectant resistance of *Listeria monocytogenes* from foods and food processing plants. *Appl. Environ. Microbiol.* 78, 6938–6945. doi: 10.1128/AEM.01553-12

Roberts, M. C., Facinelli, B., Giovanetti, E., and Varaldo, P. E. (1996). Transferable erythromycin resistance in *Listeria* spp. isolated from food. *Appl. Environ. Microbiol.* 62, 269–270.

Ryan, S., Begley, M., Gahan, C. G. M., and Hill, C. (2009). Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. *Environ. Microbiol.* 11, 432–445. doi: 10.1111/j.1462-2920.2008.01782.x

Ryan, S., Begley, M., Hill, C., and Gahan, C. G. M. (2010). A five-gene stress survival islet (SSI-1) that contributes to the growth of *Listeria monocytogenes* in suboptimal conditions. *J. Appl. Microbiol.* 109, 984–995. doi: 10.1111/j.1365-2672.2010.04726.x

Schmid, B., Klumpp, J., Raimann, E., Loessner, M. J., Stephan, R., and Tasara, T. (2009). Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. *Appl. Environ. Microbiol.* 75, 1621–1627. doi: 10.1128/AEM.02154-08

Smith, G. A., and Portnoy, D. A. (1997). How the *Listeria monocytogenes* ActA protein converts actin polymerization into a motile force. *Trends Microbiol.* 5, 272–276. doi: 10.1016/S0966-842X(97)01048-2

Srinivasan, V., Nam, H. M., Nguyen, L. T., Tamilselvam, B., Murinda, S. E., and Oliver, S. P. (2005). Prevalence of antimicrobial resistance genes in *Listeria monocytogenes* isolated from dairy farms. *Foodborne Pathog. Dis.* 2, 201–211. doi: 10.1089/fpd.2005.2.201

Stasiewicz, M. J., Oliver, H. F., Wiedmann, M., and den Bakker, H. C. (2015). Whole-genome sequencing allows for improved identification of persistent *Listeria monocytogenes* in food-associated environments. *Appl. Environ. Microbiol.* 81, 6024–6037. doi: 10.1128/AEM.01049-15

Stepanović, S., Cirković, I., Ranin, L., and Svabić-Vlahović, M. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett. Appl. Microbiol.* 38, 428–432. doi: 10.1111/j.1472-765X.2004.01513.x

Thedieck, K., Hain, T., Mohamed, W., Tindall, B. J., Nimtz, M., Chakraborty, T., et al. (2006). The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. *Mol. Microbiol.* 62, 1325–1339. doi: 10.1111/j.1365-2958.2006.05452.x

Tompkin, R. B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65, 709–725. doi: 10.4315/0362-028X-65.4.709

Travier, L., Guadagnini, S., Gouin, E., Dufour, A., Chenal-Francisque, V., Cossart, P., et al. (2013). ActA promotes *Listeria monocytogenes* aggregation, intestinal colonization and carriage. *PLoS Pathog.* 9:e1003131. doi: 10.1371/journal.ppat.1003131

Van Stelten, A., Simpson, J. M., Ward, T. J., and Nightingale, K. K. (2010). Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in inlA Are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. *Appl. Environ. Microbiol.* 76, 2783–2790. doi: 10.1128/AEM.02651-09

Véghová, A., Minarovièová, J., Koreňová, J., Drahovská, H., and Kaclíková, E. (2017). Prevalence and tracing of persistent *Listeria monocytogenes* strains in meat processing facility production chain. *J. Food Saf.* 37: e12315. doi: 10.1111/jfs.12315

Voronina, O. L., Kunda, M. S., Ryzhova, N. N., Aksenova, E. I., Semenov, A. N., Kurnaeva, M. A., et al. (2015). Regularities of the ubiquitous polyhostal microorganisms selection by the example of three taxa. *Mol. Biol.* 49, 430–441. doi: 10.7868/S0026898415030179

Xu, D., Li, Y., Zahid, M. S. H., Yamasaki, S., Shi, L., Li, J., et al. (2014). Benzalkonium chloride and heavy-metal tolerance in *Listeria monocytogenes* from retail foods. *Int. J. Food Microbiol.* 190, 24–30. doi: 10.1016/j.ijfoodmicro.2014.08.017

Xu, D., Nie, Q., Wang, W., Shi, L., and Yan, H. (2016). Characterization of a transferable bcrABC and cadAC genes-harboring plasmid in *Listeria monocytogenes* strain isolated from food products of animal origin. *Int. J. Food Microbiol.* 217, 117–122. doi: 10.1016/j.ijfoodmicro.2015.10.021

Yahara, K., Méric, G., Taylor, A. J., de Vries, S. P. W., Murray, S., Pascoe, B., et al. (2017). Genome-wide association of functional traits linked with *Campylobacter jejuni* survival from farm to fork. *Environ. Microbiol.* 19, 361–380. doi: 10.1111/1462-2920.13628

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Pasquali, Palma, Guillier, Lucchi, De Cesare and Manfreda. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

REVIEW
published: 03 April 2018
doi: 10.3389/fmicb.2018.00605

Novel Biocontrol Methods for *Listeria monocytogenes* Biofilms in Food Production Facilities

*Jessica A. Gray[1,2], P. Scott Chandry[1], Mandeep Kaur[2], Chawalit Kocharunchitt[2], John P. Bowman[2] and Edward M. Fox[1]**

[1] CSIRO Agriculture and Food, Werribee, VIC, Australia, [2] Centre for Food Safety and Innovation, Tasmanian Institute of Agriculture, University of Tasmania, Hobart, TAS, Australia

High mortality and hospitalization rates have seen *Listeria monocytogenes* as a foodborne pathogen of public health importance for many years and of particular concern for high-risk population groups. Food manufactures face an ongoing challenge in preventing the entry of *L. monocytogenes* into food production environments (FPEs) due to its ubiquitous nature. In addition to this, the capacity of *L. monocytogenes* strains to colonize FPEs can lead to repeated identification of *L. monocytogenes* in FPE surveillance. The contamination of food products requiring product recall presents large economic burden to industry and is further exacerbated by damage to the brand. Poor equipment design, facility layout, and worn or damaged equipment can result in *Listeria* hotspots and biofilms where traditional cleaning and disinfecting procedures may be inadequate. Novel biocontrol methods may offer FPEs effective means to help improve control of *L. monocytogenes* and decrease cross contamination of food. Bacteriophages have been used as a medical treatment for many years for their ability to infect and lyse specific bacteria. Endolysins, the hydrolytic enzymes of bacteriophages responsible for breaking the cell wall of Gram-positive bacteria, are being explored as a biocontrol method for food preservation and in nanotechnology and medical applications. Antibacterial proteins known as bacteriocins have been used as alternatives to antibiotics for biopreservation and food product shelf life extension. Essential oils are natural antimicrobials formed by plants and have been used as food additives and preservatives for many years and more recently as a method to prevent food spoilage by microorganisms. Competitive exclusion occurs naturally among bacteria in the environment. However, intentionally selecting and applying bacteria to effect competitive exclusion of food borne pathogens has potential as a biocontrol application. This review discusses these novel biocontrol methods and their use in food safety and prevention of spoilage, and examines their potential to control *L. monocytogenes* within biofilms in food production facilities.

Keywords: *Listeria monocytogenes*, biofilms, biocontrol, bacteriophages, bacteriocins, endolysins, competitive exclusion, essential oils

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università di Teramo, Italy

Reviewed by:
Laurent Guillier,
Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), France
Giorgia Perpetuini,
Università di Teramo, Italy
Arun K. Bhunia,
Purdue University, United States

***Correspondence:**
Edward M. Fox
edward.fox@csiro.au

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *22 December 2017*
Accepted: *15 March 2018*
Published: *03 April 2018*

Citation:
Gray JA, Chandry PS, Kaur M, Kocharunchitt C, Bowman JP and Fox EM (2018) Novel Biocontrol Methods for Listeria monocytogenes Biofilms in Food Production Facilities. Front. Microbiol. 9:605. doi: 10.3389/fmicb.2018.00605

INTRODUCTION

Listeria monocytogenes is a Gram-positive, rod shaped, facultative anaerobe capable of causing food borne illnesses particularly in high-risk population groups including the elderly, immune compromised, pregnant women, and neonates (Farber and Peterkin, 1991). While *L. monocytogenes* associated illness is not as common as that of other food borne pathogens like *Salmonella, Campylobacter*, or *Escherichia coli*, its mortality rate can be considered the highest. Approximately 30 % of invasive listeriosis cases lead to mortalities with most requiring hospitalization, and therefore demanding *L. monocytogenes* can be considered as a food borne pathogen of public health importance (Lomonaco et al., 2015; Véghová et al., 2016). Due to its ubiquitous nature, *L. monocytogenes* poses a food safety risk as it is frequently introduced into the processing environment through raw ingredients. *L. monocytogenes* can adhere to a variety of abiotic surfaces with some strains persisting for numerous years and acting as a source of continuous cross contamination (Fox E. et al., 2011; Coughlan et al., 2016; Colagiorgi et al., 2017).

Due to significant food safety risks, the control of *L. monocytogenes* has become a regulatory requirement that food business operators must adhere to. Regular cleaning, disinfecting, and sanitizing of food contact and non-food contact surfaces are required as part of a sanitation plan that also incorporates maintenance of equipment and buildings, pest control, and general hygiene. In addition, the implementation of good manufacturing practices and effective hazard analysis critical control point plan aids in reducing the risk of food borne illness (Drew and Clydesdale, 2015). However, *L. monocytogenes* is a difficult organism to eradicate and its presence still occurs even with the best management plans (Tompkin, 2002; Drew and Clydesdale, 2015).

While the exact mechanisms can be unclear for how *L. monocytogenes* is able to persist in food production environments (FPEs) so successfully, researchers have proposed that there are numerous factors at play. Poorly maintained equipment, surfaces, and unhygienic factory design can result in niches containing adequate nutrients, water, and protection from cleaning allowing bacteria to survive and grow while also introducing bacteria to subinhibitory levels of disinfectants (Carpentier and Cerf, 2011; Fox E.M. et al., 2011; Ibba et al., 2013; Møretrø et al., 2017). Typically disinfectants, when applied correctly, can sufficiently inhibit the colonization of introduced planktonic cells; however, dosing failures and applying disinfectants to wet surfaces can result in equipment being inadequately disinfected and bacteria being exposed to subinhibitory chemical levels (Martinez-Suarez et al., 2016; Møretrø et al., 2017). Incorporating desiccation processes has been shown to increase the effectiveness of disinfections procedures (Overney et al., 2017); however, an ample amount of drying time is difficult when continuous or even daily production runs are required. It is also important to note the difference between resistance, an increase in concentration or time required to exert the same reduction, and tolerance, an adaptation in a microbe's susceptibility potentially the result of exposure to subinhibitory levels (Cerf et al., 2010; Ortega Morente et al., 2013). For example, some *L. monocytogenes* strains are known to carry genes for disinfectant chemical efflux pumps, such as *qacH* and *bcrABC*. The distribution of these genes tends to vary on a strain by strain basis instead of being unique to a specific lineage or subtype (Dutta et al., 2013; Ortiz et al., 2015; Møretrø et al., 2017). Although it has been reported that these genes only result in tolerance to quaternary ammonium compounds at levels far below the concentrations actually used in the food industry (Tezel and Pavlostathis, 2015), the ability to form biofilms is also a crucial factor in the survival of *L. monocytogenes*. Biofilms are composed of numerous cells attached to each other or an abiotic surface surrounded by an extracellular matrix containing a mixture of polysaccharides, proteins, and extracellular DNA (da Silva Fernandes et al., 2015; Fagerlund et al., 2017). This extracellular matrix provides a protective barrier around the internalized microbial cells from desiccation and heat, contributes to increased adhesion, and is a reservoir of nutrients (Colagiorgi et al., 2016). In addition, biofilms can impede the activity of antimicrobial agents as the matrix limits their diffusion potential and contains cells with differing susceptibility while also allowing for the acquisition of new genetic traits like those mentioned above through horizontal gene transfer. Further, biofilms typically consist of multiple species that can allow for the colonization of transient strains or provide increased attachment and survival to strains not typically good biofilm formers (Coughlan et al., 2016).

THE BIOCONTROL METHODS MOVEMENT

While tolerance to disinfectants and sanitizers is not considered as significant an issue as antibiotic resistance, their continued use and potential ineffectiveness against biofilms warrant new strategies for the control of *L. monocytogenes*. As consumers become more conscious of food safety significance, the use of novel biocontrol methods is gaining further interest. This return to biocontrol methods of microbes and plants has the potential to relieve some of the tolerance to disinfectants and decrease some of the selective pressures that their overuse has on maintaining resistance markers (Coughlan et al., 2016). Biocontrol methods with potential to act against listerial biofilms include bacteriophages, their endolysins, competitive bacterial species and their antimicrobial products, bacteriocins, and plant-derived products and will be discussed in this review.

BACTERIOPHAGES

The most abundant microorganism on earth, bacteriophages (phages) are viruses that infect bacteria for propagation, live naturally in the environment, and anywhere host bacteria are found (Bai et al., 2016; Pérez Pulido et al., 2016). Phages are classified based upon their morphology (head and

tail, either contractile or non-contractile, or no tail), nucleic acid (double stranded or single stranded; deoxyribonucleic or ribonucleic acid), and life cycle, which is of most relevance for biocontrol. There are two types of life cycles phages can undergo after entering the bacterial cell: the lysogenic cycle (temperate phages) or the lytic cycle (**Figure 1**). Phages may be capable of a lysogenic cycle that converts to the lytic cycle in unfavorable conditions, or undergo a solely lytic life cycle. Temperate phages are not suitable as a biocontrol agent as integration into the host genome may result in increased pathogenicity through horizontal gene transfer (Hagens and Loessner, 2007; Salmond and Fineran, 2015). In comparison, lytic phages are ideal as a biocontrol agent due to their fast-lytic action.

Although identified over a hundred years ago, interest in phages has only recently been reignited with the rise of antibiotic resistance among bacteria (Hagens and Loessner, 2007). The utility of phages has included the treatment of diseases in humans and animals, typing of bacterial strains, decontaminating meat carcasses after slaughter, and targeted inactivation of pathogenic and spoilage bacteria on food contact and non-contact surfaces as well as surfaces of ready to eat products and during packaging and storage (Hagens and Loessner, 2007; Strauch et al., 2007). The application of phages as an innovative approach to control biofilms in the FPE is also beginning to be explored. While there has been great achievement in the use of phages from a therapeutic perspective, their success in the FPE is not as simple. Factors like the composition and structure of the biofilm, temperature, the metabolic state of the bacteria in the biofilm, the extracellular matrix in general, food components, and nutrients all provide additional challenges to the effectiveness of phage application (Parasion et al., 2014). While there have been some reports of phage resistance (Fister et al., 2016), it occurs more gradually than the development of antibiotic resistance as phages are able to mutate continuously, like bacteria, and resistance is further slowed by using a combination of phages active against the one bacterial species (Hagens and Loessner, 2007; Sadekuzzaman et al., 2017). There is a substantial amount of research conducted on phages' ability to protect food from *Listeria*, with two commercial *Listeria* phage products, ListShield™ and Listex™ P100 approved as food preservatives with the generally recognized as safe status since 2006. However, studies investigating the efficacy of these products and other *Listeria* phages against biofilms are few, with most having focused on Listex™ P100.

Biofilm maturity has the potential to reduce the efficacy of phage treatment, as well as any control method. Various

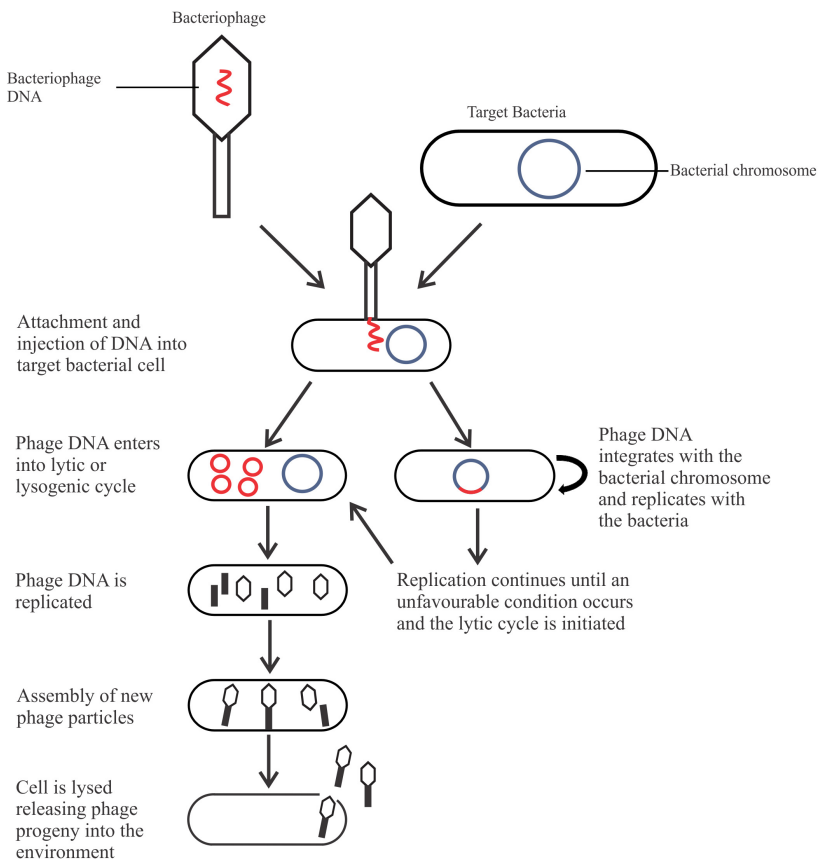


FIGURE 1 | The life cycles of a bacteriophage.

studies have examined this concept utilizing preformed biofilms at various maturity levels, ranging from 24 h to 2 weeks, with most studies reporting a minimum 1-log reduction. Most studies to date have utilized stainless steel as the surface to form *L. monocytogenes* biofilms and examine the efficacy of bacteriophage treatments. This reflects the widespread presence of these surfaces, both food contact and non-contact in food processing environments. The success of bacteriophage treatments at inactivating *L. monocytogenes* biofilms on these surfaces, however, has shown mixed results. A number of studies demonstrated promising results when Listex^{TM} P100 was applied to *L. monocytogenes* biofilms on stainless steel, with reductions in the order of 5-log being achieved (Soni and Nannapaneni, 2010; Montañez-Izquierdo et al., 2012). Both of these studies used an application treatment of 24 h at ambient room temperature. Iacumin et al. (2016) also applied Listex^{TM} P100 for 24 h at 20°C onto stainless steel wafers and report the complete elimination of *L. monocytogenes* biofilm. This prolonged treatment application, however, in many cases is not practical in an FPE. In addition, Iacumin et al. (2016) pressed the stainless steel wafer onto an agar plate to replicate the process of cross-contamination in the FPE; however, it did not take into consideration the phage products ability or inability to act on biofilms in the crevices or corners where these might be thicker than a flat surface.

A shorter treatment time of 2 h was applied by Sadekuzzaman et al. (2017) when running a similar inactivation test with ListShield^{TM}; however, this was associated with a much lower inactivation of just 2-log when applied to *L. monocytogenes* biofilm on stainless steel. This was even less effective on a rubber surface, achieving a 1-log reduction in *L. monocytogenes* cell numbers. The results of Sadekuzzaman et al. (2017) also reflect those observed by Gutiérrez et al. (2017) who saw a similarly low inactivation achieved by a 4 h ListShield^{TM} treatment, typically 1-log or less. Although the latter study did show greater inactivation with Listex^{TM} P100 under the same treatment conditions, the Listex^{TM} P100 commercial phage preparation showed a reduced activity range, only capable of infecting 7 of the 11 strains tested. An important aspect in phage application is the ratio of phage to bacteria known as the multiplicity of infectivity. To increase the likelihood the phage will infect the bacterium, the phage needs to be at a higher ratio than the number of target bacterial cells (Montañez-Izquierdo et al., 2012). High multiplicity of infectivity has been reported to result in efficient phage treatment with one study recommending a multiplicity of infectivity around five was required for adequate reductions by Listex^{TM} P100 (Montañez-Izquierdo et al., 2012).

Apart from temperature, multiplicity of infectivity, and treatment time, other factors may influence efficacy of biocontrol treatments, notably the presence of organic matter such as the food matrix. A further parameter which must be considered when examining efficacy of treatment on surfaces is the surface architecture itself, which may range from a smooth rendered surface to a scored surface with associated crevices which may be colonized by bacteria and their biofilms. Chaitiemwong et al. (2014) considered both surface crevices and food matrices (diluted food residues of ham, salmon, endive, or milk) when measuring the efficacy of Listex^{TM} P100 treatment. Results suggested deeper crevice features on the surface decreased the treatment efficacy, with inactivation in the magnitude of > 3-log achieved on 0.2 mm crevices compared to the max 1.4-log CFU/mL observed in crevice depth of 5 mm. Of particular note was the difference seen when comparing the food matrix, with lower inactivation observed for milk and vegetable when compared with meat or fish. Ganegama Arachchi et al. (2013) mimicked conditions in fish processing and demonstrated the presence of fish protein led to a lower associated biofilm density compared to control stainless steel experiments when a fish protein matrix was added to the cultivation of *L. monocytogenes* biofilm on stainless steel. This highlights the complex role the food matrix may play in both biofilm formation and subsequent efficacy of bacteriophage treatment, demonstrating the need for further studies to understand the significance of food matrix on bacteriophage treatment efficacy.

Taken together, current literature detailing phage biocontrol studies directed at *L. monocytogenes,* such as those detailed above, shows differing success in their ability to decrease established biofilms. The often low reductions achieved demonstrate the challenges biofilms pose for not only bacteriophages but all control methods, but this is not to say that there is no place for phages as a potential biocontrol method. As with many disinfection regimes, additional interventions such as steps to loosen biofilm or remove organic matter can increase the success of phage treatments (Ganegama Arachchi et al., 2013). Further research considering multi-species biofilms and in-facility application will help determine the true potential of this biocontrol approach.

ENDOLYSINS

Endolysins (lysins) are hydrolytic enzymes required for bacteriophage dissemination from the host bacterial cell. They occur at the end of the lytic cycle to release the phage virions by breaking down peptidoglycan in the bacterial cell wall in what is termed lysis from within (Chan and Abedon, 2015; Schmelcher and Loessner, 2016). Researchers have harnessed lysins through protein expression production systems, generally in *E. coli*. Following purification of the lysin, it can be applied externally to the cell wall, thus not requiring phage infection, for biopreservation and biocontrol application (García et al., 2010). Lysins are grouped based upon the cell wall component they attack with the five main classes being N-acetylglucosaminidases, endo-β-N-acetlyglucosaminidases, lytic transglycosylases, endopeptidases, and N-acetylmuramoyl-L-alanine amidases (García et al., 2010; Schmelcher and Loessner, 2016). Lysins are highly specific with a narrow spectrum of activity making them host specific with some lysins only being active on the bacterial strain the phage was isolated from (Oliveira et al., 2012). In addition, they are fast acting and no development of resistance has been reported to date (Schmelcher and Loessner, 2016). Most

research has occurred on Gram-positive bacteria using the lysis from without approach as the peptidoglycan layer is exposed. Although limited, antimicrobial activity of lysins on Gram-negative bacteria has been reported when used in conjunction with EDTA, a membrane permeablizer (Chan and Abedon, 2015).

The antimicrobial activity of lysins has mostly focused on infection control of staphylococcal bacteria. Other applications that have been considered include use in agriculture to prevent plant disease by either intense application of cell lysates expressing a chosen lysin or development of transgenic plants by incorporation of the lysin gene into the plant genome (Düring et al., 1993; Kim et al., 2004); as a rapid detection and imaging method of pathogenic bacteria (Schmelcher et al., 2010; Bai et al., 2016); and transformation of listerial bacteriophage endolysin encoding genes into dairy starter cultures as a biopreservation method (Gaeng et al., 2000). Antilisterial lysins isolated to date have predominately focused on the control of planktonic cells *in vitro* with promising results although further validation is required (**Table 1**). Only a few antilisterial lysins have been assessed in food products and the food matrix and environment have been found to affect the antimicrobial activity (Oliveira et al., 2012).

To date there is only one lysin, PlyLM, which has been tested against *L. monocytogenes* biofilms after 100 % susceptibility on planktonic *L. monocytogenes* and *Listeria innocua* cells was achieved (Simmons et al., 2012). PlyLM reduced the monolayer biofilm to the same level as the application of lysozyme and proteinase K. When used in combination with proteinase K, or both proteinase K and lysozyme, synergistic effects were observed, and the biofilm was effectively digested. However, biofilms were only grown for 24 h at 37°C, and therefore the efficacy of these enzymes under other conditions merits further investigation, for example, performance at lower temperatures which are more reflective of those of most FPEs. More research has been undertaken on staphylococcal biofilms, predominantly monospecies biofilms, which have achieved reductions in biofilm mass. Of interest is their efficacy against persister cells. Persister cells are metabolically inactive subpopulations of cells, which are "super-resistant" to antimicrobial agents such as antibiotics (Brooun et al., 2000; Wood, 2017). Studies have shown these persister cells occur as a subpopulation of bacterial biofilms, and as such can present a significant obstacle to biofilm inactivation by antimicrobials (Brooun et al., 2000; Singh et al., 2009). Several studies have shown a promising role for lysins to inactivate persister cells in biofilms (Gutiérrez et al., 2014; Schuch et al., 2017). The success being reported against staphylococcal biofilms suggests that the potential lysins may have against biofilms in a food production context, particularly in targeting *Listeria* biofilms, which are a significant problem in FPEs. Another phage enzyme, extracellular polysaccharide depolymerase, has also be shown to degrade biofilm EPS; however, they are highly specific to the strains the phage infects (Chan and Abedon, 2015). A similar approach targeting *L. monocytogenes* in biofilms could also present an alternative control measure.

COMPETITIVE BACTERIAL SPECIES

Competitive exclusion is where one bacterial species competes with another species over resources and/or space in a habitat, successfully reducing the number of cells or excluding that species (Hibbing et al., 2010). This competitive exclusion can be the result of the production of antimicrobials such as bacteriocins, organic acids either acting directly against the species it is competing with or acting on the environment altering the pH, or alternatively physically outcompeting other bacterial species for nutrients and/or space and limiting normal survival or proliferation of those competitive species. This strategy is typically categorized into three components: competition, where planktonic cells of both species are co-cultured for a period of time; exclusion, where the antagonistic species are grown to a biofilm cell density prior to the addition of planktonic cells of the target species; or displacement, in which the target species are grown to biofilm cell density prior to addition of planktonic antagonists (Woo and Ahn, 2013; Pérez-Ibarreche et al., 2016). As biofilms protect microorganisms from chemical cleaners and disinfectants, the use of non-pathogenic microorganisms may assist sanitation approaches in controlling, preventing, or eradicating unwanted species like food borne pathogens.

Competitive exclusion studies typically pit planktonic cells of the antagonist species (i.e., the species which will exert a competitive exclusion effect) against planktonic cells of the target species in a competition assay, grown together for a period of time facilitating biofilm formation. Daneshvar Alavi and Truelstrup Hansen (2013) used a short incubation time of 72 h which resulted in a 1-log decrease in *L. monocytogenes* cell density after application of *Serratia proteamaculans*. A similar reduction was also reported by Fox et al. (2014) of *L. monocytogenes* biofilm cell density after 96 h when grown in co-culture with *Janthinobacterium lividum*. However, greater reductions have been reported when cells were incubated for longer periods with results around log 4.5 and 5.5 on stainless steel coupons and polytetrafluoroethylene, respectively (Pérez-Ibarreche et al., 2016). Zhao et al. (2004) also reported higher magnitude reductions of 7.8-log reduction over 28 days at 15°C by two bacterial isolates, *Lactococcus lactis* (*Lc. lactis*) and *Enterococcus durans*. In another experiment performed at 8°C for 28 days, four isolates, including the previous two isolates were also capable of reductions around 7-log units. However, the higher reductions reported by Zhao et al. (2004) and Pérez-Ibarreche et al. (2016) were produced by lactic acid bacteria (LAB) whose inhibitory activity has been studied extensively for many years, particularly as probiotics (Jeong and Frank, 1994).

The inhibitory effect of LAB was further explored by Guerrieri et al. (2009) and Gómez et al. (2016) as a preformed biofilm preventing *L. monocytogenes* biofilm formation as part of the exclusion strategy. Gómez et al. (2016) tested a variety of LAB strains and found reductions ranged from 4- to 7-log units over 24 and 48 h; however, by 72 h, *L. monocytogenes* growth had increased by almost half fold of the control indicating that these strains were only capable of exclusion

TABLE 1 | Antilisterial lysins reported in literature, key summary, and application.

Endolysin	Reported findings	Use	Reference
Ply118	Rapidly lysed all Listeria strains tested and against three Bacillus species.	BC, IC	Loessner et al., 1995
Ply500		BC, IC	
Ply511	Rapidly lysed all Listeria strains tested against.	BC, IC	
PlyP35	Determined optimal temperature, NaCl, pH, and various ions conditions.	BC, IC	Schmelcher et al., 2012
PlyP40	Lysed *L. monocytogenes* strain and *L. innocua*.	BC, IC	Loessner and Schmelcher, 2010
PlyP825	Inhibited all growth in *L. monocytogenes* strains used.	BC, IC	Grallert et al., 2012
PlyPSA	Determined crystalized structure	RMD	Korndörfer et al., 2006
PlyP100	Lysed all *L. monocytogenes*, Listeria strains in cheese, and a *Bacillus subtilus* strain tested against	BC, BP	Van Tassell et al., 2017
LysZ5	Lysed *L. monocytogenes*, *L. innocua*, and *Listeria welshimeri*; reduced *L. monocytogenes* numbers in soya milk.	BC, BP	Zhang et al., 2012
PlyLM	Lysed all *L. monocytogenes* and *L. innocua* strains tested against; digested *L. monocytogenes* biofilms when combine with a protease.	BC	Simmons et al., 2012

BC, biocontrol; IC, infection control; RMD, rapid multiplex detection; BP, biopreservation.

within the first 24–48 h. However, *Lc. lactis* 368 strain was able to completely exclude the growth of *L. monocytogenes* for the entire period, although it should be noted that all experiments were performed at a relatively elevated temperature and as such lower temperatures reflective of many FPEs require further consideration. In comparison, Guerrieri et al. (2009) showed the potential of LAB bacteria at refrigeration temperatures with a *Lactobacillus plantarum* (*Lb. plantarum)* strain capable of a 4-log reduction over a 10-day period. Mariani et al. (2011) used the native biofilm microflora of wooden cheese ripening shelves to achieve a 1- to 2-log reduction over a 12-day period, although this reduction was less than that observed in Guerrieri et al. (2009) and Gómez et al. (2016).

The third strategy displacement, as reviewed by Woo and Ahn (2013), demonstrated that the use of planktonic antagonist LAB strains as a post-treatment control method targeting *L. monocytogenes* was less effective compared to pre-treatment, although two strains (*Lactobacillus paracasei* and *Lactobacillus rhamnosus*) were capable of a 3-log reduction in *L. monocytogenes* biofilm cell density over 24 h when incubated at 37°C.

While most studies are performed in laboratories, Zhao et al. (2006, 2013) took the concept of competitive exclusion a step further and looked at its applicability in poultry processing facilities. In a fresh poultry facility, two LAB strains (*Lc. lactis* and *E. durans*) were added to two enzyme-based cleaners and applied as a foam to selected drains four times in the first week and then two times for the following 3 weeks. Sampling continued for 18 weeks after the last treatment. Most drains experienced significant reductions within the first week after only four applications and all drains maintained lower levels of *Listeria* throughout the sampling period (Zhao et al., 2006). Importantly, two drains reported significant reductions 16 weeks after treatments finished. Similar parameters were applied to the application of the same strains at a ready to eat poultry processing facility. By the end of the first week of application, *Listeria* was not detected in five of the six drains with all drains reporting negative results between weeks 8 and 13 (Zhao et al., 2013). It should also be noted that the strains utilized were known to either possess nisin or other forms of antimicrobials; however, it was not elucidated if the inhibition was the result of the production of antimicrobials.

There have been some encouraging results in the use of LAB against *L. monocytogenes* biofilm cells in laboratory-based experiments (**Table 2**); however, very few have been trialed in actual FPEs, apart from Zhao et al. (2006, 2013). The results from their two studies have shown promising results as an alternative control method utilizing *E. durans* and *Lc. lactis*; however, further longitudinal research surrounding the in-facility application is required. In addition, the application of other bacterial species identified in some of the studies mentioned above, for example, *J. lividum* and *S. proteamaculans*, warrants in-facility testing. However, it should be noted that the LAB strains utilized for in-facility application studies were isolated from the production environment indicating that specific strains may work best in the environment they were isolated from and these strains may vary depending on the food industry.

Houry et al. (2012) reported the use of bacterial species in a novel biocontrol approach. In the study, they identified a subpopulation of bacilli known as bacterial swimmers which were capable of creating transient pores within the biofilm structure. By pre-treating *Staphylococcus aureus* biofilms with bacterial swimmers, which also produced an anti-stapylococcal bactericide, they achieved a greater inactivation of *S. aureus* in biofilm by facilitating access of toxic substances in the environment into the biofilm.

BACTERIOCINS

An important component of the competitive survival strategy of bacteria is the production of antimicrobial products. One group of ribosomally synthesized antimicrobials are the heat stable peptides known as bacteriocins (Cotter et al., 2005; Gálvez et al., 2008; Winkelströter et al., 2015). It has been suggested that most bacteria produce at least one bacteriocin and LAB are known to be prolific producers (Cotter et al., 2005). Most

TABLE 2 | Bacterial species active against *L. monocytogenes* and purported mode of action.

Bacterial species	Mode of action	Studies
S. proteamaculans	Sánchez et al. (2010) identified a bacteriocin-like substance was produced at low temperatures capable of inhibiting *L. monocytogenes*. Inhibition was suggested to be the result of Jameson effect.	Sánchez et al., 2010; Daneshvar Alavi and Truelstrup Hansen, 2013
J. lividum	Specific strain utilized not tested for antimicrobial compounds. *J. lividium* are reported to have antibacterial compounds capable of inhibiting Gram-positive bacteria (O'Sullivan et al., 1990).	Fox et al., 2014
Lc. lactis	Neither of the studies by Zhao et al. tested for production of a bacteriocin; however, this species has previously be reported to produce nisin.	Zhao et al., 2004, 2006, 2013
E. durans	Neither of the studies tested for the bacteriocin; however, this species has previously be reported to produce enterocin.	Zhao et al., 2004, 2006, 2013
Lb. plantarum 396/1	Inhibition was attributed to production of an organic acid.	Guerrieri et al., 2009
Lb. paracasei	May be the result of competition for sites and resources. As a probiotic strain it may produce bacteriocin, organic acid or hydrogen peroxide.	Woo and Ahn, 2013
Lb. rhamnosus	May be the result of competition for sites and resources. As a probiotic strain, it may produce bacteriocin, organic acid or hydrogen peroxide. A previous study isolated an antilisterial bacteriocin from this species (Jeong and Moon, 2015).	Woo and Ahn, 2013
Lb. sakei	Bacteriocin producing strain.	Pérez-Ibarreche et al., 2016
LAB – Lc. lactis 368, *Lb. helveticus 354*, *Lb. casei 40*, and *W. viridescens 113*	Not identified as bacteriocin-producing strains. May be result of biosurfactants, or exclusion by trapping (killing cells embedded in biofilm).	Gómez et al., 2016
Native microbial flora of cheese ripening wooden shelves	Established biofilms on active cheese ripening wooden shelves were used. Inhibition may have been the result of competition for sites and nutrients.	Mariani et al., 2011

bacteriocins have a narrow spectrum of activity, that is, they are active against the same species that produces them but the producer is immune to them, while some have a broad spectrum of activity acting on members within the same genus as well as other genera and species (Cotter et al., 2005). The mode of activity varies depending on the particular class of bacteriocin and can include pore formation, or inhibition of key cellular processes such as peptidoglycan production, DNA replication, mRNA, or protein synthesis, to name a few (Cotter et al., 2005). There are two main groups: Class I (also known as lantibiotics), peptides that undergo post-translational changes, and Class II, which do not (Cotter et al., 2013). Among the most well-characterized and successful bacteriocins to date is nisin, a Class I bacteriocin from *Lc. lactis* which has been approved for use in food as a preservative/additive by the World Health Organization, European Union, and the United States Food and Drug Authority (Cotter et al., 2005). A great deal of research has gone into identifying more bacteriocins active against *L. monocytogenes* planktonic cells and biofilms, an important arena as nisin resistance is slowly being reported.

Most studies can be classified into two groups based upon how the bacteriocin is applied: either as whole bacterial cells known or suspected of bacteriocin production, or alternatively the bacteriocin extract itself, applied either as a crude or semi-purified product. Their utility against preformed *L. monocytogenes* biofilms of varying times has been the subject of numerous studies, with some reporting promising results. For example, Gómez et al. (2016) assessed *Lc. lactis*, *Lactobacillus sakei*, and *Lactobacillus curvatus*, all known to produce nisin Z, sakacine A, and sakacine P, respectively, against 48 h preformed biofilms. *Lb. sakei* and *Lb. curvatus* were capable of complete inactivation over 72 h whereas the two *Lc. lactis* strains provided a 6-log reduction by the end of the test period. Winkelströter et al. (2015), however, were unable to produce results of a similar magnitude when *L. monocytogenes* was co-cultured with *Lb. paraplantarum,* only achieving 2-log inactivation at 24 and 48 h before decreasing by 72 h. Guerrieri et al. (2009) took an alternative approach and reported that *Lb. plantarum* and *Enterococcus casseliflavus* were able to inactivate *L. monocytogenes* 7-day preformed biofilms by 3.9- and 3.7-logs over a 10 day-period. Importantly, the results could be associated with bacteriocin production, as no changes to the pH were observed.

Another technique is extracting the bacteriocin in the form of cell-free supernatant (CFS), as a crude bacteriocin fermentate or semi-purifying the product. The antimicrobial activity of CFS has shown mixed success in co-inoculation studies to prevent the formation of biofilms by *L. monocytogenes*, with Camargo et al. (2016) reporting significant reductions after 24 h, whereas Bolocan et al. (2017) only observed between 1.6- and 3.6-log CFU/cm^2 reduction after 72 h depending on the media used. In the latter study, however, the CFS extract which produced the highest reduction was from an isolate known to also produce an organic acid which was not

removed, and therefore this result may not be associated solely to the antimicrobial activity of the bacteriocin. When Camargo et al. (2016) applied the CFS to 24 h preformed biofilms for 2.5 h, they found biofilm formation continued in some isolates.

Other researchers have compared the two methods, bacterial cells and extracts again with varying results. García-Almendárez et al.'s (2008) analysis on 4-day preformed biofilms demonstrated a crude bacteriocin fermentate from *Lc. lactis* known to produce nisin A was capable of a 2.7-log reduction over 24 h. However, a greater reduction over 5-logs was achieved when the *Lc. lactis* was applied for 6 h, then rinsed, and placed in a desiccator for five days. Whereas, Winkelströter et al. (2011) co-inoculated *L. monocytogenes* with *Lb. sakei* or its CFS and found that any decreases observed in the first 24 h were diminished with time, as results at 48 h were comparable to the pure culture levels. A promising approach by Pérez-Ibarreche et al. (2016) involved the supplementation of *Lb. sakei* cells with a semi-purified bacteriocin for 6 h, which resulted in a twofold reduction in *L. monocytogenes* numbers on the stainless steel surface, or an additional 1-log reduction on polytetrafluoroethylene.

As mentioned previously, the bacteriocin nisin has been approved for commercial purposes and has paved the way as an alternative biocontrol method. Research into bacteriocins has been performed with comparable results to the other non-commercial bacteriocins discussed above. Minei et al. (2008) found that nisin was capable of inhibiting *L. monocytogenes* biofilm formation for 9 h on stainless steel coupons, and although cell growth did recommence after this time, a 3.5-log inactivation was still maintained by 48 h. On the other hand, Henriques and Fraqueza (2017) shortened the treatment time to 5 min and even at the highest concentration, no activity was recorded, although activity was defined as a $\geq$ 5-log decrease.

From the above, it is obvious that results vary significantly depending on if bacteriocin producing bacterial cells or the bacteriocin extracts is used. Results from bacteriocin extracts can be correlated to the antimicrobial action of the bacteriocin with greater certainty; however, additional analysis is required particularly when whole cells are used to help ensure that the measured inhibition is not the result of competitive exclusion or the production of other antimicrobials such as organic acids. The co-inoculation and preformed biofilm studies reflect the ability of the bacteriocin to either prevent the formation or affect the removal of established biofilms in the FPE; however, the length of time the biofilms are grown for prior to the bacteriocin being applied also affects the antimicrobial activity as mature biofilms may provide better resistance. Although several studies show that promising results most require additional analysis at temperatures and other environmental conditions mirroring the FPE to identify potential candidates suitable for further testing. With the potential resistance to nisin arising, the identification of other bacteriocins is essential. In addition, the application of synergistic antimicrobials to further combat the development of resistance should be considered.

PLANT-DERIVED ANTIMICROBIAL PRODUCTS – ESSENTIAL OILS

An alternative to the use of chemicals, microorganisms, or their derivatives is the use of plant-derived antimicrobial products such as essential oils (EOs). Herbs and spices are commonly known to exhibit antimicrobial activity and have been used by various cultures for flavoring, as a food preservative or for medicinal purposes. EOs play a key role in protecting plants from bacteria, fungi, viruses, insects, and animals (Perricone et al., 2015). Traditional distillation, cold press/expressing, solvent extractions, and enfleurage methods have been used to extract EOs from plant-derived materials; more recently, modern techniques including microwave or ultra sound assisted extraction, pressurized extractions, and super critical fluid extraction have been used to obtain EOs from a variety of plant sources (including roots, wood, bark, twigs, leaves, seeds, buds, flowers, and fruits). However, the constituents and compositions of EOs vary significantly from high concentrations to trace amounts based upon the plant part, plant age, and extraction method used, in turn influencing their antimicrobial activity (Lemberkovics et al., 2004; Reyes-Jurado et al., 2014; Perricone et al., 2015; Xia et al., 2017). Key molecules in EOs with the most effective antibacterial activity are typically from aldehyde and phenol chemical classes which include compounds such as cinnamaldehyde, carvacrol, eugenol, or thymol (Bakkali et al., 2008; Perricone et al., 2015). EOs are able to permeabilize the cell membrane resulting in the leakage of ions or other cell content, and may also disrupt key genetic functions and/or cellular components like proteins, polysaccharides, phospholipids, fatty acids, and essential enzymes due to the lipophilic nature of EOs (Bakkali et al., 2008; de Oliveira et al., 2010, 2012a; Perricone et al., 2015).

While there are thousands of EOs described, it is reported around 300 of these have generally recognized as safe approval and are used commercially for flavoring or fragrance; however, more detailed information is required for their use as a biocontrol agent (Burt, 2004; Reyes-Jurado et al., 2014). Most research surrounding the antimicrobial activity of EOs focuses on their effects on planktonic cells of food spoilage and pathogenic bacteria either in standard laboratory conditions or in their application on food items. This application on food as a biocide has major limitations as higher concentrations are required potentially interfering with the sensory attributes of the food (Burt, 2004; Chorianopoulos et al., 2008). In addition, some components of food items, mainly fats, proteins, carbohydrates, water, salt, antioxidants, pH, and other preservatives or additives used may impact upon the activity of the EOs (Perricone et al., 2015). Further research is required to understand the impact EOs have on bacterial pathogens and in particular their ability to prevent or eradicate biofilms in FPEs. Some research is occurring within this space; however, there is limited research against *L. monocytogenes* biofilms with a few studies looking at the extracted EOs, the active components of specific EOs, or altering the EO chemical composition.

de Oliveira et al. (2010) assessed the EOs from fresh citronella (*Cymbopogon nardus*) and lemongrass (*Cymbopogon citratus*) leaves applied alone or in combination; however, it was the Citronella EO which demonstrated the highest reductions against both the 3 and 240 h preformed biofilms with complete reduction after 60 min of application. Similar results reported in another study by de Oliveira et al. (2012b) found 2% (vol/vol) Chinese cinnamon extract (*Cinnamomum cassia*) was capable of reducing a 48 h preformed biofilm to below the detection limit (2.84-log CFU/cm^2) after 20 min; however, both of these studies applied the EOs at temperatures above 20°C.

Essential oils contain a mixture of major and minor molecules responsible for their antimicrobial activity with some of the major components being explored further. The active components of clove (eugenol) and spearmint (carvone) EOs were tested on a 6 h preformed *L. monocytogenes* biofilm but were found to increase biofilm mass by Leonard et al. (2010). Citral and nerol, in contrast, both major components from lemongrass (*C. citratus*) and *Lippia rehmannii* (nerol only), displayed a similar reduction as the positive control ciprofloxacin.

Additional microbial species can also impact upon the activity of the EO or active component. For example, Leonard et al.'s (2010) study as mentioned above was on *L. monocytogenes* monospecies biofilms and reported a mixture of results among the EO and the various active components tested, whereas de Oliveira et al. (2012a) looked at the activity of Chinese cinnamon and its active component, Cinnamaldehyde, on a mixed biofilm of *L. monocytogenes* and enteropathogenic *E. coli* on stainless steel coupons dipped in reconstituted whole milk. The EO and cinnamaldehyde were both capable of reducing the mixed biofilm to below the detection limit of 2.84-log CFU/cm^2 whereas the EO and active components only provided reductions just over 2-logs on the *L. monocytogenes* biofilm. Chorianopoulos et al. (2008) examined the EO and hydrosol (by-product of the steam distillation) of *Satureja thymbra* (Pink Savory) and showed similar results when grown in a mixed biofilm with a food borne pathogen (*L. monocytogenes* and *Salmonella enterica*) and a spoilage bacterium (*Pseudomonas putida*). It was noted that the optimized application time was 60 min and any increase in time provided no additional reduction. The impact other microbial players may have on the activity of EOs requires further exploration in order to gain insights into the various relationships at play.

A common problem for the use of EOs as a biocontrol method on food products is the associated impacts on taste at concentrations required for appropriate antimicrobial effect. A process to concentrate the EOs for application at a lower volume with the same potentially high antimicrobial activity may be required in the case of some EOs. Krogsgård Nielsen et al. (2017) looked at emulsifying and encapsulating isoeugenol oil to increase the antimicrobial effectiveness at a smaller volume with the addition of electrostatic forces to attract negatively charged bacteria to positively charged EOs. Although the concept of emulsification and encapsulation sounds promising, the minimal biofilm eradication concentrations (MBECs) for the coated and uncoated emulsified products were only half a log lower than the pure isoeugenol when tested in standard laboratory medium at three temperatures (6, 12, and 25°C) and no difference was observed in carrot juice. This observation requires further exploration as the reductions in the MBEC did not correlate to observations under confocal microscopy. Of note was the morphological changes observed in the mixed biofilms of *Pseudomonas fluorescens* and *S. aureus* from uniform layers to clusters of numerous cells, which requires further research to determine if there are any implications.

As mentioned previously, the use of EOs at concentrations to exhibit sufficient antimicrobial activity has the potential to impart undesirable flavor and when applied in an FPE may also result in an excessive sensorial impact. In addition, the interactions of EOs with components of the food matrix from food debris may also impact on the applicability of EOs in food environments. Only a few studies have investigated the application of EOs to disrupt or prevent the formation of biofilms. Further research on parameters specific to industry will allow a better decision on the application of EOs as an alternative or supplementary biocontrol method.

CONCLUDING REMARKS

While current sanitation processes are effective against planktonic cells, the potential for tolerant strains to increase due to interactions at subinhibitory levels and the potential reliance on them as antimicrobials, as the case in the health industry, is a cause for concern. The ability to eradicate established biofilms and prevent new biofilms from being formed is a challenging task which food production managers are charged with, as biofilms can present increased food safety risks. A useful tool in understanding the microbial community is metagenomics analysis of the FPE. By understanding the FPE microbiome, valuable information can be gained regarding persistence or transience of strains. This facilitates source tracking of persistent strains, can identify other microbial species that may provide either a positive or negative effect on the target strain, and can identify strains surviving the disinfection processes (Dass and Anandappa, 2017; Doyle et al., 2017). From this information, the appropriate biocontrol method can then be determined. There have been some significant advances in the development of biocontrol methods, particularly bacteriophages that have progressed to commercial products with the results of some studies validating their progression to commercialization. The use of competitive bacterial species has also showed some promising results with the concept of utilizing antagonist strains isolated from the production environment providing individualized treatment options. Bacteriocins and endolysins have also shown their ability to significantly reduce established biofilms; however, they typically require some form of purification process to achieve these results. The sensory implications of EOs at concentrations required to exert antimicrobial effects are a limiting factor

in their use as a sole biocontrol method, and therefore they may find more appropriate utility as a supplementary method targeting non-food contact surfaces. However, like all biocontrol methods, efficacy can be impacted by a variety of factors including temperature or time the control method was applied for, the use of one species or multiple species biofilms, biofilm growth method, or surface matrix composition. Standardization in the assessment of novel biocontrol methods against biofilms is required, in addition to assessment under conditions reflective of FPEs before appropriate comparisons can be made.

AUTHOR CONTRIBUTIONS

JG and EF conceived the study and drafted the manuscript. All authors corrected and approved the manuscript.

FUNDING

This work was supported by University of Tasmania and CSIRO joint funding. JG was a recipient of University of Tasmania and CSIRO Ph.D. Scholarship.

REFERENCES

Bai, J., Kim, Y. T., Ryu, S., and Lee, J. H. (2016). Biocontrol and rapid detection of food-borne pathogens using bacteriophages and endolysins. *Front. Microbiol.* 7:474. doi: 10.3389/fmicb.2016.00474

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. (2008). Biological effects of essential oils - A review. *Food Chem. Toxicol.* 46, 446–475. doi: 10.1016/j.fct.2007.09.106

Bolocan, A. S., Pennone, V., O'connor, P. M., Coffey, A., Nicolau, A. I., Mcauliffe, O., et al. (2017). Inhibition of *Listeria monocytogenes* biofilms by bacteriocin-producing bacteria isolated from mushroom substrate. *J. Appl. Microbiol.* 122, 279–293. doi: 10.1111/jam.13337

Brooun, A., Liu, S., and Lewis, K. (2000). A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 44, 640–646. doi: 10.1128/AAC.44.3.640-646.2000

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - A review. *Int. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Camargo, A. C., de Paula, O. A., Todorov, S. D., and Nero, L. A. (2016). In vitro evaluation of bacteriocins activity against *Listeria monocytogenes* biofilm formation. *Appl. Biochem. Biotechnol.* 178, 1239–1251. doi: 10.1007/s12010-015-1941-3

Carpentier, B., and Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol.* 145, 1–8. doi: 10.1016/j.ijfoodmicro.2011.01.005

Cerf, O., Carpentier, B., and Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int. J. Food Microbiol.* 136, 247–254. doi: 10.1016/j.ijfoodmicro.2009.10.002

Chaitiemwong, N., Hazeleger, W. C., and Beumer, R. R. (2014). Inactivation of *Listeria monocytogenes* by disinfectants and bacteriophages in suspension and stainless steel carrier tests. *J. Food Prot.* 77, 2012–2020. doi: 10.4315/0362-028X.JFP-14-151

Chan, B. K., and Abedon, S. T. (2015). Bacteriophages and their enzymes in biofilm control. *Curr. Pharm. Des.* 21, 85–99. doi: 10.2174/1381612820666140905112311

Chorianopoulos, N. G., Giaouris, E. D., Skandamis, P. N., Haroutounian, S. A., and Nychas, G. J. E. (2008). Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid-base sanitizers. *J. Appl. Microbiol.* 104, 1586–1596. doi: 10.1111/j.1365-2672.2007.03694.x

Colagiorgi, A., Bruini, I., Di Ciccio, P. A., Zanardi, E., Ghidini, S., and Ianieri, A. (2017). *Listeria monocytogenes* biofilms in the wonderland of food industry. *Pathogens* 6:E41. doi: 10.3390/pathogens6030041

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., and Ianieri, A. (2016). A look inside the *Listeria monocytogenes* biofilms extracellular matrix. *Microorganisms* 4:22. doi: 10.3390/microorganisms4030022

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3, 777–788. doi: 10.1038/nrmicro1273

Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins - a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11, 95–105. doi: 10.1038/nrmicro2937

Coughlan, L. M., Cotter, P. D., Hill, C., and Alvarez-Ordóñez, A. (2016). New weapons to fight old enemies: novel strategies for the (bio)control of bacterial biofilms in the food industry. *Front. Microbiol.* 7:1641. doi: 10.3389/fmicb.2016.01641

da Silva Fernandes, M., Kabuki, D. Y., and Kuaye, A. Y. (2015). Behavior of *Listeria monocytogenes* in a multi-species biofilm with *Enterococcus faecalis* and *Enterococcus faecium* and control through sanitation procedures. *Int. J. Food Microbiol.* 200, 5–12. doi: 10.1016/j.ijfoodmicro.2015.01.003

Daneshvar Alavi, H. E., and Truelstrup Hansen, L. (2013). Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surfaces. *Biofouling* 29, 1253–1268. doi: 10.1080/08927014.2013.835805

Dass, S. C., and Anandappa, A. (2017). Food factory genomics: where big data drives quality and food safety. *Food Prot. Trends* 37, 368–374.

de Oliveira, M. M. M., Brugnera, D. F., Cardoso, M. D. G., Alves, E., and Piccoli, R. H. (2010). Disinfectant action of *Cymbopogon* sp. essential oils in different phases of biofilm formation by *Listeria monocytogenes* on stainless steel surface. *Food Control* 21, 549–553. doi: 10.1016/j.foodcont.2009.08.003

de Oliveira, M. M. M., Brugnera, D. F., Do Nascimento, J. A., Batista, N. N., and Piccoli, R. H. (2012a). Cinnamon essential oil and cinnamaldehyde in the control of bacterial biofilms formed on stainless steel surfaces. *Eur. Food Res. Technol.* 234, 821–832. doi: 10.1007/s00217-012-1694-y

de Oliveira, M. M. M., Brugnera, D. F., Do Nascimento, J. A., and Piccoli, R. H. (2012b). Control of planktonic and sessile bacterial cells by essential oils. *Food Bioprod. Process.* 90, 809–818. doi: 10.1016/j.fbp.2012.03.002

Doyle, C. J., O'toole, P. W., and Cotter, P. D. (2017). Metagenome-based surveillance and diagnostic approaches to studying the microbial ecology of food production and processing environments. *Environ. Microbiol.* 19, 4382–4391. doi: 10.1111/1462-2920.13859

Drew, C. A., and Clydesdale, F. M. (2015). New food safety law: effectiveness on the ground. *Crit. Rev. Food Sci. Nutr.* 55, 689–700. doi: 10.1080/10408398.2011.654368

Düring, K., Porsch, P., Fladung, M., and Lörz, H. (1993). Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. *Plant J.* 3, 587–598. doi: 10.1046/j.1365-313X.1993.03040587.x

Dutta, V., Elhanafi, D., and Kathariou, S. (2013). Conservation and distribution of the benzalkonium chloride resistance cassette bcrABC in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 79, 6067–6074. doi: 10.1128/AEM.01751-13

Fagerlund, A., Møretrø, T., Heir, E., Briandet, R., and Langsruda, S. (2017). Cleaning and disinfection of biofilms composed of *Listeria monocytogenes* and background microbiota from meat processing surfaces. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01046-17 [Epub ahead of print].

Farber, J. M., and Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55, 476–511.

Fister, S., Robben, C., Witte, A. K., Schoder, D., Wagner, M., and Rossmanith, P. (2016). Influence of environmental factors on phage–bacteria interaction and on the efficacy and infectivity of phage P100. *Front. Microbiol.* 7:1152. doi: 10.3389/fmicb.2016.01152

Fox, E., Hunt, K., O'brien, M., and Jordan, K. (2011). *Listeria monocytogenes* in Irish farmhouse cheese processing environments. *Int. J. Food Microbiol.* 145(Suppl. 1), S39–S45. doi: 10.1016/j.ijfoodmicro.2010.10.012

Fox, E. M., Leonard, N., and Jordan, K. (2011). Physiological and transcriptional characterization of persistent and nonpersistent *Listeria monocytogenes* isolates. *Appl. Environ. Microbiol.* 77, 6559–6569. doi: 10.1128/AEM.05529-11

Fox, E. M., Solomon, K., Moore, J. E., Wall, P. G., and Fanning, S. (2014). Phylogenetic profiles of in-house microflora in drains at a food production facility: comparison and biocontrol implications of Listeria-positive and -negative bacterial populations. *Appl. Environ. Microbiol.* 80, 3369–3374. doi: 10.1128/AEM.00468-14

Gaeng, S., Scherer, S., Neve, H., and Loessner, M. J. (2000). Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lyric enzymes in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 66, 2951–2958. doi: 10.1128/AEM.66.7.2951-2958.2000

Gálvez, A., López, R. L., Abriouel, H., Valdivia, E., and Omar, N. B. (2008). Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Crit. Rev. Biotechnol.* 28, 125–152. doi: 10.1080/07388550802107202

Ganegama Arachchi, G. J., Cridge, A. G., Dias-Wanigasekera, B. M., Cruz, C. D., Mcintyre, L., Liu, R., et al. (2013). Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. *J. Ind. Microbiol. Biotechnol.* 40, 1105–1116. doi: 10.1007/s10295-013-1313-3

García, P., Rodríguez, L., Rodríguez, A., and Martínez, B. (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends Food Sci. Technol.* 21, 373–382. doi: 10.1016/j.tifs.2010.04.010

García-Almendárez, B. E., Cann, I. K. O., Martin, S. E., Guerrero-Legarreta, I., and Regalado, C. (2008). Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. *Food Control* 19, 670–680. doi: 10.1016/j.foodcont.2007.07.015

Gómez, N. C., Ramiro, J. M. P., Quecan, B. X. V., and de Melo Franco, B. D. G. (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157: H7 biofilms formation. *Front. Microbiol.* 7:863. doi: 10.3389/fmicb.2016.00863

Grallert, H., Molinario, S., and Kaps, J. (2012). Novel Listeria bacteriophage p825n and uses thereof. U.S. Patent No. WO 2012159774 A1.

Guerrieri, E., De Niederhäusern, S., Messi, P., Sabia, C., Iseppi, R., Anacarso, I., et al. (2009). Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes* in a small-scale model. *Food Control* 20, 861–865. doi: 10.3389/fmicb.2016.00863

Gutiérrez, D., Rodríguez-Rubio, L., Fernández, L., Martínez, B., Rodríguez, A., and García, P. (2017). Applicability of commercial phage-based products against *Listeria monocytogenes* for improvement of food safety in Spanish dry-cured ham and food contact surfaces. *Food Control* 73, 1474–1482. doi: 10.1016/j.foodcont.2016.11.007

Gutiérrez, D., Ruas-Madiedo, P., Martínez, B., Rodríguez, A., and García, P. (2014). Effective removal of staphylococcal biofilms by the endolysin LysH5. *PLoS One* 9:e107307. doi: 10.1371/journal.pone.0107307

Hagens, S., and Loessner, M. J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Appl. Microbiol. Biotechnol.* 76, 513–519. doi: 10.1007/s00253-007-1031-8

Henriques, A. R., and Fraqueza, M. J. (2017). Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain. *LWT Food Sci. Technol.* 81, 180–187. doi: 10.1016/j.lwt.2017.03.045

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25. doi: 10.1038/nrmicro2259

Houry, A., Gohar, M., Deschamps, J., Tischenko, E., Aymerich, S., Gruss, A., et al. (2012). Bacterial swimmers that infiltrate and take over the biofilm matrix. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13088–13093. doi: 10.1073/pnas.1200791109

Iacumin, L., Manzano, M., and Comi, G. (2016). Phage inactivation of *Listeria monocytogenes* on San Daniele dry-cured ham and elimination of biofilms from equipment and working environments. *Microorganisms* 4:E4. doi: 10.3390/microorganisms4010004

Ibba, M., Cossu, F., Spanu, V., Virdis, S., Spanu, C., Scarano, C., et al. (2013). *Listeria monocytogenes* contamination in dairy plants: evaluation of *Listeria monocytogenes* environmental contamination in two cheese-making plants using sheeps milk. *Ital. J. Food Saf.* 2, 109–112. doi: 10.4081/ijfs.2013.e31

Jeong, D. K., and Frank, J. F. (1994). Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganisms isolated from meat and dairy processing environments. *J. Food Prot.* 57, 576–586. doi: 10.4315/0362-028X-57.7.576

Jeong, Y.-J., and Moon, G.-S. (2015). Antilisterial bacteriocin from *Lactobacillus rhamnosus* CJNU 0519 presenting a narrow antimicrobial spectrum. *Korean J. Food Sci. Anim. Resour.* 35, 137–142. doi: 10.5851/kosfa.2015.35.1.137

Kim, W. S., Salm, H., and Geider, K. (2004). Expression of bacteriophage phiEa1h lysozyme in *Escherichia coli* and its activity in growth inhibition of *Erwinia amylovora*. *Microbiology* 150, 2707–2714. doi: 10.1099/mic.0.27224-0

Korndörfer, I. P., Danzer, J., Schmelcher, M., Zimmer, M., Skerra, A., and Loessner, M. J. (2006). The crystal structure of the bacteriophage PSA endolysin reveals a unique fold responsible for specific recognition of *Listeria* cell walls. *J. Mol. Biol.* 364, 678–689. doi: 10.1016/j.jmb.2006.08.069

Krogsgård Nielsen, C., Kjems, J., Mygind, T., Snabe, T., Schwarz, K., Serfert, Y., et al. (2017). Antimicrobial effect of emulsion-encapsulated isoeugenol against biofilms of food pathogens and spoilage bacteria. *Int. J. Food Microbiol.* 242, 7–12. doi: 10.1016/j.ijfoodmicro.2016.11.002

Lemberkovics, É., Kéry, Á., Simándi, B., Kakasy, A., Balázs, A., Héthelyi, É., et al. (2004). Influence of extraction methods on the composition of essential oils. *Acta Pharm. Hung.* 74, 166–170.

Leonard, C. M., Virijevic, S., Regnier, T., and Combrinck, S. (2010). Bioactivity of selected essential oils and some components on *Listeria monocytogenes* biofilms. *South Afr. J. Bot.* 76, 676–680. doi: 10.1016/j.sajb.2010.07.002

Loessner, M., and Schmelcher, M. (2010). New endolysin plyp40. U.S. Patent No. US20140079727A1.

Loessner, M. J., Wendlinger, G., and Scherer, S. (1995). Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Mol. Microbiol.* 16, 1231–1241. doi: 10.1111/j.1365-2958.1995.tb02345.x

Lomonaco, S., Nucera, D., and Filipello, V. (2015). The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. *Infect. Genet. Evol.* 35, 172–183. doi: 10.1016/j.meegid.2015.08.008

Mariani, C., Oulahal, N., Chamba, J. F., Dubois-Brissonnet, F., Notz, E., and Briandet, R. (2011). Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. *Food Control* 22, 1357–1362. doi: 10.1016/j.ijfoodmicro.2008.06.028

Martinez-Suarez, J. V., Ortiz, S., and Lopez-Alonso, V. (2016). Potential impact of the resistance to quaternary ammonium disinfectants on the persistence of *Listeria monocytogenes* in food processing environments. *Front. Microbiol.* 7:638. doi: 10.3389/fmicb.2016.00638

Minei, C. C., Gomes, B. C., Ratti, R. P., D'angelis, C. E. M., and De Martinis, E. C. P. (2008). Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *J. Food Prot.* 71, 634–638. doi: 10.4315/0362-028X-71.3.634

Montañez-Izquierdo, V. Y., Salas-Vázquez, D. I., and Rodríguez-Jerez, J. J. (2012). Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling *Listeria monocytogenes* biofilms on stainless steel surfaces. *Food Control* 23, 470–477. doi: 10.1016/j.foodcont.2011.08.016

Møretrø, T., Schirmer, B. C. T., Heir, E., Fagerlund, A., Hjemli, P., and Langsrud, S. (2017). Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. *Int. J. Food Microbiol.* 241, 215–224. doi: 10.1016/j.ijfoodmicro.2016.10.025

Oliveira, H., Azeredo, J., Lavigne, R., and Kluskens, L. D. (2012). Bacteriophage endolysins as a response to emerging foodborne pathogens. *Trends Food Sci. Technol.* 28, 103–115. doi: 10.1016/j.tifs.2012.06.016

Ortega Morente, E., Fernandez-Fuentes, M. A., Grande Burgos, M. J., Abriouel, H., Perez Pulido, R., and Galvez, A. (2013). Biocide tolerance in bacteria. *Int. J. Food Microbiol.* 162, 13–25. doi: 10.1016/j.ijfoodmicro.2012.12.028

Ortiz, S., Lopez-Alonso, V., Rodriguez, P., and Martinez-Suarez, J. V. (2015). The connection between persistent, disinfectant-resistant *Listeria monocytogenes* strains from two geographically separate Iberian pork processing plants: evidence from comparative genome analysis. *Appl. Environ. Microbiol.* 82, 308–317. doi: 10.1128/AEM.02824-15

O'Sullivan, J., Mccullough, J., Johnson, J. H., Bonner, D. P., Clark, J. C., Dean, L., et al. (1990). Janthinocins A, B and C, novel peptide lactone antibiotics produced by *Janthinobacterium lividum*: I. taxonomy, fermentation, isolation, physico-chemical and biological characterization. *J. Antibiot.* 43, 913–919. doi: 10.7164/antibiotics.43.913

Overney, A., Jacques-André-Coquin, J., Ng, P., Carpentier, B., Guillier, L., and Firmesse, O. (2017). Impact of environmental factors on the culturability and viability of *Listeria monocytogenes* under conditions encountered in food processing plants. *Int. J. Food Microbiol.* 244, 74–81. doi: 10.1016/j.ijfoodmicro.2016.12.012

Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., and Malm, A. (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Pol. J. Microbiol.* 63, 137–145.

Pérez Pulido, R., Grande Burgos, M. J., Gálvez, A., and Lucas López, R. (2016). Application of bacteriophages in post-harvest control of human pathogenic and food spoiling bacteria. *Crit. Rev. Biotechnol.* 36, 851–861. doi: 10.3109/07388551.2015.1049935

Pérez-Ibarreche, M., Castellano, P., Leclercq, A., and Vignolo, G. (2016). Control of *Listeria monocytogenes* biofilms on industrial surfaces by the bacteriocin-producing *Lactobacillus sakei* CRL1862. *FEMS Microbiol. Lett.* 363, 1–6. doi: 10.1093/femsle/fnw118

Perricone, M., Arace, E., Corbo, M. R., Sinigaglia, M., and Bevilacqua, A. (2015). Bioactivity of essential oils: a review on their interaction with food components. *Front. Microbiol.* 6:76. doi: 10.3389/fmicb.2015.00076

Reyes-Jurado, F., Franco-Vega, A., Ramírez-Corona, N., Palou, E., and López-Malo, A. (2014). Essential oils: antimicrobial activities, extraction methods, and their modeling. *Food Eng. Rev.* 7, 275–297. doi: 10.1007/s12393-014-9099-2

Sadekuzzaman, M., Yang, S., Mizan, M. F. R., Kim, H. S., and Ha, S. D. (2017). Effectiveness of a phage cocktail as a biocontrol agent against *L. monocytogenes* biofilms. *Food Control* 78, 256–263. doi: 10.1016/j.foodcont.2016.10.056

Salmond, G. P. C., and Fineran, P. C. (2015). A century of the phage: past, present and future. *Nat. Rev. Microbiol.* 13, 777–786. doi: 10.1038/nrmicro3564

Sánchez, L. A., Hedström, M., Delgado, M. A., and Delgado, O. D. (2010). Production, purification and characterization of serraticin A, a novel cold-active antimicrobial produced by *Serratia proteamaculans* 136. *J. Appl. Microbiol.* 109, 936–945. doi: 10.1111/j.1365-2672.2010.04720.x

Schmelcher, M., and Loessner, M. J. (2016). Bacteriophage endolysins: applications for food safety. *Curr. Opin. Biotechnol.* 37, 76–87. doi: 10.1016/j.copbio.2015.10.005

Schmelcher, M., Shabarova, T., Eugster, M. R., Eichenseher, F., Tchang, V. S., Banz, M., et al. (2010). Rapid multiplex detection and differentiation of *Listeria* cells by use of fluorescent phage endolysin cell wall binding domains. *Appl. Environ. Microbiol.* 76, 5745–5756. doi: 10.1128/AEM.00801-10

Schmelcher, M., Waldherr, F., and Loessner, M. J. (2012). *Listeria* bacteriophage peptidoglycan hydrolases feature high thermoresistance and reveal increased activity after divalent metal cation substitution. *Appl. Microbiol. Biotechnol.* 93, 633–643. doi: 10.1007/s00253-011-3372-6

Schuch, R., Khan, B. K., Raz, A., Rotolo, J. A., and Wittekind, M. (2017). Bacteriophage lysin CF-301, a potent antistaphylococcal biofilm agent. *Antimicrob. Agents Chemother.* 61:e02666-16. doi: 10.1128/AAC.02666-16

Simmons, M., Morales, C. A., Oakley, B. B., and Seal, B. S. (2012). Recombinant expression of a putative amidase cloned from the genome of *Listeria monocytogenes* that lyses the bacterium and its monolayer in conjunction with a protease. *Probiot. Antimicrob. Proteins* 4, 1–10. doi: 10.1007/s12602-011-9084-5

Singh, R., Ray, P., Das, A., and Sharma, M. (2009). Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an in vitro study. *J. Med. Microbiol.* 58, 1067–1073. doi: 10.1099/jmm.0.009720-0

Soni, K. A., and Nannapaneni, R. (2010). Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *J. Food Prot.* 73, 1519–1524. doi: 10.4315/0362-028X-73.8.1519

Strauch, E., Hammerl, J. A., and Hertwig, S. (2007). Bacteriophages: new tools for safer food? *J. Verbrauchersch. Lebensm.* 2, 138–143. doi: 10.4067/S0716-10182015000700010

Tezel, U., and Pavlostathis, S. G. (2015). Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. *Curr. Opin. Biotechnol.* 33, 296–304. doi: 10.1016/j.copbio.2015.03.018

Tompkin, R. B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65, 709–725. doi: 10.4315/0362-028X-65.4.709

Van Tassell, M. L., Ibarra-Sanchez, L. A., Hoepker, G. P., and Miller, M. J. (2017). Hot topic: antilisterial activity by endolysin PlyP100 in fresh cheese. *J. Dairy Sci.* 100, 2482–2487. doi: 10.3168/jds.2016-11990

Véghová, A., Minarovièová, J., Koreňová, J., Drahovská, H., and Kaclíková, E. (2016). Prevalence and tracing of persistent *Listeria monocytogenes* strains in meat processing facility production chain. *J. Food Saf.* 37:e12315. doi: 10.1111/jfs.12315

Winkelströter, L. K., Gomes, B. C., Thomaz, M. R. S., Souza, V. M., and De Martinis, E. C. P. (2011). *Lactobacillus sakei* 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. *Food Control* 22, 1404–1407. doi: 10.1016/j.foodcont.2011.02.021

Winkelströter, L. K., Tulini, F. L., and De Martinis, E. C. P. (2015). Identification of the bacteriocin produced by cheese isolate *Lactobacillus paraplantarum* FT259 and its potential influence on *Listeria monocytogenes* biofilm formation. *LWT Food Sci. Technol.* 64, 586–592. doi: 10.1016/j.lwt.2015.06.014

Woo, J., and Ahn, J. (2013). Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. *Lett. Appl. Microbiol.* 56, 307–313. doi: 10.1111/lam.12051

Wood, T. K. (2017). Strategies for combating persister cell and biofilm infections. *Microb. Biotechnol.* 10, 1054–1056. doi: 10.1111/1751-7915.12774

Xia, X., Li, G., Luo, D., Xia, J., Zheng, J., and Kan, J. (2017). Analysis of essential oil extracted by different methods from *Osmanthus fragrans*. *J. Chin. Cereals Oils Assoc.* 32, 67–73.

Zhang, H., Bao, H., Billington, C., Hudson, J. A., and Wang, R. (2012). Isolation and lytic activity of the *Listeria* bacteriophage endolysin LysZ5 against *Listeria monocytogenes* in soya milk. *Food Microbiol.* 31, 133–136. doi: 10.1016/j.fm.2012.01.005

Zhao, T., Doyle, M. P., and Zhao, P. (2004). Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. *Appl. Environ. Microbiol.* 70, 3996–4003. doi: 10.1128/AEM.70.7.3996-4003.2004

Zhao, T., Podtburg, T. C., Zhao, P., Chen, D., Baker, D. A., Cords, B., et al. (2013). Reduction by competitive bacteria of *Listeria monocytogenes* in biofilms and Listeria bacteria in floor drains in a ready-to-eat poultry processing plant. *J. Food Prot.* 76, 601–607. doi: 10.4315/0362-028X.JFP-12-323

Zhao, T., Podtburg, T. C., Zhao, P., Schmidt, B. E., Baker, D. A., Cords, B., et al. (2006). Control of *Listeria* spp. by competitive-exclusion bacteria in floor drains of a poultry processing plant. *Appl. Environ. Microbiol.* 72, 3314–3320. doi: 10.1128/AEM.72.5.3314-3320.2006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GP and handling Editor declared their shared affiliation.

Copyright © 2018 Gray, Chandry, Kaur, Kocharunchitt, Bowman and Fox. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 27 April 2018
doi: 10.3389/fmicb.2018.00616

Complete Genomic Analysis of a *Salmonella enterica* Serovar Typhimurium Isolate Cultured From Ready-to-Eat Pork in China Carrying One Large Plasmid Containing *mcr-1*

Wei Wang[1†], Zulqarnain Baloch[2†], Mingyuan Zou[3†], Yinping Dong[1], Zixin Peng[1], Yujie Hu[1], Jin Xu[1], Nafeesa Yasmeen[4], Fengqin Li[1] and Séamus Fanning[1,5,6]**

[1] Key Laboratory of Food Safety Risk Assessment, Ministry of Health, China National Center for Food Safety Risk Assessment, Beijing, China, [2] College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [3] Heilongjiang Provincial Center for Disease Control and Prevention, Harbin, China, [4] Institute of Microbiology, University of Agriculture Faisalabad, Faisalabad, Pakistan, [5] UCD-Centre for Food Safety, School of Public Health, Physiotherapy and Sports Science, University College Dublin, Dublin, Ireland, [6] Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, Ireland

One *mcr-1*-carrying ST34-type *Salmonella* Typhimurium WW012 was cultured from 3,200 ready-to-eat (RTE) pork samples in 2014 in China. Broth dilution method was applied to obtain the antimicrobial susceptibility of *Salmonella* Typhimurium WW012. Broth matting assays were carried out to detect transferability of this phenotype and whole-genome sequencing was performed to analyze its genomic characteristic. Thirty out of 3,200 RTE samples were positive for *Salmonella* and the three most frequent serotypes were identified as *S*. Derby (*n* = 8), *S*. Typhimurium (*n* = 6), and *S*. Enteritidis (*n* = 6). One *S*. Typhimurium isolate (*S*. Typhimurium WW012) cultured from RTE prepared pork was found to contain the *mcr-1* gene. *S*. Typhimurium WW012 expressed a level of high resistance to seven different antimicrobial compounds in addition to colistin (MIC = 8 mg/L). A single plasmid, pWW012 (151,609-bp) was identified and found to be of an IncHI2/HI2A type that encoded a *mcr-1* gene along with six additional antimicrobial resistance genes. Plasmid pWW012 contained an IS*30-mcr-1-orf-orf*-IS*30* composite transposon that can be successfully transferred to *Escherichia coli* J53. When assessed further, the latter demonstrated considerable similarity to three plasmids pHYEC7-*mcr-1*, pSCC4, and pHNSHP45-2, respectively. Furthermore, plasmid pWW012 also contained a multidrug resistance (MDR) genetic structure IS*26-aadA2-cmlA2-aadA1*-IS*406-sul3*-IS*26-dfrA12-aadA2*-IS*26*, which showed high similarity to two plasmids, pHNLDF400 and pHNSHP45-2, respectively. Moreover, genes mapping to the chromosome (4,991,167-bp) were found to carry 28 mutations, related to two component regulatory systems (*pmrAB, phoPQ*) leading to modifications of lipid A component of the lipopolysaccharide structure. Additionally, one mutation (D87N) in the quinolone resistance determining region (QRDR) gene of *gyrA* was

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università di Teramo, Italy

Reviewed by:
Haijian Zhou, Chinese Center for Disease Control and Prevention, China
Rong Zhang, Second Affiliated Hospital of Zhejiang University School of Medicine, China
Maria Schirone, Università di Teramo, Italy

***Correspondence:**
Fengqin Li
lifengqin@cfsa.net.cn
Séamus Fanning
sfanning@ucd.ie

† These authors have contributed equally to this work as co-first authors.

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *12 December 2017*
Accepted: *16 March 2018*
Published: *27 April 2018*

Citation:
Wang W, Baloch Z, Zou M, Dong Y, Peng Z, Hu Y, Xu J, Yasmeen N, Li F and Fanning S (2018) Complete Genomic Analysis of a Salmonella enterica Serovar Typhimurium Isolate Cultured From Ready-to-Eat Pork in China Carrying One Large Plasmid Containing mcr-1. Front. Microbiol. 9:616. doi: 10.3389/fmicb.2018.00616

identified in this *mcr-1* harboring *S*. Typhimurium. In addition, various virulence factors and heavy metal resistance-encoding genes were also identified on the genome of *S*. Typhimurium WW012. This is the first report of the complete nucleotide sequence of *mcr-1*-carrying MDR *S*. Typhimurium strain from RTE pork in China.

Keywords: MDR *Salmonella enteric* serovar Typhimurium, conjugation, mcr-1, *phoP/Q*, *pmrA/B*, plasmids, ready-to-eat pork

INTRODUCTION

Salmonella is often acquired from contaminated food, and is an important zoonotic bacterium linked to cases of gastroenteritis and bacteremia. This bacterium is now the leading cause of global bacterial food poisoning out breaks and is associated with increased morbidity and mortality (Kirk et al., 2015). There are estimated to be 94 million cases of gastroenteritis globally per year, with 155,000 deaths attributed to this bacterium (Majowicz et al., 2010; Gallati et al., 2013). It was reported that between 1994 and 2005, approximately 22% of food borne infections in China were caused by *Salmonella* (Wang et al., 2007). Of note, *Salmonella* Typhimurium is one of the most prevalent serovars identified among these food borne illnesses in China (Zhang et al., 2014). *Salmonella* Typhimurium strains are most commonly isolated from retail meat particularly pork in China, and both sporadic and outbreak-associated cases of human salmonellosis caused by this bacterium often need clinical therapy (Zhang et al., 2016). Moreover, this bacterium has the potential to act as a reservoir for different antimicrobial resistance-encoding genes (Zhang et al., 2016).

The relevance of *S.* Typhimurium is also marked by its capability to acquire resistance determinants, to various drug classes, especially those that are third-generation cephalosporins, tetracyclines, (fluoro) quinolones, folate pathway inhibitors, phenicols, penicillines, aminoglycosides and macrolides (Wong et al., 2014; Yi et al., 2017). Resistance to polymyxin in bacteria including *Salmonella* is commonly due to chromosomal mutations in two component regulatory systems (e.g., *pmrAB* and *phoPQ*) resulting in structural modification of lipid A. Therefore, bacterial resistant to polymyxin was historically thought to be rare (Lee et al., 2014). However, recently, a new plasmid-encoding polymyxin resistance gene, *mcr-1*, was discovered from bacterial isolated from the environment, animals, and humans in China (Liu et al., 2016). Subsequently, occurrence of the *mcr-1* gene has been reported worldwide, indicating a newly recognized global distribution. Furthermore, the *mcr-1*-bearing plasmids were found to possess the ability to disseminate between food-producing animal and human bacteria and were also highly stable in these backgrounds, even in the absence of polymyxin selection (Anjum et al., 2016; Kempf et al., 2016; Liu et al., 2016; El Garch et al., 2017).

The *mcr-1* gene in *Salmonella* was originally detected from human and food in England and Wales, including 8 *S*. Typhimurium, 1 *S*. Paratyphi B var Java and 1 *S*. Virchow strains (Doumith et al., 2016). Since then, this gene was reported from *Salmonella* isolates in Europe, the United States, and China, from humans, food-producing animals, and their surrounding environment, and retail meat (Anjum et al., 2016; Campos et al., 2016; Figueiredo et al., 2016; Quesada et al., 2016; Vinueza-Burgos et al., 2016; Yang et al., 2016). Nonetheless, to date, few reports of the detection of *mcr-1* have been cited in *Salmonella* from ready-to-eat (RTE) pork meat in China. In this study, we report for the first time, the prevalence of *Salmonella* species among RTE pork in China, and while one *S*. Typhimurium isolate was then found to be positive for the *mcr-1* gene, which was located on a large transferable plasmid, along with multiple antimicrobial resistance genes. The complete genome sequence of both the plasmid and chromosome was determined to shed insight into the diversity and complexity of this interesting strain.

MATERIALS AND METHODS

Strains Isolation

A total of 3,200 RTE pork samples were collected from 32 retail outlets and 32 commercial hypermarkets, in 32 provincial capitals of China in 2014 (**Figure 1**). Fifty RTE pork samples were collected at each sampling site and all were stored inside tightly sealed aseptic bags, surrounded by a biological ice bag, and then placed in a box maintained at a temperature lower than 4°C. Samples were immediately transported to the laboratory and subjected to microbiological analysis within 2 h. All samples were subjected to qualitative analysis for *Salmonella* using an enrichment method described by the National Food Safety Standard of China-Food microbiological examination, *Salmonella* (GB 4789.4-2010). Finally, presumptive *Salmonella* were selected for biochemical confirmation using API 20E test identification test strips (bioMérieux, Marcy l′ Etoile, France), as well as for molecular identification using PCR assay targeting the *invA* gene (Malorny et al., 2003). For all of the confirmed *Salmonella* isolates, serotypes were determined by the slide agglutination test, using *Salmonella* antisera (Statens Serum Institute, Denmark) according to the Kauffmann–White scheme. All confirmed *Salmonella* isolates were stored in brain heart infusion broth with 40% [v/v] glycerol (Land Bridge, Beijing, China) at −80°C. Each sample retained was represented by at least one bacterial isolate.

Detection of *mcr-1* Gene

The frozen strains were cultured overnight at 37°C in brain heart infusion broth. A TIANamp Bacterial DNA

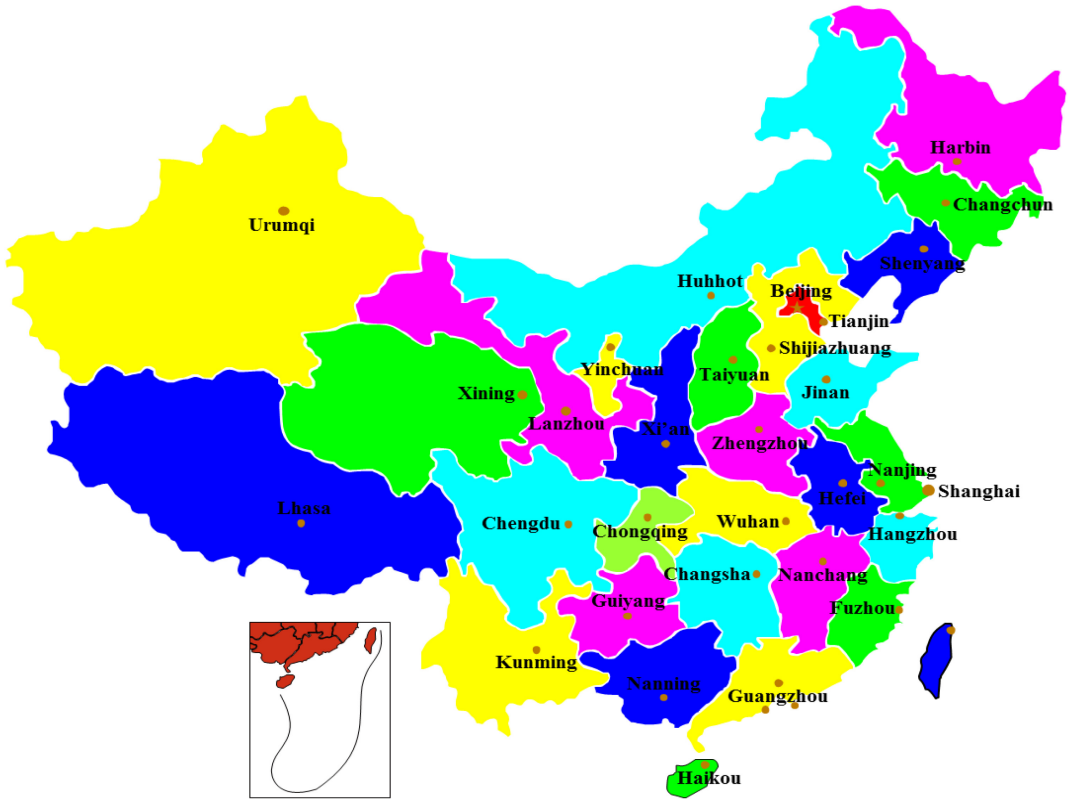


FIGURE 1 | Map of China showing the locations of the 32 metropolitan cities from where the *Salmonella* isolates were collected.

extraction kit (TianGen DNA Kit DP302, Beijing, China) was used to extract the genomic DNA from the culture according to the manufacturer's instructions. A NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, NH, United States) was then used to evaluate the quality of DNA. PCR was performed to confirm the presence of the *mcr-1* gene among the studied strains using primers targeting *mcr-1* as reported previously (Liu et al., 2016). The genetic identity of all amplicons was subsequently confirmed by nucleotide sequencing (Liu et al., 2016). Finally, one *S.* Typhimurium isolate designated as *S.* Typhimurium WW012 was determined to harbor the *mcr-1* gene and this isolate was selected to perform the follow-up experiments.

Antimicrobial Susceptibility Tests (AST)

Antimicrobial susceptibility testing (AST) of the *S.* Typhimurium WW012 isolate was performed using broth dilution method, using the Biofosun® Gram-negative panel (Fosun Diagnostics, Shanghai, China). Data obtained was interpreted according to standards and guidelines described by Clinical and Laboratory Standards Institute [CLSI] (2016). Additionally, we also used the National Antimicrobial Resistance Monitoring System (NARMS) protocol when CLSI standards were not available. The panel of 21 antimicrobial compounds as shown in **Table 1** was tested Reference *Escherichia coli* strain ATCC™25922 was used as the quality control.

Conjugal Plasmid Transfer and Characterization

The *mcr-1* harboring *S.* Typhimurium WW012 isolate was analyzed for its ability to transfer the colistin resistance phenotype using broth matting to the plasmid-free recipients, *E. coli* J53. Transfer of *mcr-1* to transconjugants was confirmed by PCR (Cui et al., 2017). Plasmid DNA profiles of both the donor and transconjugants were carried out using the S1-nuclease digestion pulsed-field gel electrophoresis (S1-PFGE) molecular sub-typing method (Liu et al., 2016).

Whole Genome Sequencing and Annotation

To assess the genomic background of *S.* Typhimurium WW012, whole-genome sequencing (WGS) was performed using the SMRT® Pacific Biosciences RS II platform (Pacific Biosciences, Menlo Park, CA, United States). Annotation of the genomes was performed using RAST[1], BLASTn and BLASTp[2] programs. The open reading frame (ORF) Finder program[3] was used to identify features of public health interest. PlasmidFinder-1.3[4]

[1]http://rast.nmpdr.org

[2]http://blast.ncbi.nlm.nih.gov/Blast.cgi

[3]http://www.ncbi.nlm.nih.gov/orffinder

[4]https://cge.cbs.dtu.dk/services/PlasmidFinder/

TABLE 1 | Antimicrobial resistance test of *S.* Typhimurium WW012 to a panel of 21 antimicrobial agents*.

Class	Antimicrobial agent[a]	MIC (mg/L)	R/I/S
Penicillins	Ampicillin (AMP)	1	S
Cephalosporins	Cefepime (FEP)	0.125	S
	Cefoxitin (CFX)	2	S
	Cefazolin (CFZ)	1	S
	Cefotaxime (CTX)	0.125	S
	Ceftazidime (CAZ)	0.25	S
	Ceftiofur (TIO)[b,€]	0.25	S
Carbapenems	Imipenem (IMI)	0.5	S
	Meropenem (MEM)	0.125	S
Aminoglicosides	Gentamicin (GEN)	1	S
	Streptomycin (STR)[b]	> 32	R
Polymyxin	Colistin (CT)[€]	8	R
beta-lactamase inhibitor combinations	Amoxicillin/ Clavulanic Acid (AMC)	>64/32	R
Folate pathway inhibitors	Trimethoprim/ sulfamethoxazole (SXT)	>8/152	R
Tetracyclines	Tetracycline (TET)	>32	R
Phenicols	Chloramphenicol (CHL)	32	R
	Florfenicol (FFC)[€]	>16	R
Fluoroquinolones	Nalidixic acid(NAL)	>128	R
	Ciprofloxacin (CIP)	0.12	I
	Enrofloxacin (ENO)[€]	0.125	S
Macrolides	Azithromycin (AMZ)[€]	4	S

**Data was interpreted according to (a) CLSI; (b) NARMS; €Used as a feed additive in animal production.*

was used to identify plasmid replicon types. The comprehensive antibiotic resistance database (CARD)[5] was used to identify antimicrobial resistance genes. Virulence factor database (VFDB)[6] was queried to predict the presence of virulence factors.

Three complete genomes, *Salmonella* Typhimurium LT2 (Accession number AE006468.1), *Salmonella* Typhi CT18 (AL513382.1), and *Salmonella* Indiana C629 (Accession number CP015724) were available from GenBank and used to compare the *Salmonella* pathogenicity islands (SPIs) with *S.* Typhimurium WW012 in this study by SPIFinder[7]. Finally, the antibacterial biocide and metal resistance genes database (BacMet)[8] was used to predict the presence of any metal-resistance genes.

Nucleotide Accession Numbers

The complete genome of *S.* Typhimurium WW012 was deposited at GenBank under the Accession number CP022168 (chromosome) and CP022169 (plasmid p WW012).

[5]https://card.mcmaster.ca/analyze

[6]http://www.mgc.ac.cn/VFs/main.htm

[7]http://cge.cbs.dtu.dk/services/SPIFinder

[8]http://bacmet.biomedicine.gu.se/

RESULTS

Prevalence of *mcr-1*-Positive *Salmonella*

In this study, we collected and tested a total of 3,200 RTE pork samples from 32 provincial capitals in China in 2014. Thirty (30/3,200, 0.94%) samples were positive for *Salmonella*; one isolate from each sample was selected for subsequent analyses. In total, thirty isolates were recovered (comprising 10 serotypes), and the three most frequent serotypes among RTE pork samples covering 66.7% of the total, were identified as *S.* Derby (n = 8), *S.* Typhimurium (n = 6), and *S.* Enteritidis (n = 6). One (1/30, 3.3%) *S.* Typhimurium isolate (designated as *S.* Typhimurium WW012) was cultured from RTE prepared pork collected from a convenience market in Nanning city, Guangxi Province, and found to contain the *mcr-1* gene. The sampling details of isolates are shown in **Supplementary Table S1**.

Susceptibility to Antimicrobial Compounds

In this study, *S.* Typhimurium WW012 expressed a level of high resistance to seven different antimicrobial compounds in addition to colistin (MIC = 8 mg/L) (**Table 1**). The isolate was susceptible to all tested penicillins, cephalosporins, carbapenems, and macrolides (**Table 1**). Moreover, *S.* Typhimurium WW012 was also found to be susceptible to enrofloxacin, whilst expressing an intermediate resistant phenotype to ciprofloxacin (**Table 1**).

Horizontal Transfer of *mcr-1* Genes and Associated Determinants

One plasmid was identified from *S.* Typhimurium WW012 by S1-PFGE and the conjugation assay showed that it could be transferred to a plasmid-free recipient *E. coli* J53 with a conjugation frequency of 1.2×10^{-6} as determined under laboratory conditions (**Table 2** and **Supplementary Figure S1**). MIC value of the transconjugant were recorded at 8mg/L using broth dilution test, which showed 64-fold increase in when compared with the recipient *E. coli* J53 (0.125 mg/L). This transconjugant expressed a MDR phenotype and was found to be resistance to five antimicrobial compounds including of trimethoprim, sulfamethoxazole, streptomycin, tetracycline, chloramphenicol, and florfenicol, in addition to colistin.

Genome Sequence Information

The *S.* Typhimurium WW012 genome consisted of a single circular chromosome and a circular plasmid (**Table 2** and **Supplementary Figure S2**). The chromosome consisted of 4,991,167 bp with 4,684 predicted ORFs along with 110 RNAs. Meanwhile, a single circular extra-chromosomal element was identified and denoted as plasmid pWW012 of IncHI2 and IncHI2A replicon types. The total size of plasmid pWW012 was 151,609 bp including 168 predicted ORFs with the average GC content of 45.0% (**Table 2**). Moreover, the multilocus sequence type (MLST) of *S.* Typhimurium WW012 was identified as ST34 (**Table 2**).

TABLE 2 | The summary of the features associated with the genome and plasmid identified in *S.* Typhimurium WW012*.

Location	Size (bp)	G+C%	ORFs	RNAs	ST/Inc type	S1-PFGE	Conjugation frequency	Antimicrobial resistance phenotype	Antimicrobial resistance genes
Chromosome	4,991,167	52.1%	4,684	110	ST34	–	–	Streptomycin	*strA*,*strB*
								Sulfamethoxazole	*sul2*
								Tetracycline	*tet(B)*
								Nalidixic acid	*gyrA* (D87N)
								Colistin	PhoPQ *phoP* (S131P), *phoQ* (T168A); *ArnBCADTEF*operson *arnA* (L48Q, G51E, N184D, A195S, I247M, G284D, C303R, D337N, E554D, D631G),*arnC*(S320P), *arnD* (P164S, V216A); PmerCAB *pmrC* (I81V, Q415E), *pmrA* (S89T, R211G), *pmrB* (T18M, S76G, V86I, T114A); *pmrD* (L85S)
Plasmid	151,609	45.0%	168	–	*IncHI2*, *IncHI2A*	∼150 kb	1.2×10^{-6}	Streptomycin	*aadA1*, *aadA2*
								Sulfamethoxazole	*sul3*
								Trimethoprim	*dfrA12*
								Colistin	*mcr-1*
								Chloramphenicol, florfenicol	*cmlA1*

**No features were identified.*

Acquired Antimicrobial Resistance-Encoding Genes Identified in the Bacterial Genome

The CARD database was queried to identify resistance related genotypes on the genome of *S.* Typhimurium WW012. A total of 21 antimicrobial resistance genes (two genes were identified as *aadA2*) were identified, which encoded resistance to 11 antimicrobial agents of 10 different classes, including 14 genes on the chromosome and 7 on the plasmid (**Table 2** and **Supplementary Figure S2**). In detail, when compared with *S.* Typhimurium LT2, one missense mutation in *gyrA* (D87N) and a total of 24 missense mutations in the PmrAB and PhoPQ systems were found within the chromosome of *S.* Typhimurium WW012. Moreover, four and five additional antimicrobial resistance genes of *strA/B*, *sul2*, *tet(B)* were also found within the chromosome and plasmid, respectively, (**Table 2** and **Supplementary Figure S2**).

Virulence Factors Annotated in the Bacterial Genome

To identify potential virulence-encoding genes in *S.* Typhimurium WW012, the genome was used to query those factors listed in the VFDB. These were aligned to the ORF protein sequences using BLASTP and filtered with 90% identity and match length. Using this approach, 201 virulence factors were identified on the chromosome, among which 66 genes belonged to type-three secretion system (T3SS) encoding genes (**Supplementary Figure S2** and **Supplementary Table S2**). The genomes of *S.* Typhimurium WW012 were also compared with those of *S.* Typhimurium LT2, *S.* Typhi CT18, and *S.* Indiana C629 in NCBI to identify the presence of SPIs. It was found that *S.* Typhimurium WW012 possessed SPI-3, SPI-4, SPI-5, SPI-13, SPI-14, and C63PI (**Table 3**) in addition to SPI-1 and SPI-2. Moreover, 63 fimbrial adherence-encoding genes and other virulence-encoding genes were identified on the chromosome of *S.* Typhimurium WW012 (**Supplementary Figure S2** and **Supplementary Table S2**).

Metal Resistance Genes Annotated in the Bacterial Genome

The antibacterial biocide and metal resistance-encoding gene database (BacMet) was used to predict the presence of metal-resistance genes encoded on the genome of *S.* Typhimurium WW012. Twenty-eight resistance genes related to arsenic, copper, iron, zinc, magnesium, cobalt, mercury, molybdenum, nickel, and silver, were located on the chromosome, whilst 7 tellurium resistance encoding genes were identified as *terA/B/C/D/E/W/Z* (**Supplementary Figure S2** and **Supplementary Table S3**) on plasmid pWW012.

Comparative Analysis of Plasmid pWW012

The *mcr-1* gene located on plasmid pWW012 was bracketed by two IS*30* elements that were located in the same orientation. Similar structures were found in plasmids pHYEC7-*mcr-1*

TABLE 3 | Distribution of SPIs in four representative genomes of *Salmonella* stains*.

Genomic Island	*S.* Typhimurium WW012	*S.* Typhimurium LT2	*S.* Typhi CT18	*S.* Indiana C629
SPI-1	+	+	+	+
SPI-2	+	+	+	+
SPI-3	+	+	+	-
SPI-4	+	+	+	+
SPI-5	+	+	+	+
SPI-6	−	−	+	−
SPI-7	−	−	+	−
SPI-8	−	−	+	−
SPI-9	−	−	+	−
SPI-10	−	−	+	−
SPI-12	−	−	+	−
SPI-13	+	+	−	−
SPI-14	+	+	−	−
C63PI	+	+	−	+

*Not identified.

(KX518745), pHNSHP45-2 (KU341381), and pSCC4 (CP021078) recovered previously from *E. coli* and *Citrobacter braakii*, respectively, (**Supplementary Figure S3A**). The insertion element IS*30* showed 100% (95% query coverage) of homologous sequence with IS*Apl1* in plasmid pHNSHP45-2, where in 17 amino acids were missing at the 5′-end comparing with the latter (**Supplementary Figure S3A**). In addition to the *mcr-1* gene locus, plasmid pWW012 also contained an additional MDR gene cassette embedded in a complex class 1 integron of 15.2-kbp (**Supplementary Figure S3B**) bracketed by two IS*26* elements in but found in inverted orientations directions, at a distance from the *mcr-1* gene locus (**Supplementary Figure S2**). The class 1 integron was preceded by a copy of insertion element IS*26*, and two transposase-encoding genes *tnpR* and *tnpM*, followed by the *aadA2*, *cmlA1*, and *aadA1* genes. Downstream of these resistance genes, an interesting resistance gene locus consisting of IS*406*–*sul3*-*orf*-*orf*-*orf*-IS*26* was identified. This locus was followed by one integrase, two resistance genes of *dfrA12* and *aadA2*, and the IS*26* element. This structure has also been identified in two *E. coli* originated plasmids designated as pHNLDF400 (KV019258.1) and pHNSHP45-2 (KU341381) (**Supplementary Figure S3B**).

DISCUSSION

In the past, *Salmonella* has attracted much attention owing to its important role as a food-borne pathogen. Recent studies reported on the prevalence of *Salmonella* and plasmid-encoded polymyxin resistance genes, containing *mcr-1*, among humans, food-producing animals and food samples in China and elsewhere (Anjum et al., 2016; Campos et al., 2016; Doumith et al., 2016; El Garch et al., 2016; Figueiredo et al., 2016; Kempf et al., 2016; Liu et al., 2016; Quesada et al., 2016; Yang et al., 2016). However, limited comprehensive epidemiological data are available describing the prevalence of *Salmonella* and *mcr-1* gene among RTE pork samples in China, although pork and pork-related products are the main source of animal protein for Chinese consumers. To the best of our knowledge, the current study was the first report on the epidemiological prevalence and detection of *Salmonella* and *mcr-1* gene among RTE pork samples in China. Generally, the prevalence rate of *Salmonella* and the *mcr-1* gene among RTE pork samples was relatively low in this study, while similar results were observed from clinical, pigs and retail pork in previous studies (Figueiredo et al., 2016; Quesada et al., 2016; Cui et al., 2017). However, considering the fact that these RTE pork samples are positive for *Salmonella* and therefore would not be heated or cooked the infectious risk would increase. In China, only prepackaged RTE foodstuffs have a pathogen limiting standard applied at National level. In this study all RTE pork samples were collected from marketing sites and nearly 87% of the samples that were positive for *Salmonella* were unpackaged. Although these pork samples were permitted for sale by the food hygiene bureau at the beginning, unpacked treatment may later increase the probability of the contamination of *Salmonella* during their shelf-life. Therefore, limited standard and effective legislation in RTE pork to prevent and control the contamination of *Salmonella* should now be considered.

In this study, we successfully recovered and characterized one *Salmonella* isolate (*S.* Typhimurium WW012) that harbored a *mcr-1*-bearing plasmid from a RTE prepared pork sample in China. The genus *Salmonella* consists of a number of serovars, where in it is reported that the most often detected serovar harboring the *mcr-1*-was Typhimurium (Doumith et al., 2016; Cui et al., 2017), suggesting that the acquisition and prevalence of *mcr-1*-bearing mobile elements may require a specific genetic background. The relationship between *S.* Typhimurium and *mcr-1*-bearing plasmids warrants further investigation (Doumith et al., 2016). This RTE pork isolate expressed a MDR phenotype in addition to colistin. Notably, all of the resistant antimicrobial agents tested in this study are widely used at human and veterinary clinics. Carnevali et al. (2016) screened for the *mcr-1* gene in 4,473 *Salmonella* isolates from human, food-producing animal, food and environment samples and identified 25 positive isolates of human and veterinary origin, all of which were susceptible to broad-spectrum cephalosporins. The *S.* Typhimurium WW012 isolate in this study was also susceptible to all tested cephalosporin antimicrobials. Similar results were reported from human, food-producing animals in China (Li et al., 2016; Yi et al., 2017).

A laboratory-based conjugation assay revealed that the *mcr-1* gene was transferrable to the plasmid-free recipient *E. coli* J53, with a frequency of 1.2×10^{-6}. In contrast, the transfer frequency of the IncI2-type plasmid carrying the *mcr-1* gene reported in the original study was surprisingly higher, ranging from 10^{-1} to 10^{-3} between *E. coli* strains (Liu et al., 2016). In this study these data showed that the transfer of the *mcr-1* gene from *S.* Typhimurium WW012 to *E. coli* had a frequency that was lower. Nonetheless, it was confirmed that the *mcr-1*-carrying IncHI2-type plasmid in this study can be transferred between different genera of Enterobacteriaceae.

Of note, according to previous reports, the IncHI2 replicon was one of the most common found in *Salmonella* (Campos et al., 2016; Doumith et al., 2016). Moreover, several reports confirmed *mcr-1*-positive *Salmonella* that were expressing a MDR phenotype (Doumith et al., 2016; Figueiredo et al., 2016; Yang et al., 2016). Notably, the current data showed that this transconjugant showed resistance to five antimicrobial agents in addition to colistin, indicating that *S.* Typhimurium WW012 could not only transfer the *mcr-1* gene but also other MDR-encoding genes into other Enterobacteriaceae strains. Therefore, once this clone carrying *mcr-1* gene disseminates from RTE pork to humans via the food chain, it could be expected to promote the forward dissemination of the latter marker and other members of the Enterobacteriaceae. In this study *S.* Typhimurium WW012 belonged to ST34, which is the predominant sequence type in southern China (Sun et al., 2014). Recently, the *mcr-1* gene was also detected in MDR *S.* Typhimurium (ST34) in China and Europe (Doumith et al., 2016; Li et al., 2016).

In this study, the plasmid mediated colistin resistance gene *mcr-1* was found to be located in a composite transposon with the structure IS*30*-*mcr-1*-*orf*-*orf*-IS*30*, which was similar to those found previously on two *E. coli* plasmids pHYEC7-*mcr-1* (KX518745) and pHNSHP45-2 (KU341381), and one *C. braakii* plasmid pSCC4 (CP021078, unpublished) (Liu et al., 2016; Wang et al., 2018). Unlike plasmid pHNSHP45-2, the original IncHI2-type *mcr-1*-carrying episome, the *mcr-1* gene located on plasmids pWW012, pHYEC7-*mcr-1* and pSCC4 was bracketed by IS*30* elements. According to a latest report, IS*Apl1* is a member of the IS*30* family of insertion sequences, which utilize a 'copy-out, paste-in' mechanism with a targeted transposition pathway requiring the formation of a synaptic complex between an inverted repeat (IR) in the transposon circle and an IR-like sequence in the target (Wang et al., 2018). IS*30* is related to site-specific recombination, which could mediate the transmission of *mcr-1* among different bacterial species. Moreover, it was found that several resistance genes were also found to be present and located between two IS*26* elements on plasmid pWW012, in which a class 1 integron similar to that found in plasmids pHNSHP45-2 and pHNLDF400 (KY019258.1) also existed (Liu et al., 2016; Wang et al., 2017). The recovery of plasmid pWW012 that harbored structurally similar mobile elements to these contained in these reported plasmids implied that genetic exchange between such plasmids was common, and that such plasmids could readily be transferred to other bacteria, constituting one major evolution and resistance development route for bacterial pathogens.

In addition to the plasmid-mediated colistin resistance gene *mcr-1*, genes found on chromosome containing mutations, encoding two component regulatory systems (*pmrAB, phoPQ*), were also found in this study. These chromosomal mutations mediated mechanisms were thought to lead to the modification of lipid A, which anchors the lipopolysaccharide molecule to the outer membrane, resulting in reduction in the affinity to colistin (Lee et al., 2014). Moreover, a target gene mutation in the quinolone resistance determining region (QRDR) on the chromosome were identified in *gyrA* mutation (giving rise to D87N amino acid substitution) in this *mcr-1* harboring *S.* Typhimurium. The latter is frequently reported in other MDR *Salmonella* species in China (Wong et al., 2014). In addition, *strA/B* encoding streptomycin resistance and *sul2* encoding sulfamethoxazole resistances were also detected to locate on the chromosome of *S.* Typhimurium WW012.

In this study, 201 virulence genes were found on the chromosome of *S.* Typhimurium WW012 when queried through VFDB. Type-three secretion systems (T3SS) encoding genes related to SPI-1 and SPI-2 were commonly detected. Both SPI-1 and SPI-2 contained two independent type three secretion systems (denoted as TTSS SPI-1 and TTSS SPI-2, respectively) that can inject effect or proteins into host cells, which are crucial for various stages of infection (Weening et al., 2005). Comparison of the genomes *S.* Typhimurium WW012 with the reference or complete sequences in NCBI for the presence of SPIs showed that SPIs in *S.* Typhimurium WW012 were closely related to their equivalents in *S.* Typhimurium LT2 (McClelland et al., 2001), and shared some of those in *S.* Typhi CT18 and *S.* Indiana C629 (Parkhill et al., 2001; Wang et al., 2016). Additionally, 63 fimbrial adherence encoding genes on the chromosome of *S.* Typhimurium WW012 were also identified in this study. Several *in vitro* studies have suggested that fimbrial-encoding genes found in *Salmonella* could mediate the attachment of this bacterium to epithelial cells in the host (Leclerc et al., 2016; Peters et al., 2017). The latter phenotype may support long-term intestinal carriage of this bacterium.

Heavy metal compounds as well as antimicrobial agents are widely used as feed additives in food-producing animals for therapy, and growth purposes in China (Hu et al., 2017). Because of their stable and persistent characteristics, when heavy metals accumulate to specific concentrations, they potentially result in resistance among bacteria cultured from food-producing animals. Heavy metal resistance-encoding genes have also been identified in different environments (Deng et al., 2017). Recent research data showed that a relationship between the acquisition of heavy metal resistance genes and antimicrobial resistance genes, and antimicrobial resistance may arise through co-resistance or cross-resistance to metals or co-regulation of resistance pathways (Argudín et al., 2016; Deng et al., 2017). In this study, 28 and 7 heavy metal resistance genes were found on the chromosome and plasmid, respectively. These heavy metal resistance genes might increase antimicrobial resistance capacity within this bacterium, through co-selection of determinants.

CONCLUSION

This study firstly reported the epidemiological prevalence and detection of *Salmonella* and *mcr-1* gene among RTE pork samples in China. These data highlight the importance of role played by *S.* Typhimurium in the dissemination of MDR genes. Although the mechanisms remain to be further described, the emergence of MDR genes particularly *mcr-1*, along with various virulence factors and heavy metal resistance genes, on the chromosome and

plasmid from *S*. Typhimurium, will challenge therapeutic options for clinicians and others. Moreover, successful transmission of the *mcr-1* gene poses a challenge to those with an interest in the protection of public health.

AVAILABILITY OF DATA AND MATERIALS

The aggregate data supporting findings contained within this manuscript will be shared upon request submitted to the corresponding author.

AUTHOR CONTRIBUTIONS

WW, ZB, SF, and FL designed the experiments and wrote the manuscript. MZ, YD, YH, ZP, and JX carried out the experiments. ZB, WW, and NY analyzed the experimental results.

FUNDING

This study was funded by the National Key R&D Program of China (2016YFD0401102) and China Food Safety Talent Competency Development Initiative: CFSA 523 Program.

ACKNOWLEDGMENTS

We sincerely thank all the participants who took part in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00616/full#supplementary-material

FIGURE S1 | S1-PFGE indicating the transfer of single plasmid harboring *mcr-1*. Lane 1, *Escherichia coli* J53 (recipient); Lane 2, *S.* Typhimurium WW012 (donor); Lane 3, CT-*E. coli* J53(transconjugant); Lane 4, H-9812(PFGE marker strain, *S*.BraenderupH9812).

FIGURE S2 | A schematic representation of the complete chromosome **(A)** and plasmid **(B)** of *S.* Typhimurium WW012. Each ring, beginning from the inside to out represents the following features: GC content (black); GC skew (green and purple); CDS (blue); Antimicrobial resistance genes (shown in blue colored font), metal resistance genes (shown in green colored font), and virulence factor genes (shown in black colored font).

FIGURE S3 | Major structural features of plasmid pWW012 compared with several other plasmids identified from NCBI. **(A)** Major structural features of plasmid pWW012 compared with plasmids pHYEC7-mcr-1, pSCC4, and pHNSHP45-2 identified in *E. coli* and *Citrobacter braakii*, respectively. **(B)** Major structural features of plasmid pWW012 compared with plasmids pHNLDF400 and pHNSHP45-2 both identified in *E. coli*. ORFs are shown with arrows (blue arrows, transposase; red arrows, antibiotic resistance genes; green arrows, integrase; gray arrows, member protein; white arrows, hypothetical protein–see the key to the right hand side of the panel). The missing amino acids in IS*30* comparing with IS*Apl1* were showed in red box.

TABLE S1 | Sampling information of *Salmonella* isolates in this study.

TABLE S2 | Virulence resistance-encoding genes identified in *S*. Typhimurium WW012 when comparing the genome of the latter against the current version of the VFDB database.

TABLE S3 | Metal resistance genes of *S*. Typhimurium WW012 with the current version of the BacMet database.

REFERENCES

Anjum, M. F., Duggett, N. A., AbuOun, M., Randall, L., Nunez-Garcia, J., Ellis, R. J., et al. (2016). Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. *J. Antimicrob. Chemother.* 71, 2306–2313. doi: 10.1093/jac/dkw149

Argudín, M. A., Lauzat, B., Kraushaar, B., Alba, P., Agerso, Y., Cavaco, L., et al. (2016). Heavy metal and biocidal agent resistance genes among livestock-associated methicillin-resistant *Staphylococcus aureus* isolates. *Vet. Microbiol.* 191, 88–95. doi: 10.1016/j.vetmic.2016.06.004

Campos, J., Cristino, L., Peixe, L., and Antunes, P. (2016). MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S. Rissen* clones in Portugal, 2011 to 2015. *Euro Surveill.* 21. doi: 10.2807/1560-7917.ES.2016.21.26.30270

Carnevali, C., Morganti, M., Scaltriti, E., Bolzoni, L., Pongolini, S., and Casadei, G. (2016). Occurrence of *mcr-1* in colistin-resistant *Salmonella enterica* isolates recovered from humans and animals in Italy, 2012 to 2015. *Antimicrob. Agents Chemother.* 60, 7532–7534. doi: 10.1128/AAC.01803-16

Clinical and Laboratory Standards Institute [CLSI] (2016). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Sixth Informational Supplement. CLSI Document M*100-S26. Wayne, PA: Clinical and Laboratory Standards Institute.

Cui, M., Zhang, J., Gu, Z., Li, R., Chan, E. W., Yan, M., et al. (2017). Prevalence and molecular characterization of mcr-1-positive *Salmonella* strains recovered from clinical specimens in China. *Antimicrob. Agents Chemother.* 61, e02471–16. doi: 10.1128/AAC.02471-16

Deng, W., Quan, Y., Yang, S., Guo, L., Zhang, X., Liu, S., et al. (2017). Antibiotic resistance in *Salmonella* from retail foods of animal origin and its association with disinfectant and heavy metal resistance. *Microb. Drug Resist.* doi: 10.1089/mdr.2017.0127 [Epub ahead of print].

Doumith, M., Godbole, G., Ashton, P., Larkin, L., Dallman, T., Day, M., et al. (2016). Detection of the plasmid-mediated *mcr-1* gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J. Antimicrob. Chemother.* 71, 2300–2305. doi: 10.1093/jac/dkw093

El Garch, F., de Jong, A., Simjee, S., Moyaert, H., Klein, U., Ludwig, C., et al. (2016). Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009–2012: VetPath results. *Vet Microbiol.* 194, 11–22. doi: 10.1016/j.vetmic.2016.04.009

El Garch, F., Sauget, M., Hocquet, D., LeChaudee, D., Woehrle, F., and Bertrand, X. (2017). *mcr-1* is borne by highly diverse *Escherichia coli* isolates since 2004 in food-producing animals in Europe. *Clin. Microbiol. Infect.* 23, 51.e1–51.e4. doi: 10.1016/j.cmi.2016.08.033

Figueiredo, R., Card, R. M., Nunez, J., Pomba, C., Mendonca, N., Anjum, M. F., et al. (2016). Detection of an *mcr-1*-encoding plasmid mediating colistin resistance in *Salmonella enterica* from retail meat in Portugal. *J. Antimicrob. Chemother.* 71, 2338–2340. doi: 10.1093/jac/dkw240

Gallati, C., Stephan, R., Hächler, H., Malorny, B., Schroeter, A., and Nüesch-Inderbinen, M. (2013). Characterization of *Salmonella enterica* subsp. *Enterica serovar* 4,[5],12:i:- clones isolated from human and other sources in Switzerland between 2007 and 2011. *Foodborne Pathog. Dis.* 10, 549–554. doi: 10.1089/fpd.2012.1407

Hu, Y., Cheng, H., and Tao, S. (2017). Environmental and human health challenges of industrial livestock and poultry farming in China and their mitigation. *Environ. Int.* 107, 111–130. doi: 10.1016/j.envint.2017.07.003

Kempf, I., Jouy, E., and Chauvin, C. (2016). Colistin use and colistin resistance in bacteria from animals. *Int. J. Antimicrob. Agents* 48, 598–606. doi: 10.1016/j.ijantimicag.2016.09.016

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World Health Organization estimates of the global and regional disease burden of 22 a foodborne bacterial, protozoal, and viral Diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Leclerc, J. M., Quevillon, E. L., Houde, Y., Paranjape, K., Dozois, C. M., and Daigle, F. (2016). Regulation and production of Tcf, a cable-like fimbriae from *Salmonella enterica* serovar Typhi. *Microbiology* 162, 777–788. doi: 10.1099/mic.0.000270

Lee, J. Y., Chung, E. S., Na, I. Y., Kim, H., Shin, D., and Ko, K. S. (2014). Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 69, 2966–2971. doi: 10.1093/jac/dku238

Li, X. P., Fang, L. X., Song, J. Q., Xia, J., Huo, W., Fang, J. T., et al. (2016). Clonal spread of mcr-1 in PMQR-carrying ST34 *Salmonella* isolates from animals in China. *Sci. Rep.* 6:38511. doi: 10.1038/srep38511

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/S1473-3099(15)00424-7

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., et al. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* 50, 882–889. doi: 10.1086/650733

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., et al. (2003). Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. *Int. J. Food Microbiol.* 89, 241–249. doi: 10.1016/S0168-1605(03)00154-5

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., et al. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 413, 852–856. doi: 10.1038/35101614

Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D., Wain, J., et al. (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 413, 848–852. doi: 10.1038/35101607

Peters, B., Stein, J., Klingl, S., Sander, N., Sandmann, A., Taccardi, N., et al. (2017). Structural and functional dissection reveals distinct roles of Ca2+-binding sites in the giant adhesin *SiiE* of *Salmonella enterica*. *PLoS Pathog.* 13:e1006418. doi: 10.1371/journal.ppat.1006418

Quesada, A., Ugarte-Ruiz, M., Iglesias, M. R., Porrero, M. C., Martinez, R., Florez-Cuadrado, D., et al. (2016). Detection of plasmid mediated colistin resistance (MCR-1) in *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine in Spain. *Res. Vet. Sci.* 105, 134–135. doi: 10.1016/j.rvsc.2016.02.003

Sun, J., Ke, B., Huang, Y., He, D., Li, X., Liang, Z., et al. (2014). The molecular epidemiological characteristics and genetic diversity of *Salmonella* Typhimurium in Guangdong, China, 2007–2011. *PLoS One* 9:e113145. doi: 10.1371/journal.pone.0113145

Vinueza-Burgos, C., Cevallos, M., Ron-Garrido, L., Bertrand, S., and De Zutter, L. (2016). Prevalence and diversity of *Salmonella* serotypes in ecuadorian broilers at slaughter age. *PLoS One* 11:e0159567. doi: 10.1371/journal.pone.0159567

Wang, J., Zhi, C. P., Chen, X. J., Guo, Z. W., Liu, W. L., Luo, J., et al. (2017). Characterization of *oqxAB* in *Escherichia coli* isolates from animals, retail meat, and human patients in Guangzhou, China. *Front. Microbiol.* 8:1982. doi: 10.3389/fmicb.2017.01982

Wang, R. B., van Dorp, L., Shaw, L. P., Bradley, P., Wang, Q., Wang, X. J., et al. (2018). The global distribution and spread of the mobilized colistin resistance gene *mcr-1*. *Nat. Commun.* 9:1179. doi: 10.1038/s41467-018-03205-z

Wang, S., Duan, H., Zhang, W., and Li, J. W. (2007). Analysis of bacterial foodborne disease outbreaks in China between 1994 and 2005. *FEMS Immunol. Med. Microbiol.* 51, 8–13. doi: 10.1111/j.1574-695X.2007.00305.x

Wang, W., Liu, F., Peng, Z., Li, F., and Ma, A. (2016). Complete genome sequence of *Salmonella enterica* subsp. Enterica serovar Indiana C629, a carbapenem-resistant bacterium isolated from chicken carcass in China. *Genome Announc.* 4, e00662–16. doi: 10.1128/genomeA.00662-1

Weening, E. H., Barker, J. D., Laarakker, M. C., Humphries, A. D., Tsolis, R. M., and Bäumler, A. J. (2005). The *Salmonella enterica* serotype Typhimurium *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* fimbrial operons are required for intestinal persistence in mice. *Infect. Immun.* 73, 3358–3366. doi: 10.1128/IAI.73.6.3358-3366.2005

Wong, M. H., Yan, M., Chan, E. W., Biao, K., and Chen, S. (2014). Emergence of clinical *Salmonella enterica* serovar Typhimurium isolates with concurrent resistance to ciprofloxacin, ceftriaxone, and azithromycin. *Antimicrob. Agents Chemother.* 58, 3752–3756. doi: 10.1128/AAC.02770-13

Yang, Y. Q., Zhang, A. Y., Ma, S. Z., Kong, L. H., Li, Y. X., Liu, J. X., et al. (2016). Co-occurrence of *mcr-1* and ESBL on a single plasmid in *Salmonella enterica*. *J. Antimicrob. Chemother.* 71, 2336–2338. doi: 10.1093/jac/dkw243

Yi, L., Wang, J., Gao, Y., Liu, Y., Doi, Y., Wu, R., et al. (2017). *mcr-1*–harboring *Salmonella enterica* serovar Typhimurium sequence type 34 in pigs, China. *Emerg. Infect. Dis.* 23, 291–295. doi: 10.3201/eid2302.161543

Zhang, J., Jin, H., Hu, J., Yuan, Z., Shi, W., Ran, L., et al. (2014). Serovars and antimicrobial resistance of non-typhoidal *Salmonella* from human patients in Shanghai, China, 2006-2010. *Epidemiol. Infect.* 142, 826–832. doi: 10.1017/S0950268813001659

Zhang, W. H., Lin, X. Y., Xu, L., Gu, X. X., Yang, L., Li, W., et al. (2016). CTX-M-27 producing *Salmonella enterica* serotypes Typhimurium and Indiana are prevalent among food-producing animals in China. *Front. Microbiol.* 7:436. doi: 10.3389/fmicb.2016.00436

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MS and handling Editor declared their shared affiliation.

Copyright © 2018 Wang, Baloch, Zou, Dong, Peng, Hu, Xu, Yasmeen, Li and Fanning. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 June 2018
doi: 10.3389/fmicb.2018.01103

Virulence Gene Sequencing Highlights Similarities and Differences in Sequences in *Listeria monocytogenes* Serotype 1/2a and 4b Strains of Clinical and Food Origin From 3 Different Geographic Locations

Sofia V. Poimenidou[1], Marion Dalmasso[2†], Konstantinos Papadimitriou[3], Edward M. Fox[4], Panagiotis N. Skandamis[1] and Kieran Jordan[2]*

[1] Laboratory of Food Quality Control and Hygiene, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece, [2] Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, Ireland, [3] Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece, [4] CSIRO Agriculture and Food, Werribee, VIC, Australia

The *prfA*-virulence gene cluster (*p*VGC) is the main pathogenicity island in *Listeria monocytogenes*, comprising the *prfA, plcA, hly, mpl, actA,* and *plcB* genes. In this study, the *p*VGC of 36 *L. monocytogenes* isolates with respect to different serotypes (1/2a or 4b), geographical origin (Australia, Greece or Ireland) and isolation source (food-associated or clinical) was characterized. The most conserved genes were *prfA* and *hly*, with the lowest nucleotide diversity (π) among all genes ($P < 0.05$), and the lowest number of alleles, substitutions and non-synonymous substitutions for *prfA*. Conversely, the most diverse gene was *actA*, which presented the highest number of alleles ($n = 20$) and showed the highest nucleotide diversity. Grouping by serotype had a significantly lower π value ($P < 0.0001$) compared to isolation source or geographical origin, suggesting a distinct and well-defined unit compared to other groupings. Among all tested genes, only *hly* and *mpl* were those with lower nucleotide diversity in 1/2a serotype than 4b serotype, reflecting a high within-1/2a serotype divergence compared to 4b serotype. Geographical divergence was noted with respect to the *hly* gene, where serotype 4b Irish strains were distinct from Greek and Australian strains. Australian strains showed less diversity in *plcB* and *mpl* relative to Irish or Greek strains. Notable differences regarding sequence mutations were identified between food-associated and clinical isolates in *prfA, actA,* and *plcB* sequences. Overall, these results indicate that virulence genes follow different evolutionary pathways, which are affected by a strain's origin and serotype and may influence virulence and/or epidemiological dominance of certain subgroups.

Keywords: *Listeria monocytogenes*, virulence, gene sequencing, diversity, *prfA*, *hly*, *actA*

OPEN ACCESS

Edited by:
Maria Schirone,
Università di Teramo, Italy

Reviewed by:
Arun K. Bhunia,
Purdue University, United States
Valentina Bernini,
Università degli Studi di Parma, Italy
Hongxia Wang,
University of Alabama at Birmingham, United States

***Correspondence:**
Kieran Jordan
kieran.jordan@teagasc.ie

†Present Address:
Marion Dalmasso,
Normandie Univ, UNICAEN, ABTE, Caen, France

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 September 2017*
Accepted: *08 May 2018*
Published: *05 June 2018*

Citation:
Poimenidou SV, Dalmasso M, Papadimitriou K, Fox EM, Skandamis PN and Jordan K (2018) Virulence Gene Sequencing Highlights Similarities and Differences in Sequences in Listeria monocytogenes Serotype 1/2a and 4b Strains of Clinical and Food Origin From 3 Different Geographic Locations. Front. Microbiol. 9:1103. doi: 10.3389/fmicb.2018.01103

INTRODUCTION

Listeria monocytogenes is a facultative intracellular foodborne pathogen, with pregnant women and neonates, immunocompromised individuals, and the elderly representing high risk groups for infection (Farber and Peterkin, 1991; EFSA ECDC, 2015). It is equally capable of both a saprophytic lifecycle in the environment and human infection causing the severe disease of listeriosis (Gray et al., 2006). Due to its wide variety of reservoirs (Farber and Peterkin, 1991; Lianou and Sofos, 2007), its ability to colonize abiotic surfaces (Møretrø and Langsrud, 2004; Poimenidou et al., 2016b) and to withstand environmental stresses (Hill et al., 2002; Poimenidou et al., 2016a), it is frequently implicated in food processing plant contamination, where it is able to persist for several months or years (Halberg Larsen et al., 2014), thus raising the risk to food safety. After transmission via contaminated food to humans, *L. monocytogenes* cells may cause illnesses such as gastroenteritis or invasive listeriosis following intestinal translocation. It may then be carried by blood or lymph fluid and reach the mesenteric lymph nodes, spleen and/or the liver, leading to subclinical pyogranulomatous hepatitis, meningoencephalitis, septicemia, placentitis, abortion, or neonatal septicemia (Vázquez-Boland et al., 2001b). Within the host, *L. monocytogenes* parasitizes macrophages and invades non-phagocytic cells, utilizing its virulence factors to mediate cell-to-cell spread (de las Heras et al., 2011).

The virulence potential of *L. monocytogenes* relies on several molecular determinants (Camejo et al., 2011), which play key roles at different stages of the infection process. Among the early stages of the infection process, genes including the internalins (*inlA*, *inlB*, *inlF*, *inlJ*) play key roles in adhesion and invasion. Intracellular pathogenesis heavily relies on factors transcribed by genes located in the major *prfA*-regulated virulence gene cluster (*p*VGC), also referred to as *Listeria* pathogenicity island 1 or LIPI-1 (Vázquez-Boland et al., 2001a; Ward et al., 2004). *p*VGC genes facilitate the intracellular growth and spread of the bacterium in the host and consist of a monocistron *hly*, which occupies the central position in the locus, a lecithinase operon comprising *mpl, actA,* and *plcB* genes, which is located downstream from *hly* and transcribed in the same orientation, and the *plcA-prfA* operon located upstream from *hly* and transcribed in the reverse direction (Portnoy et al., 1992; Vázquez-Boland et al., 2001b; Roberts and Wiedmann, 2003). The *prfA* gene encodes the PrfA protein, which is required for the transcription of *p*VGC, and *prfA* itself. Listeriolysin O (LLO) encoded by the *hly* gene is a pore-forming toxin that mediates lysis of bacterium-containing phagocytic vacuole, resulting in the release of bacterial cells into the host cytoplasm. *plcA* and *plcB* encode the phosphatidylinositol-specific phospholipase C (PI-PLC) and zinc-dependent broad-spectrum phospholipase C (PC-PLC), respectively, which synergistically with LLO mediate the escape of the pathogen from the single- and double-membrane-bound vacuoles. After lysis, the intracellular motility and cell-to-cell spread are mediated by the surface protein actin A (ActA) through actin polymerization, for which additional functions (i.e., role in invasion, aggregation, colonization and persistence in the gut lumen) have been reported (Suárez et al., 2001; Travier et al., 2013). *mpl* encodes a zinc metalloproteinase needed to activate PC-PLC in order to initiate a new infection cycle.

Listeria monocytogenes is a genetically diverse species; its isolates form a structured population and are differentiated into four distinct lineages and 13 serotypes (Orsi et al., 2011), with the majority of isolates clustering into lineage I (serotypes 1/2b, 3b, 3c, 4b) and lineage II (serotypes 1/2a, 1/2c, 3a). Serotypes 4b and 1/2a are overrepresented among isolates associated with human listeriosis cases and food environment isolates, respectively (McLauchlin, 1990, 2004; Schuchat et al., 1991; Norton et al., 2001; Jacquet et al., 2002; Mereghetti et al., 2002; Gray et al., 2004; Lukinmaa et al., 2004; Gilbreth et al., 2005; Kiss et al., 2006; Swaminathan and Gerner-Smidt, 2007; Ebner et al., 2015). Additionally, various *L. monocytogenes* strains have presented diversity in virulence potential (Brosch et al., 1993; Chakraborty et al., 1994; Jaradat and Bhunia, 2003; Roche et al., 2003; Neves et al., 2008). Defective forms of virulence determinants were identified as the source of such virulence attenuation (Olier et al., 2002, 2003; Roberts et al., 2005; Roche et al., 2005; Témoin et al., 2008; Van Stelten et al., 2011).

The reasons that 1/2a serotype strains predominate among food environment isolates and 4b serotype strains among human listeriosis isolates are under investigation, with no clear inference made so far (Jaradat et al., 2002; Larsen et al., 2002; Gray et al., 2004; Jensen et al., 2007, 2008; Neves et al., 2008; Houhoula et al., 2012). On the other hand, there are indications of selective pressure for maintenance or specific adaptation of the *p*VGC genes in particular environments (Roberts et al., 2005; Orsi et al., 2008; Travier et al., 2013). Comparative genotyping could contribute to identifying unique genetic determinants toward the intraspecific pathogenic characteristics of *L. monocytogenes* isolates. Considering the above, the objective of this study was to examine the nucleotide diversity of the *p*VGC genes of *L. monocytogenes* strains isolated from human clinical cases and food or food-related environments, which belonged to the serotypes 4b and 1/2a and originated from three distinct geographical locations (i.e., Australia, Greece, and Ireland). Studying these variations may provide valuable information toward understanding the significance of virulence gene variation and the influence of environmental pressures acting on the genes.

MATERIALS AND METHODS

Bacterial Strains

A total of 36 *Listeria monocytogenes* strains (**Table 1**) were analyzed in this study. The strains represented three distinct geographically dispersed regions (Australia, Greece, Ireland), two serotypes (serotype 4b and 1/2a) and two isolation sources (clinical and food–related isolates). The clinical strains were kindly provided by Dr. Josheph Papaparaskevas (Houhoula et al., 2012) and Prof. Martin Cormican (University College Hospital, Galway, Ireland). The food-associated isolates were obtained from food and the food-processing environment. The strains were serotyped using a combination of antisera specific to the *L. monocytogenes* somatic O-antigen (Denka Seiken Co., Ltd., Tokyo, Japan), in tandem with a PCR-based serovar

TABLE 1 | Origins and characteristics of 36 *L. monocytogenes* strains used in the study.

Country	Isolate	Origin	Date	Serotype
Ireland	IR_227	Dairy processing environment	2008	1/2a
Ireland	IR_728	Dairy processing environment	2012	1/2a
Ireland	IR_872	Dairy processing environment	2013	1/2a
Ireland	IR_873	Clinical isolate	2013	1/2a
Ireland	IR_874	Clinical isolate	2013	1/2a
Ireland	IR_875	Clinical isolate	2013	1/2a
Ireland	IR_250	Dairy processing environment	2008	4b
Ireland	IR_338	Dairy processing environment	2008	4b
Ireland	IR_355	Dairy processing environment	2008	4b
Ireland	IR_876	Clinical isolate	2013	4b
Ireland	IR_877	Clinical isolate	2013	4b
Ireland	IR_878	Clinical isolate	2013	4b
Greece	GR_PL11	Chicken	2007	1/2a
Greece	GR_PL18	Chicken	2007	1/2a
Greece	GR_PL37	Clinical isolate	2009	1/2a
Greece	GR_PL38	Clinical isolate	2009	1/2a
Greece	GR_PL44	Clinical isolate	2013	1/2a
Greece	GR_PL50	Food isolate	2013	1/2a
Greece	GR_PL4	Dairy farm environment	2007	4b
Greece	GR_PL13	Chicken	2007	4b
Greece	GR_PL32	Clinical isolate	2009	4b
Greece	GR_PL41	Clinical isolate	2009	4b
Greece	GR_PL46	Clinical isolate	2013	4b
Greece	GR_FL78	Meat	2012	4b
Australia	AU_2884	Seafood	2009	1/2a
Australia	AU_2919	Meat processing environment	2013	1/2a
Australia	AU_2942	Dairy food	2009	1/2a
Australia	AU_2994	Vegetable	2011	1/2a
Australia	AU_2998	Meat	2011	1/2a
Australia	AU_Lm14-002	Dairy food	2014	1/2a
Australia	AU_2473	Dairy food	1998	4b
Australia	AU_2544	Clinical isolate	1994	4b
Australia	AU_2727	Meat	1988	4b
Australia	AU_2948	Dairy food	2010	4b
Australia	AU_2993	Dairy processing environment	2009	4b
Australia	AU_2995	Dairy processing environment	2009	4b

determination assay (Doumith et al., 2004), as described by Fox et al. (2009). Bacterial strains were stored at −80°C in Tryptic Soy broth (TSB) containing 20% glycerol and were cultured in TSB supplemented with 0.6% yeast extract (YE) at 37°C overnight, prior to pulsed-field gel electrophoresis (PFGE) and DNA extraction.

PFGE of *L. monocytogenes* Isolates

PFGE was carried out using the International Standard PulseNet protocol (Pulsenet USA, 2009). Two restriction enzymes, *Asc*I and *Apa*I, were used and band patterns were analyzed using Bionumerics version 5.10 software (Applied Maths, Belgium), as previously described (Fox et al., 2012). Briefly, band matching was performed using the DICE coefficient, with both optimization and tolerance settings of 1%. Dendrograms were created using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Strains were considered to be indistinguishable when their pulsotypes displayed 100% similarity on the dendrogram and after confirmation by visual examination of the bands. To help support population diversity, all isolates were confirmed as having a unique pulsotype relative to any other isolate included in this study.

DNA Extraction

Following overnight culture of each strain, DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, UK) for strains from both Greece and Australia, or the QIAmp DNA mini kits (Qiagen) for strains from Ireland. A cell lysis step preceded DNA extraction and consisted in incubation of the cells in lysis buffer (20 mM TrisHCl, pH 8; 2 mM EDTA, pH 8; 1.2% Triton® −100; 20 mg/ml lysozyme) for 1 h at 37°C. DNA was stored at −20°C before use.

Nucleotide Sequencing of *actA, hly, mpl, plcA, plcB, prfA*

PCR amplification of the targeted genes was performed using genomic DNA extracted as described above. Primer design was based on available sequences of the targeted genes in public databases using Primer3Plus software version 2.3.5 (Untergasser et al., 2012). The primers and PCR conditions, all including 35 cycles, are described in **Table 2**. Phusion® High-Fidelity DNA polymerase (New England Biolabs® Inc, USA) and AccuTaq™ LA DNA polymerase (Sigma, USA) were used for PCR reactions on 50 ng DNA for strains from Greece and Ireland, respectively. Following amplification, PCR products were purified using MinElute Gel Extraction kit (Qiagen). DNA sequencing was performed using external forward and reverse PCR primers at CEMIA SA (Larisa, Greece) and Source Biosciences (Dublin, Ireland) for Greece and Ireland PCR products, respectively. In the case of Australian isolates, sequences were extracted *in silico* from draft genomes using the same primer sets (**Table 2**) with Geneious® software version 9 (Kearse et al., 2012). DNA sequencing chromatograms were saved as ABI files for analysis.

Data Analysis

Sequence assembly was performed using SeqMan Pro application in Lasergene® Genomics suite (DNASTAR, USA). Geneious® software version 9 (Kearse et al., 2012) was used to construct translation alignments for each gene separately and the *p*VGC (a concatenated sequence comprising the *prfA, plcA, hly, mpl, actA, plcB* sequences).

Descriptive Analysis

Number of polymorphic sites (S), nucleotide diversity (π; average pairwise nucleotide differences per site), number of segregating sites (θ), and Tajima's *D* for neutrality were calculated using DnaSP software version 5 (Librado and Rozas, 2009). Number of polymorphic sites, number of substitutions, number of synonymous substitutions (SS) and non-synonymous substitutions (NSS), and the G + C content

TABLE 2 | Primer sequences and PCR conditions for each virulence gene target.

Gene	Strains ID	Primer sequence (5′-3′)	Hybridization temperature (°C)	Elongation time (min)
actA	All strains	Forward[a], GTATTAGCGTATCACGAGGA	60	1
		Reverse[a], CAAGCACATACCTAGAACCA		
		Forward[b], AAGMGTCAGTTRYGGATRCT	57	1
		Reverse[b], CCCGCATTTCTTGAGTGTTT		
hly	227, 728, 872, 873, 874, 875, 876	Forward[a], GGCCCCCTCCTTTGATTAGT	60	2
		Reverse[a], GCCTCTTTCTACATTCTTCACAAA		
	355	Forward[a], TATGCTTTTCCGCCTAATGG	57	1
		Reverse[a], CGTGTGTGTTAAGCGGTTT		
	250, 338, 877, 878	Forward[a], AAAAGAGAGGGGTGGCAAAC	60	2
		Reverse[a], GCCTCTTTCTACATTCTTCACAAA		
	All strains	Forward[b], CCAGGTGCTCTCGTRAAAGC	57	1
		Reverse[b], RCCGTCGATGATTTGAACTT		
mpl	All strains	Forward[a], GCCACCTATAGTTTCTACTGCAAA	57	1
		Reverse[a], TGRAGAATTAAKTTTTCTCTAACATTT		
	All strains	Forward[b], ATACGCTCGCGCTAAGTTCT	60	1
		Reverse[b], GCTTCTTATTCGCCCATCTCG		
plcA	All strains	Forward[a], ATCAAAGGAGGGGGCCATT	60	1
		Reverse[a], CCGAGGTTGCTCGGAGATATAC		
plcB	All strains	Forward[a], ATTGGCGTGTTCTCTTTAGG[c]	57	1
		Reverse[a], CAAAGAAAAAGATTAACCTCCCTTT		
prfA	All strains	Forward[a], TTCAGGTCCKGCTATGAAAC	57	1
		Reverse[a], AACTCCATCGCTCTTCCAGA		

[a] *External primers located in upstream and downstream regions surrounding the targeted gene.* [b] *Internal primers.* [c] *Roche et al., 2005.*

(%) were defined using Geneious software version 9 (Kearse et al., 2012). The d_N/d_S ratios or ω [number of non-synonymous substitutions/nonsynonymous sites (d_N) to the number of synonymous substitutions/synonymous sites (d_S)] were calculated using the Datamonkey online platform (Kosakovsky Pond and Frost, 2005). 3D scatterplots were created using 'Excel 3D Scatter Plot' version 2.1 (available at: http://www.doka.ch/Excel3Dscatterplot.htm).

Phylogenetic Analysis

Phylogenetic trees were generated using the NeighborNet algorithm (Bryant and Moulton, 2004) as adopted in SplitsTree software (Huson, 1998).

Statistical Analysis

Descriptive analysis data calculated for individual genes were used in order to compare π, θ, and ω parameters for the *p*VGC with regard to different serotypes, geographical origin or isolation source using Student's *t* test (JMP version 9.0); significance level was set at $\alpha = 0.05$.

RESULTS

Among the 36 strain sequences analyzed, representing distinct PFGE profiles (Supplementary Material), 26 unique alleles were identified for *p*VGC (**Table 3**, Supplementary Dataset S1). Twenty-three isolates harbored a full length cluster of 7,503 nucleotides; 12 isolates had a 105 bp deletion in their *actA* sequence and as such had a 7,398 bp *p*VGC; one isolate had a single nucleotide deletion in its *actA* gene sequence and thus a 7,502 bp *p*VGC.

The *p*VGC contained 439 polymorphic sites, with 281 synonymous and 182 non-synonymous substitutions. The G + C% content was 37.2%. The overall nucleotide diversity was $\pi = 0.02427$ and $\theta = 0.01601$. Although π and θ values for serotype 1/2a strains were higher than for 4b strains, the difference was not significant ($P > 0.05$). No significant π difference was observed among strains of different geographical origin, or between food environment and clinical origin. Comparing groupings by serotype, geographical origin or isolation source, grouping by serotype had a significantly lower π value ($P < 0.0001$). Serotype groups also exhibited distinct clustering on the 3D-scatter plot (**Figure 1A**), showing divergence from the other groupings. Divergence between the two serotypes in d_N/d_S ratio was also observed, suggesting different selective pressure acting on the two serotypes, with higher values among the serotype 1/2a group. The *p*VGC phylogenetic tree (**Figure 2**) showed two major distinct clusters representing the two serotypes, 1/2a and 4b. No specific pattern of origin-based classification was observed, with strains isolated in different countries or from different sources (i.e., food-associated or clinical) sharing an identical nucleotide sequence. In each serotype group, stains were clustered in short distances to each other, with only strain GR_PL50 distant to the others.

TABLE 3 | Sequence diversity analysis of virulence genes sequences.

Gene (length in nt)	Strains	Polymorphic sites	Substitutions	Alleles	G + C content (%)	SynSubs[a]	Non-SynSubs[b]	π/site[c]	θ/site[d]	Tajima's *D* value[e]	d_N/d_S
*p*VGC (7503)	36	439	463	26	37.2	281	182	0.02427	0.01601	2.05558**	0.188618
Serotype 1/2a	18	243	258	14	37.2	124	134	0.00913	0.01059	−0.62147	0.230547
Serotype 4b	18	75	75	12	37.2	24	51	0.00411	0.00345	0.89916	0.169137
Food associated	23	433	456	19	37.2	276	180	0.025	0.01736	1.85522*	0.181073
Clinical	13	351	359	11	37.2	223	136	0.02393	0.0174	1.81904*	0.168402
Australian	12	405	424	10	37.2	258	166	0.02593	0.01978	1.55772	0.183604
Greek	12	373	374	8	37.2	237	137	0.02513	0.01971	1.5115	0.175085
Irish	12	384	395	11	37.2	245	150	0.0243	0.01823	1.61429	0.173244
prfA (711)	36	24	24	8	33.4	20	4	0.01551	0.01296	1.03014	0.0842314
Serotype 1/2a	18	6	6	5	33.4	3	3	0.00336	0.00403	−1.14554	0.26142
Serotype 4b	18	2	2	3	33.2	1	1	0.00187	0.00187	NA[f]	0.259992
Food associated	23	24	24	8	33.2	20	4	0.01551	0.01296	1.03014	0.0842313
Clinical	13	19	19	4	33.3	19	0	0.01727	0.01451	1.94585	$5.00E-09$
Australian	12	21	22	5	33.4	19	3	0.01653	0.01412	1.26346	0.0464163
Greek	12	20	20	5	33.3	19	1	0.01625	0.01345	1.54012	0.0312951
Irish	12	20	20	5	33.4	19	1	0.01597	0.01345	1.38611	0.0314107
plcA (951)	36	57	58	13	35.8	41	17	0.02215	0.01925	0.67574	0.166368
Serotype 1/2a	18	38	39	10	35.7	26	13	0.01624	0.01408	0.74193	0.193846
Serotype 4b	18	6	6	3	36.1	5	1	0.00419	0.00419	NA	0.0560234
Food associated	23	56	57	11	35.8	41	16	0.02314	0.02004	0.73176	0.152218
Clinical	13	50	50	7	35.8	36	14	0.02296	0.02139	0.42397	0.16682
Australian	12	51	52	8	35.8	38	14	0.02242	0.02062	0.47162	0.141993
Greek	12	43	43	5	35.7	32	11	0.02432	0.02164	0.93385	0.123106
Irish	12	54	55	8	35.9	39	16	0.02538	0.02183	0.87667	0.153798
hly (1587)	36	57	59	19	36	53	6	0.01409	0.01044	1.43362	0.0660299
Serotype1/2a	18	22	23	11	36	19	4	0.00453	0.00472	−0.18884	0.106618
Serotype 4b	18	31	31	8	36	26	5	0.00984	0.00752	1.63658	0.0630305
Food associated	23	55	57	16	36	51	6	0.01388	0.01061	1.30974	0.0681005
Clinical	13	51	52	9	36	48	4	0.01544	0.0118	1.57407	0.0634322
Australian	12	53	54	8	36	49	5	0.01388	0.0131	0.32216	0.0474897
Greek	12	51	51	6	36	46	5	0.01694	0.01405	1.3193	0.0498417
Irish	12	48	48	9	36	44	4	0.01328	0.01111	0.99492	0.0458643
mpl (1497)	36	86	87	14	38.1	58	29	0.02413	0.01873	1.16267	0.133937
Serotype1/2a	18	12	12	10	38.2	9	3	0.0026	0.00283	−0.37581	0.0731896
Serotype 4b	18	9	9	4	37.9	6	3	0.00345	0.00328	0.52223	0.118136
Food associated	23	86	87	11	38.1	58	29	0.02633	0.01984	1.56388	0.136483
Clinical	13	79	79	7	38.1	52	27	0.02418	0.02154	0.71271	0.144224
Australian	12	81	82	8	37.1	54	28	0.02221	0.02113	0.27887	0.141179
Greek	12	82	82	6	38.1	54	28	0.03059	0.02399	1.77607	0.148356
Irish	12	78	78	8	38.1	52	26	0.02667	0.0201	1.77542*	0.136472
actA (1890)	36	174	190	20	40.1	82	108	0.03782	0.029	1.26319	0.288458

(Continued)

TABLE 3 | Continued

Gene (length in nt)	Strains	Polymorphic sites	Substitutions	Alleles	G + C content (%)	SynSubs[a]	Non-SynSubs[b]	π/site[c]	θ/site[d]	Tajima's *D* value[e]	d_N/d_S
Serotype1/2a	18	86	93	13	38.8	44	49	0.01819	0.01594	0.64095	0.276396
Serotype 4b	18	24	25	7	40.2	13	12	0.0055	0.00572	−0.21918	0.248332
Food associated	23	169	184	16	40.1	81	103	0.03939	0.03005	1.35214	0.280161
Clinical	13	140	152	8	40.1	61	91	0.03874	0.03549	0.50002	0.320893
Australian	12	154	167	10	40.2	72	95	0.04118	0.03228	1.37403	0.305107
Greek	12	135	142	7	40.1	61	81	0.03727	0.03156	1.06034	0.284034
Irish	12	161	175	8	40.1	78	97	0.04197	0.03614	0.88101	0.288721
plcB (870)	36	45	46	12	36	26	20	0.02254	0.01751	1.31427	0.189206
Serotype1/2a	18	8	8	8	35.8	4	4	0.00271	0.00355	−1.14142	0.246995
Serotype 4b	18	4	4	4	36.3	2	2	0.0023	0.00251	−0.78012	0.251206
Food associated	23	45	46	11	35.9	26	20	0.02082	0.01805	0.72105	0.188388
Clinical	13	41	42	6	36	25	17	0.02713	0.02114	1.80741*	0.16398
Australian	12	42	43	8	35.9	24	19	0.02011	0.01906	0.29607	0.189685
Greek	12	43	44	6	36.1	26	18	0.02797	0.02215	1.67957	0.168418
Irish	12	40	41	6	36.1	24	17	0.02674	0.02064	1.88777	0.169274

*[a]Number of synonymous substitutions. [b]Number of non-synonymous substitutions. [c]Average pairwise nucleotide difference per site. [d]Index of the number of segregating sites (mutation rate). [e]Tajima's D-values significantly different from 0 are indicated with * ($0.05 < P < 0.1$) or with ** ($P < 0.05$). [f]The value is not available, as it could be not evaluated due to the low number of alleles (4 or more sequences needed).*

Eight haplotypes among the 36 strains were recovered for the *prfA* gene (5 for 1/2a serotype and 3 for 4b serotype). This gene possessed the lowest number of polymorphic sites ($n = 24$) with the lowest number of substitutions ($n = 24$) and non-synonymous substitutions ($n = 4$) compared to all fragments tested (**Table 3**). The overall nucleotide diversity was $\pi = 0.01551$ and $\theta = 0.01296$. Groups containing strains of different geographical origin were clustered closely to each other (**Figure 1B**), while food isolates were distinct from the clinical isolates with respect to π values. Divergence in d_N/d_S, π and θ parameters resulted in distinct clustering of serotype groups compared to other groupings. The phylogenetic tree of *prfA* gene (**Figure 3**) showed the lowest degree of divergence among all tested genes, with longer branch lengths observed for 1/2a serotype isolates than for 4b isolates, which is in accordance with the higher nucleotide diversity within 1/2a serotype than 4b serotype (**Table 3**). Among the 19 substitutions observed for clinical isolates, none of them were non-synonymous.

The nucleotide sequence of the *plcA* gene (13 haplotypes; $\pi = 0.02215$) was diversified into 10 unique alleles of 1/2a serotype strains ($\pi = 0.01624$) and 3 alleles of 4b serotype strains ($\pi = 0.0419$). Serotype 4b strains had the lowest number of substitutions ($n = 6$) compared to the other subgroups ($n = 39$–57), which resulted in the lowest nucleotide diversity. Serotype 1/2a strains differed from the other groups in d_N/d_S ratio values and serotype 4b strains in θ values, resulting in distinct clustering on the 3D-scatter plot (**Figure 1C**). The phylogenetic tree of the *plcA* gene (**Figure 4**) showed that isolates of the 1/2a serotype were highly divergent with more distant branches compared to 4b serotype strains. Unique sequence types in the group of 1/2a serotype belonged to Australian or Irish origin strains.

Analysis of the *hly gene* showed 19 haplotypes among the 36 strains with overall nucleotide diversity $\pi = 0.01409$ and $\theta = 0.01044$. Higher diversity was observed among 1/2a serotype than 4b serotype strains (11 and 8 unique alleles, respectively). Groups of different geographical origin or groups of different isolation source (i.e., food environment or clinical) were clustered closely to each other (**Figure 1D**), in contrast to different serotypes, where the two groups (i.e., 1/2a and 4b serotypes) clustered apart along the d_N/d_S ratio axis showing a diverse selective pressure acting on the gene within each serotype. As illustrated in **Figure 5**, a high divergence in *hly* gene sequences among strains of 4b serotype was observed; two subpopulations were identified, one of which only included Irish isolates. The second subpopulation contained two sets of strains with shared sequences between Australian and Greek strains, and three unique alleles (i.e., one Greek strain and two Irish).

The *mpl* gene was represented by 14 unique alleles, 10 for 1/2a serotype and 4 for 4b serotype, with $\pi = 0.02413$ and $\theta = 0.01873$. Grouping according to serotypes resulted in distinct clusters compared to the other groupings (**Figure 1E**), due to lower π values, while additionally the two serotype groups (i.e., 1/2a and 4b) differed in their d_N/d_S ratio demonstrating diverse selective pressure acting on the strains of each serotype within this gene. The phylogenetic tree for the *mpl* gene (**Figure 6**)

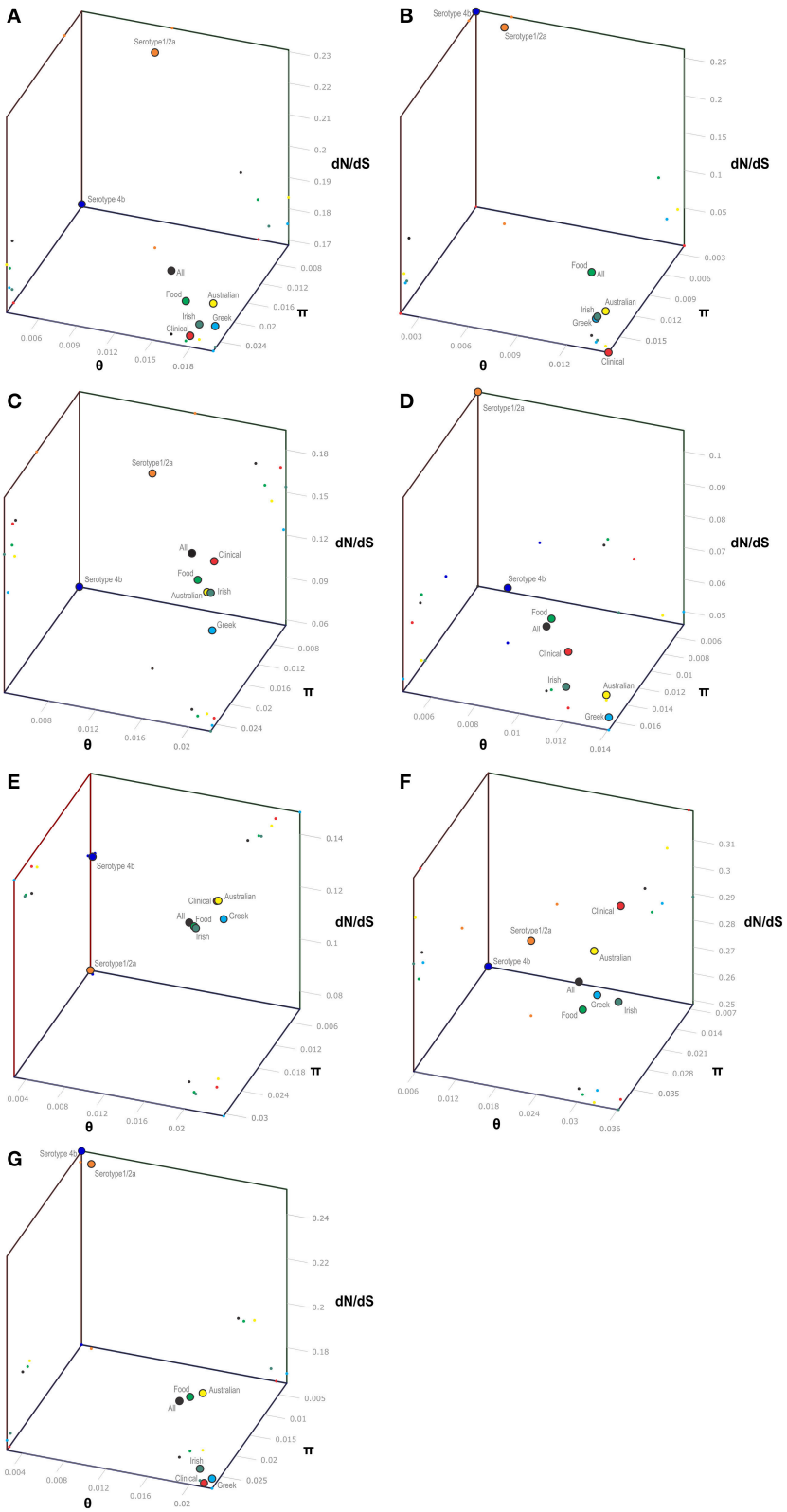


FIGURE 1 | 3-D scatter-plot illustration of nucleotide diversity parameters (π, θ) and d_N/d_S ratio (ω) for the *p*VGC **(A)**, *prfA* **(B)**, *plcA* **(C)**, *hly* **(D)**, *mpl* **(E)**, *actA* **(F)**, and *plcB* **(G)** genes. Within each gene, colored dots represent the *L. monocytogenes* population grouping based on serotype (4b and 1/2a), geographical origin (Australian, Greek, and Irish strains), source of isolation (clinical or food environment), and as a whole (All strains).

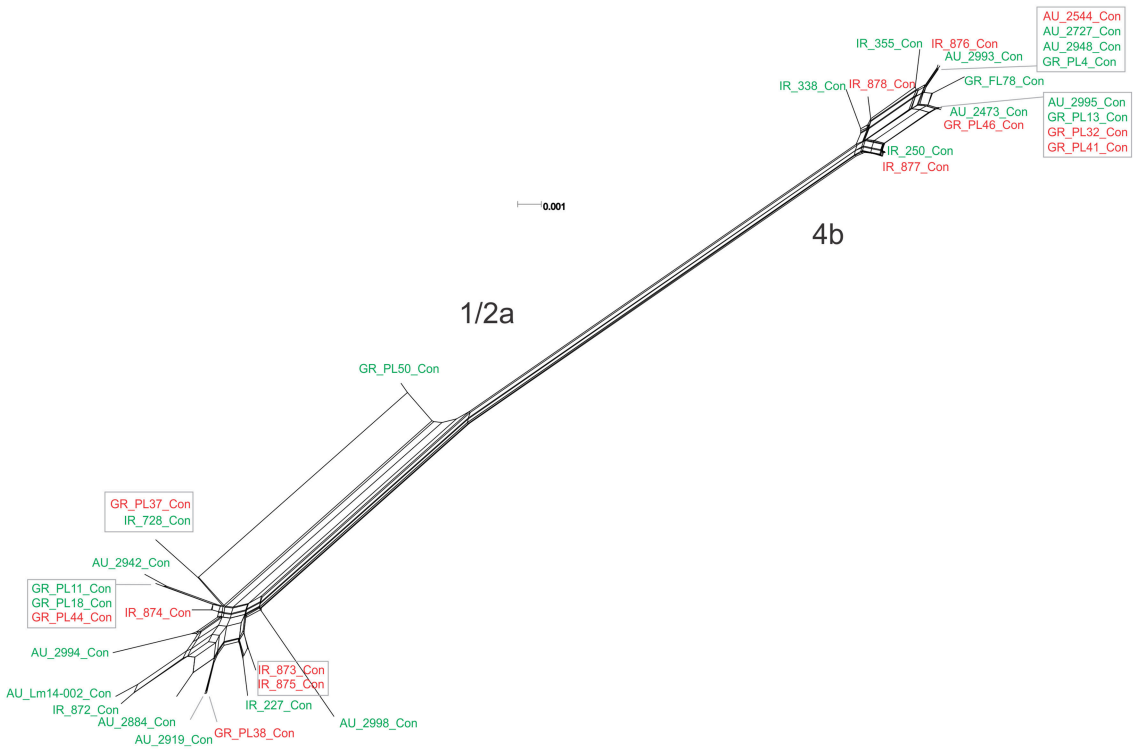


FIGURE 2 | Phylogenetic network applied to virulence gene cluster *prfA* (*p*VGC; concatenated genes *prfA, plcA, hly, mpl, actA,* and *plcB*) using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

showed a similar clustering of the strains between the two serotypes with respect to branch lengths, and higher divergence within the 1/2a serotype compared to 4b serotype, in terms of unique alleles.

The *actA* gene was represented by 20 unique alleles, the highest number among any of the *p*VGC genes, with overall nucleotide diversity $\pi = 0.03782$ and $\theta = 0.029$. Groups containing strains of various origins or serotypes were highly variant, as illustrated in **Figure 1F**, confirming the diversity of this particular gene. Strains of serotype 1/2a were more diverse ($\pi = 0.01819, \theta = 0.01594$) than serotype 4b strains ($\pi = 0.0055$, $\theta = 0.00572$). This was also evident from the phylogenetic tree (**Figure 7**), where 13 different nucleotide sequences were found among 18 isolates of 1/2a serotype, with longer branch lengths compared 4b serotype strains. Food isolates had the highest number of non-synonymous substitutions ($n = 103$) among all subgroups within this gene and clinical isolates the lowest ($n = 12$). A large variation between the d_N/d_S ratio values was observed for food and clinical isolates, suggesting a different selective pressure acting on these two groups. Divergence in d_N/d_S was also observed between Australian and Greek or Irish isolates. Twelve isolates, representing 5 unique alleles, had a 105-bp deletion in their sequences; 8 of these isolates were of food environment origin and 4 of clinical origin. The isolate (AU_Lm14-002) that had a single nucleotide deletion was of food origin.

For the *plcB* gene, 12 haplotypes were observed among the 36 strains, with nucleotide diversity $\pi = 0.02254$ and $\theta = 0.01751$. Serotype 1/2a strains were more diverse than 4b strains, represented by higher numbers of unique alleles (8 and 4, respectively), and higher π and θ values. Food-related strains differed from clinical strains, and Australian strains clustered apart from Greek and Irish strains (**Figure 1G**), showing lower nucleotide diversity and thus, a higher genetic uniformity within the former groups (i.e., food or Australian) compared to the latter (i.e., clinical, Greek, or Irish). In the phylogenetic tree (**Figure 8**), the short length of the branches indicated the small divergence level among strains within each serotype.

Comparing all genes, the most diverse gene was *actA* ($\pi = 0.03782$) and the most conserved *hly* ($\pi = 0.01409$) and *prfA* ($\pi = 0.01551$); the π value of *actA* was significantly higher compared to *hly* ($P = 0.0095$) or *prfA* ($P = 0.0088$). Additionally, for *p*VGC no significant difference in nucleotide diversity was observed between the two serotype groupings, the two isolation sources or the three geographical origin groups. Higher nucleotide diversity in serotype 4b vs. serotype 1/2a was only observed for *mpl* and *hly* genes. Regarding the selective pressure acting on the genes, the highest values of the d_N/d_S ratio were observed for *actA* and the lowest on *prfA* and *hly* genes ($P < 0.05$).

Tajima's *D*-test for neutrality (Tajima, 1989; Simonsen et al., 1995), which examines whether the occurring mutations are

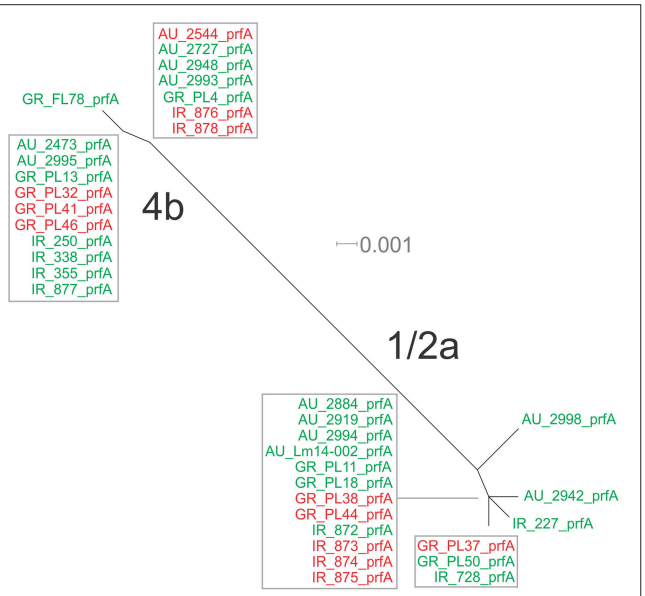


FIGURE 3 | Phylogenetic network applied to virulence gene *prfA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

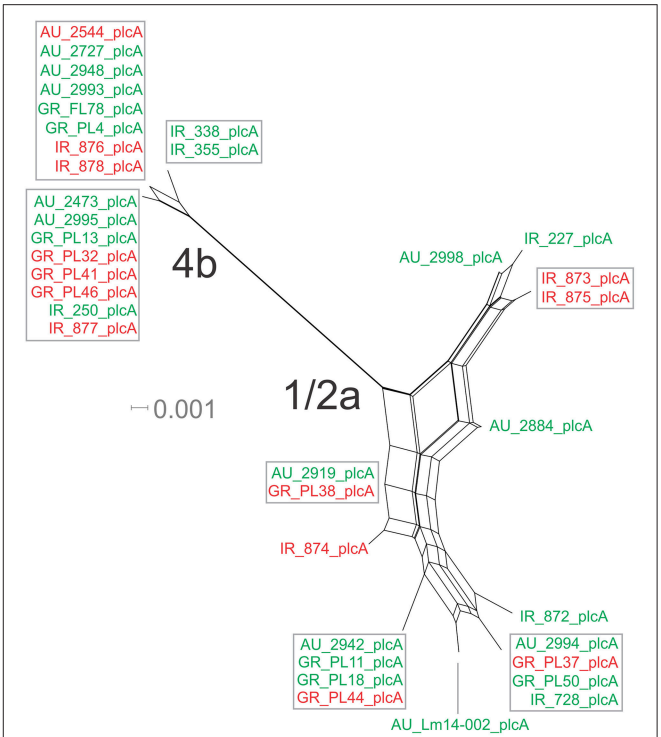


FIGURE 4 | Phylogenetic network applied to virulence gene *plcA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

a result of selection or random (neutral) evolution, showed a significantly positive value for the test for the *p*VGC (**Table 3**). This suggests that the gene evolution deviates significantly from the standard neutral model and is under balancing selection, decrease in population size or a subdivision of the population structure. High Tajima's *D*-values ($0.1 > P > 0.05$) were also observed for food and clinical isolates in the *p*VGC, for Irish isolates in the *mpl* gene and for clinical isolates in the *plcB* gene. Negative values were observed for serotype 1/2a strains in the *p*VGC, *prfA*, and *plcB* genes, and for 4b serotype in *plcB*; however, these were not statistically significant and therefore are unlikely to represent a population bottleneck, a selective sweep or purifying selection.

DISCUSSION

In the present study, the intraspecies variations in the *prfA* virulence gene cluster among 36 *L. monocytogenes* strains, with respect to different serotype (i.e., 1/2a and 4b), geographical origin (Australian, Greek, and Irish isolates), or isolation source (i.e., food environment or clinical isolates) was investigated. Consistent with previous classification studies (Ward et al., 2004; Orsi et al., 2008), within all six virulence genes analyzed and the *p*VGC, strains were divided into two major clusters, each representing one serotype, i.e., 4b and 1/2a serotype, which belong to lineage I and II, respectively. *L. monocytogenes* is a highly diverse species and lineages I and II are considered to be deeply separated evolutionary lineages (Nightingale et al., 2005). Significant association between lineage and the origin of the strains has been reported (Wiedmann et al., 1997), while additionally, molecular types of the strains were shown to be associated with specific food types (Gray et al., 2004). Strains of different lineages are also divergent in terms of their virulence potential. While higher virulence associated with the lineage I population relative to that of lineage II has been reported, (Wiedmann et al., 1997; Norton et al., 2001; Gray et al., 2004; Jensen et al., 2007), others found no statistical correlation between virulence of the strains and their serotypes (Conter et al., 2009). Therefore, molecular typing and a better understanding of virulence stratification among serotypes and lineages are essential in epidemiological surveys and risk estimation procedures. The analysis in this study also showed that 4b serotype strains exhibited lower diversity than the 1/2a strains. This is consistent with previous findings where lineage II strains were genetically more diverse compared to lineage I, based on molecular typing of seven genetic loci including four housekeeping genes, two virulence genes and stress response *sigB* gene (den Bakker et al., 2008), ribotyping and random multiprimer PCR analysis (Mereghetti et al., 2002), or analysis of the *prfA* virulence gene cluster (Orsi et al., 2008). In addition to these reports, it was shown here that ω values were similar between the serotype groups for *prfA* and *plcB*, while varied largely for the *p*VGC, *plcA*, *hly*, *mpl*, and *actA*, indicating a different selective pressure

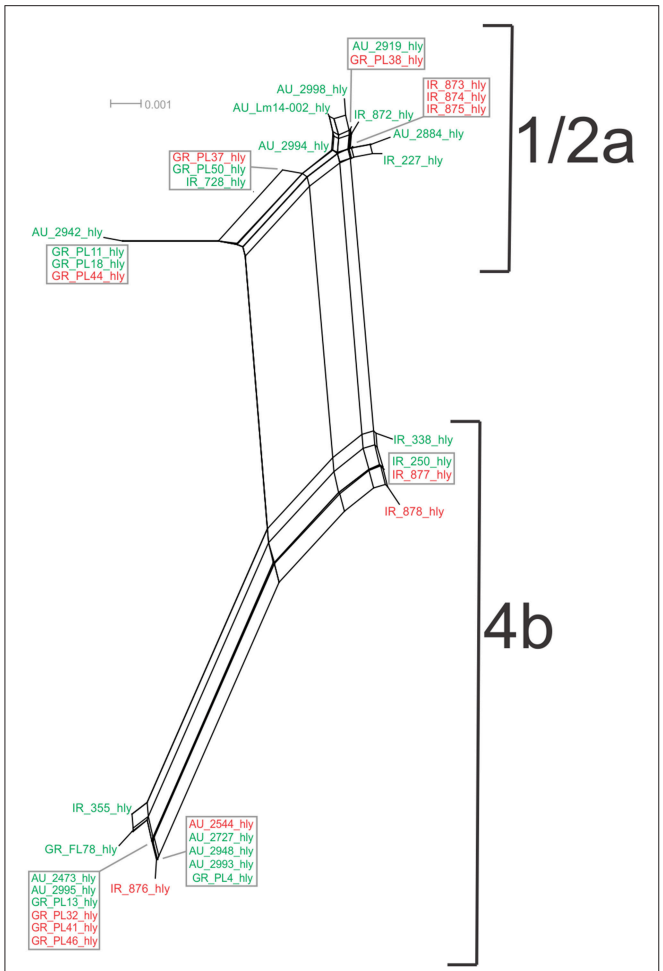


FIGURE 5 | Phylogenetic network applied to virulence gene *hly* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

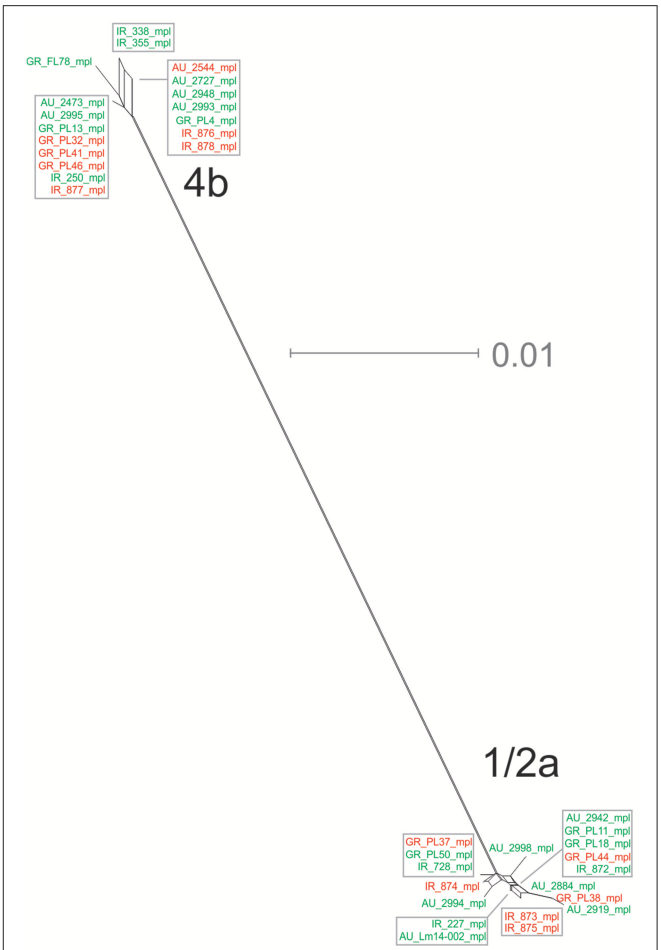


FIGURE 6 | Phylogenetic network applied to virulence gene *mpl* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

acting on these genes within each serotype. Furthermore, the opposite (i.e., negative vs. positive) Tajima's *D* values for the serotype groups within *p*VGC, *hly, mpl,* and *actA* suggest that these genes follow a different evolutionary pathway across serotypes.

Results of this study showed that among the six genes examined, only the *hly* gene of 4b serotype strains was partially correlated with geographical origin, with strains separating into two distinct subpopulations: one containing only Irish strains, the other containing Greek and Australian strains and two Irish strains. Since serotype 4b strains have been found as the etiological agent of the majority of epidemic or sporadic human listeriosis cases in many countries, including Ireland (Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007; Fox et al., 2012), and *hly* is a key gene for the virulence potential of *L. monocytogenes* (Gaillard et al., 1986; Roberts et al., 2005), the correlation between Irish strains and *hly* could imply a possible impact of geographical-specific diversification. Previous studies have shown no polymorphism in LLO protein among 150 strains of food and human origin, while slight changes in the *hly* gene did not imply alterations on LLO molecular weight (Jacquet et al., 2002). Furthermore, no significant differences in the LLO protein among different serotypes 4b and 1/2a were reported (Matar et al., 1992; Jacquet et al., 2002). Nonetheless, Gray et al. (2004) reported a significant correlation between *hly* allelic types and origin of the strains (i.e., food vs. human isolates); *hly* type 1 was significantly more common among human isolates and was associated with larger plaque forming, indicative of *in vitro* cytopathogenicity, compared with other *hly* types (Gray et al., 2004). Therefore, such correlation of origin and *hly* types might be important in epidemiological studies. Additionally, studies based on ribotype analysis showed no specific clustering among *L. monocytogenes* strains distributed across different geographical locations, and therefore no significant effect of geographical distribution on their genetic diversity (Gendel and

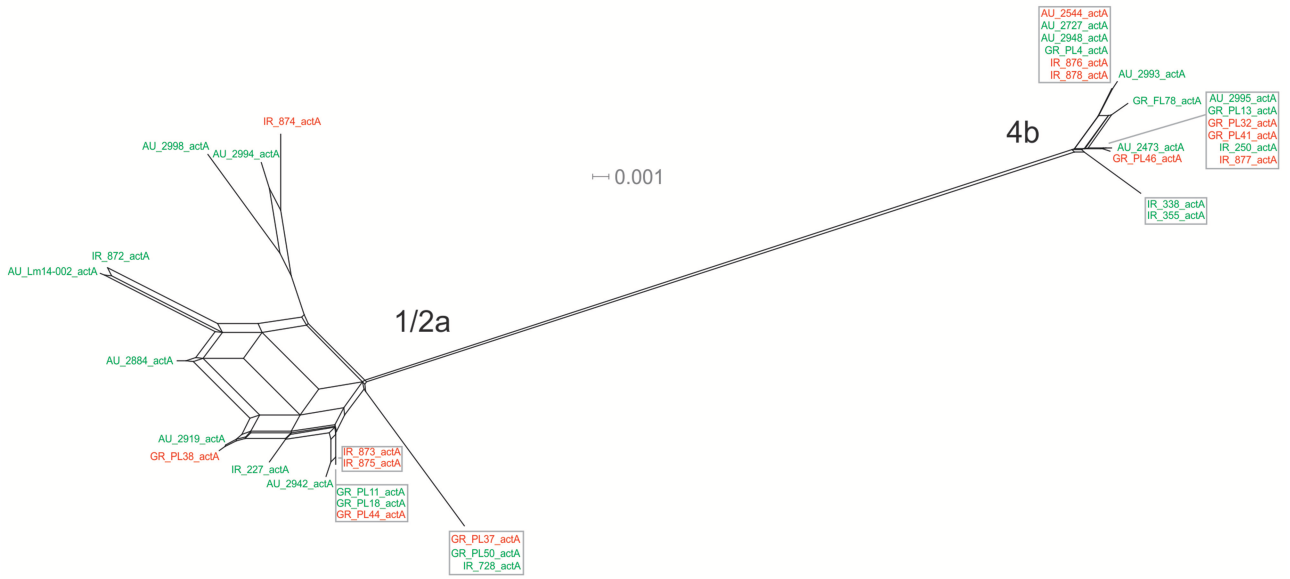


FIGURE 7 | Phylogenetic network applied to virulence gene *actA* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

Ulaszek, 2000; Jaradat et al., 2002; Mereghetti et al., 2002). The sequence diversity analysis in the current study showed that the groups of Greek, Australian and Irish isolates within the *p*VGC form distinct clusters based on parameters π, θ, and ω, which may underlie diverse evolutionary pathways for each group; this was also observed for all individual genes except the *prfA* gene. Origin-based pattern in nucleotide diversity was observed for Australian strains, which showed less diversity in *plcB* and *mpl* sequences relative to their Irish or Greek counterparts. The Tajima's *D*-values for Australian isolates were close to 0 contrary to Greek and Irish isolates with increased Tajima's *D*-values. This indicates a differentiation in the evolutionary pathway of Australian compared to Greek and Irish isolates within these genes.

Although serotype 4b strains predominate among human clinical isolates and serotype 1/2a strains among food isolates, gene-specific pattern between clinical isolates and 4b serotype strains or between food isolates and 1/2a serotype strains were not observed; food and clinical isolates could share alleles for all genes tested. However, descriptive analysis revealed that food and clinical isolates formed distinct clusters regarding their π and ω parameters for all the genes tested, with larger variations within *prfA*, *actA,* and *plcB* genes. This divergence might indicate that these genes were adapted differentially within each group, and this adaptation correlated with their prevalence in food or virulence phenotype, respectively. Previous studies investigating the correlation of isolation source and virulence of strains yielded differing conclusions. Some showed lower virulence potential for strains isolated from food environments compared to human clinical isolates (Norton et al., 2001; Jensen et al., 2008). Conversely, Larsen et al. (2002) reported no significant correlation between food or human origin of strains and invasiveness in the Caco-2 cell infection model, while all strains managed equally to multiply once inside the host cells when an *in vivo* test was used. Similarly others found no systematic differences in virulence between food or clinical isolates (Brosch et al., 1993; Gray et al., 2004; Neves et al., 2008; Bueno et al., 2010).

The results of this study also showed that the most conserved genes were *prfA* and *hly* and the most diverse was *actA*. Proteins PrfA, LLO and ActA are considered essential virulence factors (Gaillard et al., 1986; Nishibori et al., 1995; Vázquez-Boland et al., 2001a; Travier and Lecuit, 2014). It seems that there is a selective pressure on *L. monocytogenes* to maintain the former genes, while the increased diversity of *actA* compared to the other genes is consistent with previous findings (Orsi et al., 2008) and is attributed to increased recombination events occurring in *actA*, and to evolution by positive selection in both lineages I and II. Rapid PCR-based methods utilize species-specific genes to detect *L. monocytogenes* in food samples, aiming at preventing the unnecessary recalls of food products. It is of great importance to use target sequences of highly conserved regions rather than genes prone to genetic variability (Rodríguez-Lázaro et al., 2004). Virulence associated genes (e.g. *actA, hly, inlA, inlB, prfA, plcA, plcB*) and 16S/23S rRNA genes have been studied toward the development of such methods (Liu, 2006). The results indicated that due to the diversity seen, PCR assays based on *prfA* or *hly* as opposed to *actA* would be more reliable, covering isolates of different origin, serotype or isolation source.

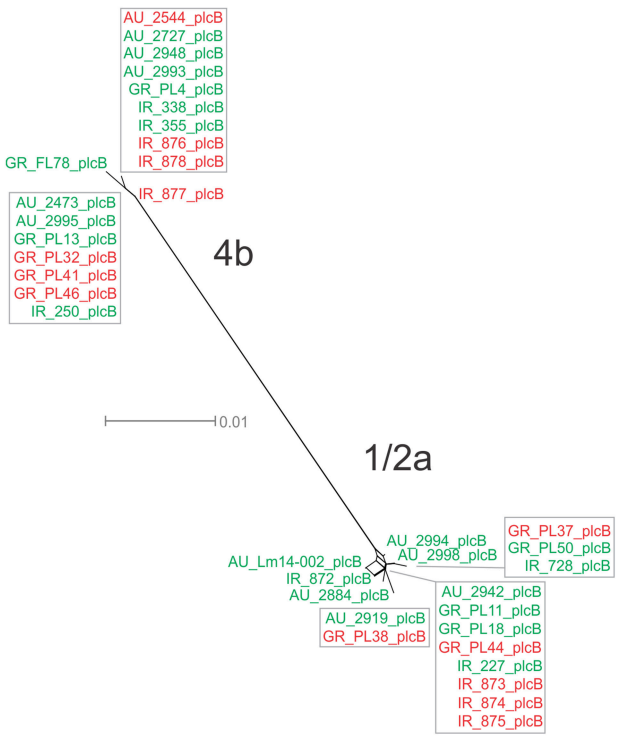


FIGURE 8 | Phylogenetic network applied to virulence gene *plcB* using the Neighbor-Net algorithm. *L. monocytogenes* strains represented food environment isolates (green color) or clinical isolates (red color), isolated in Ireland (IR), Greece (GR), and Australia (AU). Strains clustered together in a box represent identical nucleotide sequence.

In the current study, *actA* showed the highest number of alleles among all genes tested; 13 alleles were observed for serotype 1/2a strains and 7 alleles for serotype 4b strains. One food isolate (AU_Lm14-002) had a single nucleotide deletion. Although this deletion would lead to a premature stop codon and a predicted truncated 487 amino acid protein, it was located immediately upstream of a poly(A) tract of 7 adenine residues. These mutations may have a role in influencing gene regulation, which allows phase switching and inactivation, and may be influencing *actA* transcription in this isolate, whereby a full length ActA may still be synthesized (Orsi et al., 2010). Twelve isolates representing 5 unique alleles had a 105 bp deletion, which comprises a 35 amino acid Proline-Rich Repeats (PRRs) fragment (Wiedmann et al., 1997; Jacquet et al., 2002; Orsi et al., 2008; Holen et al., 2010); the encoded proteins possess 3 instead of 4 PRRs. The number of PRRs contributes to bacterial movement (Lasa et al., 1995; Smith et al., 1996), however no significant effect on virulence potential of the strains has been shown (Roberts and Wiedmann, 2006; Holen et al., 2010). Among the isolates tested in this study, the 105 bp deletion was observed for 4 out of 18 isolates of 1/2a serotype and 8 out of 18 isolates of 4b serotype. Of these, 8 strains (which includes 3 alleles) were isolated from the food environment and 4 strains (2 alleles) were clinical isolates. Similar results were demonstrated by Wiedmann et al. (1997), who observed a predominance of 3-PRRs *actA* sequence among lineage I isolates compared to isolates of lineage II. This could indicate that this deletion does not influence the pathogenic potential of *L. monocytogenes*. Jacquet et al. (2002) observed that polymorphism in ActA proteins was rather correlated with origin (human or food isolates) than with serotype of the strains, while Conter et al. (2009) could not correlate *actA* polymorphism to the virulence of the strains. Based on the sequence analysis in the current study, no clear driving factor appeared to influence the nucleotide sequence or mutations in this gene, as all of the groups were dispersed regarding the parameters π, θ, and ω, while phylogenetic trees showed no consistent pattern between origin or environment of the strains and their genetic polymorphisms. These findings, along with the adapting character to certain functions previously suggested for this gene, and the increased recombination events (Orsi et al., 2008) might imply its multi-functionality recently reported (Travier et al., 2013).

Overall, this study provides insights into the selective pressures acting on the main virulence gene cluster of *L. monocytogenes*, and suggests differences based on serotype, geographic location and source. The selective pressure to minimize diversification was noted with the key virulence regulatory gene *prfA*, therefore results of this study support the key role of the global regulator *prfA* in the lifecycle of *L. monocytogenes*. In contrast to this, conservation of the *actA* gene sequence was lowest, with a greater sequence variation and number of alleles. Broadly speaking, higher conservation was noted among isolates sharing a serotype when compared with other groupings such as geographical location or source. Food and clinical isolates largely varied with respect to nucleotide diversity within *prfA*, *actA*, and *plcB* genes, possibly suggesting that a particular adaptation correlated with their prevalence in food or virulence phenotype, respectively. Geographical divergence was noted with respect to the *hly* gene, with serotype 4b Irish strains distinct to Greek and Australian strains. Future studies will be needed in order to clarify the correlation of geographical distribution of strains and their *hly* sequence, as well as the impact of such correlation on LLO functionality. Additionally, *actA* polymorphism should be further evaluated for other phenotypes that might result from its increased diversity among strains and diverse origins. In the present study, strains were selected to represent the distribution of *L. monocytogenes* based on prevalent serotypes and clinical or food associated origin. Further, a larger data set comprising strains of more serotypes, geographical or isolation origin and year of isolation should be investigated in order to infer significant conclusions regarding the impact of these parameters on LIPI-1 evolution and its correlation to virulence potential of the pathogen.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by KJ, PS, and EF. All authors contributed to acquisition,

analysis, and interpretation of the data. The work was drafted and revised by SP and EF. All authors approved and agreed in the final version of the manuscript.

FUNDING

This study was supported by the 7th Framework Programme projects PROMISE, contract number 265877.

ACKNOWLEDGMENTS

SP would like to acknowledge the Greek State Scholarships Foundation (IKY) for providing her a Ph.D. fellowship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01103/full#supplementary-material

REFERENCES

Brosch, R., Catimel, B., Milon, G., Buchrieser, C., Vindel, E., and Rocourt, J. (1993). Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompetent mice and its association with typing characteristics. *J. Food Prot.* 56, 296–301. doi: 10.4315/0362-028X-56.4.297

Bryant, D., and Moulton, V. (2004). Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. *Mol. Biol. Evol.* 21, 255–265. doi: 10.1093/molbev/msh018

Bueno, V. F., Banerjee, P., Banada, P. P., José De Mesquita, A., Lemes-Marques, E. G., and Bhunia, A. K. (2010). Characterization of *Listeria monocytogenes* isolates of food and human origins from Brazil using molecular typing procedures and *in vitro* cell culture assays. *Int. J. Environ. Health Res.* 20, 43–59. doi: 10.1080/09603120903281283

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S., and Cabanes, D. (2011). The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. *Virulence* 2, 379–394. doi: 10.4161/viru.2.5.17703

Chakraborty, T., Ebel, F., Wehland, J., Dufrenne, J., and Notermans, S. (1994). Naturally occurring virulence-attenuated isolates of *Listeria monocytogenes* capable of inducing long term protection against infection by virulent strains of homologous and heterologous serotypes. *FEMS Immunol. Med. Microbiol.* 10, 1–9. doi: 10.1111/j.1574-695X.1994.tb00004.x

Conter, M., Vergara, A., Di Ciccio, P., Zanardi, E., Ghidini, S., and Ianieri, A. (2009). Polymorphism of *actA* gene is not related to *in vitro* virulence of *Listeria monocytogenes*. *Int. J. Food Microbiol.* 137, 100–105. doi: 10.1016/j.ijfoodmicro.2009.10.019

de las Heras, A., Cain, R. J., and Bielecka, M. K., Vázquez-Boland, J. (2011). Regulation of *Listeria* virulence: PrfA master and commander. *Curr. Opin. Microbiol.* 14, 118–127. doi: 10.1016/j.mib.2011.01.005

den Bakker, H. C., Didelot, X., Fortes, E. D., Nightingale, K. K., and Wiedmann, M. (2008). Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. *BMC Evol. Biol.* 8:277. doi: 10.1186/1471-2148-8-277

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., and Martin, P. (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.* 42, 3819–3822. doi: 10.1128/JCM.42.8.3819-3822.2004

Ebner, R., Stephan, R., Althaus, D., Brisse, S., Maury, M., and Tasara, T. (2015). Phenotypic and genotypic characteristics of *Listeria monocytogenes* strains isolated during 2011-2014 from different food matrices in Switzerland. *Food Control* 57, 321–326. doi: 10.1016/j.foodcont.2015.04.030

EFSA ECDC (2015). EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA J.* 13:4329. doi: 10.2903/j.efsa.2015.4329

Farber, J. M., and Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55, 476–511.

Fox, E. M., Garvey, P., Mckeown, P., Cormican, M., Leonard, N., and Jordan, K. (2012). PFGE analysis of *Listeria monocytogenes* isolates of clinical, animal, food and environmental origin from Ireland. *J. Med. Microbiol.* 61, 540–547. doi: 10.1099/jmm.0.036764-0

Fox, E., O'Mahony, T., Clancy, M., Dempsey, R., Brien, M. O., and Jordan, K. (2009). *Listeria monocytogenes* in the Irish dairy farm environment. *J. Food Prot.* 72, 1450–1456. doi: 10.4315/0362-028X-72.7.1450

Gaillard, J. L., Berche, P., and Sansonetti, P. (1986). Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. *Infect. Immun.* 52, 50–55.

Gendel, S. M., and Ulaszek, J. (2000). Ribotype analysis of strain distribution in *Listeria monocytogenes*. *J. Food Prot.* 63, 179–185. doi: 10.4315/0362-028X-63.2.179

Gilbreth, S. E., Call, J. E., Wallace, F. M., Scott, V. N., Chen, Y., and Luchansky, J. B. (2005). Relatedness of *Listeria monocytogenes* isolates recovered from selected ready-to-eat foods and listeriosis patients in the United States. *Appl. Environ. Microbiol.* 71, 8115–8122. doi: 10.1128/AEM.71.12.8115-8122.2005

Gray, M. J., Freitag, N. E., and Boor, K. J. (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect. Immun.* 74, 2505–2512. doi: 10.1128/IAI.74.5.2505-2512.2006

Gray, M. J., Zadoks, R. N., Fortes, E. D., Dogan, B., Cai, S., Chen, Y., et al. (2004). *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Appl. Environ. Microbiol.* 70, 5833–5841. doi: 10.1128/AEM.70.10.5833-5841.2004

Halberg Larsen, M., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., et al. (2014). Persistence of foodborne pathogens and their control in primary and secondary food production chains. *Food Control* 44, 92–109. doi: 10.1016/j.foodcont.2014.03.039

Hill, C., Cotter, P. D., Sleator, R. D., and Gahan, C. G. M. (2002). Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. *Int. Dairy, J.* 12, 273–283. doi: 10.1016/S0958-6946(01)00125-X

Holen, A., Gottlieb, C. T., Larsen, M. H., Ingmer, H., and Gram, L. (2010). Poor invasion of trophoblastic cells but normal plaque formation in fibroblastic cells despite acta deletion in a group of *Listeria monocytogenes* strains persisting in some food processing environments. *Appl. Environ. Microbiol.* 76, 3391–3397. doi: 10.1128/AEM.02862-09

Houhoula, D., Peirasmaki, D., Konteles, S. J., Kizis, D., Koussissis, S., Bratacos, M., et al. (2012). High level of heterogeneity among *Listeria monocytogenes* isolates from clinical and food origin specimens in Greece. *Foodborne Pathog. Dis.* 9, 848–852. doi: 10.1089/fpd.2012.1187

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* 14, 68–73. doi: 10.1093/bioinformatics/14.1.68

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P., and Rocourt, J. (2002). Expression of ActA, Ami, InlB, and Listeriolysin O in *Listeria monocytogenes* of human and food origin. *Appl. Environ. Microbiol.* 68, 616–622. doi: 10.1128/AEM.68.2.616-622.2002

Jaradat, Z. W., and Bhunia, A. K. (2003). Adhesion, invasion and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Appl. Environ. Microbiol.* 69, 3640–3645. doi: 10.1128/AEM.69.6.3640-3645.2003

Jaradat, Z. W., Schutze, G. E., and Bhunia, A. K. (2002). Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. *Int. J. Food Microbiol.* 76, 1–10. doi: 10.1016/S0168-1605(02)00050-8

Jensen, A., Larsen, M. H., Ingmer, H., and Vogel, B. F. (2007). Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. *J. Food Prot.* 70, 592–599. doi: 10.4315/0362-028X-70.3.592

Jensen, A., Thomsen, L. E., Jørgensen, R. L., Larsen, M. H., Roldgaard, B. B., Christensen, B. B., et al. (2008). Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. *Int. J. Food Microbiol.* 123, 254–261. doi: 10.1016/j.ijfoodmicro.2008.02.016

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Kiss, R., Tirczka, T., Szita, G., Bernáth, S., and Csikó, G. (2006). *Listeria monocytogenes* food monitoring data and incidence of human listeriosis in Hungary, (2004). *Int. J. Food Microbiol.* 112, 71–74. doi: 10.1016/j.ijfoodmicro.2006.06.013

Kosakovsky Pond, S. L., and Frost, S. D. W. (2005). Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinforma*. 21, 2531–2533. doi: 10.1093/bioinformatics/bti320

Larsen, C. N., Nørrung, B., Sommer, H. M., and Jakobsen, M. (2002). *In vitro* and *in vivo* invasiveness of different pulsed-field gel electrophoresis types of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 68, 5698–5703. doi: 10.1128/AEM.68.11.5698-5703.2002

Lasa, I., David, V., Gouin, E., Marchand, J. B., and Cossart, P. (1995). The amino-terminal part of ActA is critical for the actin-based motility of *Listeria monocytogenes*; the central proline-rich region acts as a stimulator. *Mol. Microbiol.* 18, 425–436. doi: 10.1111/j.1365-2958.1995.mmi_18030425.x

Lianou, A., and Sofos, J. N. (2007). A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *J. Food Prot.* 70, 2172–2198. doi: 10.4315/0362-028X-70.9.2172

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *J. Med. Microbiol.* 55, 645–659. doi: 10.1099/jmm.0.46495-0

Lukinmaa, S., Aarnisalo, K., Suihko, M. L., and Siitonen, A. (2004). Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. *Clin. Microbiol. Infect.* 10, 562–568. doi: 10.1111/j.1469-0691.2004.00876.x

Matar, G. M., Bibb, W. F., Helsel, L., Dewitt, W., and Swaminathan, B. (1992). Immunoaffinity purification, stabilization and comparative characterization of listeriolysin O from *Listeria monocytogenes* serotypes 1/2a and 4b. *Res. Microbiol.* 143, 489–498. doi: 10.1016/0923-2508(92)90095-6

McLauchlin, J. (1990). Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 9, 210–213. doi: 10.1007/BF01963840

McLauchlin, J. (2004). *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *Int. J. Food Microbiol.* 92, 15–33. doi: 10.1016/S0168-1605(03)00326-X

Mereghetti, L., Lanotte, P., Savoye-Marczuk, V., Marquet-van Der Mee, N., Audurier, A., and Quentin, R. (2002). Combined ribotyping and random multiprimer DNA analysis to probe the population structure of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 68, 2849–2857. doi: 10.1128/AEM.68.6.2849-2857.2002

Møretrø, T., and Langsrud, S. (2004). *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. *Biofilms* 1, 107–121. doi: 10.1017/S1479050504001322

Neves, E., Silva, A. C., Roche, S. M., Velge, P., and Brito, L. (2008). Virulence of *Listeria monocytogenes* isolated from the cheese dairy environment, other foods and clinical cases. *J. Med. Microbiol.* 57, 411–415. doi: 10.1099/jmm.0.47672-0

Nightingale, K. K., Windham, K., and Wiedmann, M. (2005). Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods. *J. Bacteriol.* 187, 5537–5551. doi: 10.1128/JB.187.16.5537-5551.2005

Nishibori, T., Cooray, K., Xiong, H., Kawamura, I., Fujita, M., and Mitsuyama, M. (1995). Correlation between the presence of virulence-associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp. *Microbiol. Immunol.* 39, 343–349. doi: 10.1111/j.1348-0421.1995.tb02211.x

Norton, D. M., Scarlett, J. M., Horton, K., Sue, D., Thimothe, J., Boor, K. J., et al. (2001). Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. *Appl. Environ. Microbiol.* 67, 646–653. doi: 10.1128/AEM.67.2.646-653.2001

Olier, M., Pierre, F., Lemaître, J. P., Divies, C., Rousset, A., and Guzzo, J. (2002). Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. *Microbiology* 148, 1855–1862. doi: 10.1099/00221287-148-6-1855

Olier, M., Pierre, F., Rousseaux, S., Lemaître, J. P., Rousset, A., Piveteau, P., et al. (2003). Expression of truncated internalin A is involved in impaired internalization of some *Listeria monocytogenes* isolates carried asymptomatically by humans. *Infect. Immun.* 71, 1217–1224. doi: 10.1128/IAI.71.3.1217-1224.2003

Orsi, R. H., Bowen, B. M., and Wiedmann, M. (2010). Homopolymeric tracts represent a general regulatory mechanism in prokaryotes. *BMC Genomics* 11:102. doi: 10.1186/1471-2164-11-102

Orsi, R. H., den Bakker, H. C., and Wiedmann, M. (2011). *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. *Int. J. Med. Microbiol.* 301, 79–96. doi: 10.1016/j.ijmm.2010.05.002

Orsi, R. H., Maron, S. B., Nightingale, K. K., Jerome, M., Tabor, H., and Wiedmann, M. (2008). Lineage specific recombination and positive selection in coding and intragenic regions contributed to evolution of the main *Listeria monocytogenes* virulence gene cluster. *Infect. Genet. Evol.* 8, 566–576. doi: 10.1016/j.meegid.2008.04.006

Poimenidou, S. V., Chatzithoma, D.-N., Nychas, G.-J., and Skandamis, P. N. (2016a). Adaptive response of *Listeria monocytogenes* to heat, salinity and low pH, after habituation on cherry tomatoes and lettuce leaves. *PLoS ONE* 11:e0165746. doi: 10.1371/journal.pone.0165746

Poimenidou, S. V., Chrysadakou, M., Tzakoniati, A., Bikouli, V. C., Nychas, G.-J., and Skandamis, P. N. (2016b). Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. *Int. J. Food Microbiol.* 237, 164–171. doi: 10.1016/j.ijfoodmicro.2016.08.029

Portnoy, D. A., Chakraborty, T., Goebel, W., and Cossart, P. (1992). Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. *Immun.* 60, 1263–1267.

Roberts, A. J., and Wiedmann, M. (2003). Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. *Cell. Mol. Life Sci.* 60, 904–918. doi: 10.1007/s00018-003-2225-6

Roberts, A. J., and Wiedmann, M. (2006). Allelic exchange and site-directed mutagenesis probe the contribution of ActA amino-acid variability to phosphorylation and virulence-associated phenotypes among *Listeria monocytogenes* strains. *FEMS Microbiol. Lett.* 254, 300–307. doi: 10.1111/j.1574-6968.2005.00041.x

Roberts, A., Chan, Y., and Wiedmann, M. (2005). Definition of genetically distinct attenuation mechanisms in naturally virulence-attenuated *Listeria monocytogenes* by comparative cell culture and molecular characterization. *Appl. Environ. Microbiol.* 71, 3900–3910. doi: 10.1128/AEM.71.7.3900-3910.2005

Roche, S. M., Gracieux, P., Albert, I., Gouali, M., Jacquet, C., Martin, P. M. V., et al. (2003). Experimental validation of low virulence in field strains of *Listeria monocytogenes*. *Infect. Immun.* 71, 3429–3436. doi: 10.1128/IAI.71.6.3429-3436.2003

Roche, S. M., Gracieux, P., Milohanic, E., Albert, I., Témoin, S., Grépinet, O., et al. (2005). Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 71, 6039–6048. doi: 10.1128/AEM.71.10.6039-6048.2005

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J. A., and Pla, M. (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap*, and *lin02483* targets and amplifluor technology. *Appl. Environ. Microbiol.* 70, 1366–1377. doi: 10.1128/AEM.70.3.1366-1377.2004

Schuchat, A., Swaminathan, B., and Broome, C. V. (1991). Epidemiology of human listeriosis. *Clin. Microbiol. Rev.* 4, 169–183. doi: 10.1128/CMR.4.2.169

Simonsen, K. L., Churchill, G. A., and Aquadro, C. F. (1995). Properties of statistical tests of neutrality for DNA polymorphism data. *Genetics* 141, 413–429.

Smith, G. A., Theriot, J. A., and Portnoy, D. A. (1996). The tandem repeat domain in the *Listeria monocytogenes* ActA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. *J. Cell Biol.* 135, 647–660. doi: 10.1083/jcb.135.3.647

Suárez, M., González-Zorn, B., Vega, Y., Chico-Calero, I., and Vázquez-Boland, J. A. (2001). A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. *Cell. Microbiol.* 3, 853–864. doi: 10.1046/j.1462-5822.2001.00160.x

Swaminathan, B., and Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes Infect.* 9, 1236–1243. doi: 10.1016/j.micinf.2007.05.011

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123, 585–595.

Témoin, S., Roche, S. M., Grépinet, O., Fardini, Y., and Velge, P. (2008). Multiple point mutations in virulence genes explain the low virulence of *Listeria monocytogenes* field strains. *Microbiology* 154, 939–948. doi: 10.1099/mic.0.2007/011106-0

Travier, L., and Lecuit, M. (2014). *Listeria monocytogenes* ActA: A new function for a "classic" virulence factor. *Curr. Opin. Microbiol.* 17, 53–60.

Travier, L., Guadagnini, S., Gouin, E., Dufour, A., Chenal-Francisque, V., Cossart, P., et al. (2013). ActA promotes *Listeria monocytogenes* aggregation, intestinal colonization and carriage. *PLoS Pathog.* 9:e1003131. doi: 10.1371/journal.ppat.1003131

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3 — new capabilities and interfaces. *Nucleic Acids Res.* 40, 1–12. doi: 10.1093/nar/gks596

Van Stelten, A., Simpson, J. M., Chen, Y., Scott, V. N., Whiting, R. C., Ross, W. H., et al. (2011). Significant shift in median guinea pig infectious dose shown by an outbreak-associated *Listeria monocytogenes* epidemic clone strain and a strain carrying a premature stop codon mutation in inlA. *Appl. Environ. Microbiol.* 77, 2479–2487. doi: 10.1128/AEM.02626-10

Vázquez-Boland, J. A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., and Goebel, W. (2001a). Pathogenicity islands and virulence evolution in *Listeria*. *Microbes Infect.* 3, 571–584. doi: 10.1016/S1286-4579(01)01413-7

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., et al. (2001b). *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14, 584–640. doi: 10.1128/CMR.14.3.584-640.2001

Ward, T. J., Gorski, L., Borucki, M. K., Robert, E., Hutchins, J., Pupedis, K., et al. (2004). Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. *J. Bacteriol.* 186, 4994–5002. doi: 10.1128/JB.186.15.4994-5002.2004

Wiedmann, M., Bruce, J. L., Keating, C., Johnson, A. E., McDonough, P. L., and Batt, C. A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect. Immun.* 65, 2707–2716.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Poimenidou, Dalmasso, Papadimitriou, Fox, Skandamis and Jordan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 June 2018
doi: 10.3389/fmicb.2018.01204

Enhanced Biofilm Formation by Ferrous and Ferric Iron Through Oxidative Stress in *Campylobacter jejuni*

*Euna Oh, Katelyn J. Andrews and Byeonghwa Jeon**

School of Public Health, University of Alberta, Edmonton, AB, Canada

Campylobacter is a leading foodborne pathogen worldwide. Biofilm formation is an important survival mechanism that sustains the viability of *Campylobacter* under harsh stress conditions. Iron affects biofilm formation in some other bacteria; however, the effect of iron on biofilm formation has not been investigated in *Campylobacter*. In this study, we discovered that ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron stimulated biofilm formation in *Campylobacter jejuni*. The sequestration of iron with an iron chelator prevented the iron-mediated biofilm stimulation. The level of total reactive oxygen species (ROS) in biofilms was increased by iron. However, the supplementation with an antioxidant prevented the total ROS level from being increased in biofilms by iron and also inhibited iron-mediated biofilm stimulation in *C. jejuni*. This suggests that iron promotes biofilm formation through oxidative stress. Based on the results of fluorescence microscopic analysis, Fe^{2+} and Fe^{3+} enhanced both microcolony formation and biofilm maturation. The levels of extracellular DNA and polysaccharides in biofilms were increased by iron supplementation. The effect of iron on biofilm formation was also investigated with 70 *C. jejuni* isolates from raw chicken. Regardless of the inherent levels of biofilm formation, iron stimulated biofilm formation in all tested strains; however, there were strain variations in iron concentrations affecting biofilm formation. The biofilm formation of 92.9% (65 of 70) strains was enhanced by either 40 μM Fe^{2+} or 20 μM Fe^{3+} or both (the iron concentrations that enhanced biofilm formation in *C. jejuni* NCTC 11168), whereas different iron concentrations were required to promote biofilms in the rest of the strains. The findings in this study showed that Fe^{2+} and Fe^{3+} contributed to the stimulation of biofilm formation in *C. jejuni* through oxidative stress.

Keywords: *Campylobacter*, biofilms, oxidative stress, iron, survival mechanisms

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università di Teramo, Italy

Reviewed by:
Odile Tresse,
INRA – Centre Angers-Nantes, France
Giorgia Perpetuini,
Università di Teramo, Italy

****Correspondence:***
Byeonghwa Jeon
bjeon@ualberta.ca

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *15 January 2018*
Accepted: *17 May 2018*
Published: *06 June 2018*

Citation:
Oh E, Andrews KJ and Jeon B (2018) Enhanced Biofilm Formation by Ferrous and Ferric Iron Through Oxidative Stress in Campylobacter jejuni. Front. Microbiol. 9:1204. doi: 10.3389/fmicb.2018.01204

INTRODUCTION

Campylobacter is a leading bacterial cause of gastroenteritis and is responsible for approximately 166 million diarrheal cases and 37,600 deaths worldwide per year (Kirk et al., 2015). In addition to gastrointestinal infections, in some cases, *Campylobacter jejuni* may result in the development of Guillain–Barré syndrome (GBS), an acute flaccid paralysis (Willison et al., 2016). Although *C. jejuni* is isolated from a wide range of domestic, companion, and wild

animals (Huang et al., 2015), poultry is considered as the most important reservoir for foodborne transmission of *C. jejuni* to humans (Hermans et al., 2012). Compared to other foodborne pathogens, such as *Salmonella* and pathogenic *Escherichia coli*, *C. jejuni* is physiologically unique (e.g., microaerophilic and asaccharolytic) and fastidious to culture (Silva et al., 2011). Thus, specific culture conditions are required for the growth of *C. jejuni*. For example, low oxygen concentrations (e.g., 5% O_2) and high growth temperatures (e.g., 37∼42°C) are needed for the optimal growth of *C. jejuni* (Davis and DiRita, 2008). As a capnophile, additionally, *C. jejuni* requires CO_2, and carbonic anhydrase that is encoded by *canB* contributes to *C. jejuni* growth under low (such as 1%) CO_2 conditions (Al-Haideri et al., 2016).

A biofilm is microbial communities that are encased in a matrix of self-produced extracellular polymeric substance (EPS), including extracellular DNA (eDNA), polysaccharides, and proteins (Flemming et al., 2016). *C. jejuni* is capable of forming biofilms on various abiotic surfaces and frequently isolated from environmental samples (Kemp et al., 2005; Jokinen et al., 2011). Particularly, biofilm formation is deemed as an important survival mechanism in *C. jejuni* (Buswell et al., 1998; Murphy et al., 2006). As bacteria are usually found in biofilms in natural settings (Branda et al., 2005), *Campylobacter* is also found in biofilms on the surface of river rock and wood in the environment (Maal-Bared et al., 2012).

Several environmental factors affecting biofilm formation in *C. jejuni* have been reported. The biofilm formation of this microaerophilic bacterium is enhanced under aerobic conditions (Reuter et al., 2010; Turonova et al., 2015). Oxygen-rich conditions enhance the expression of membrane proteins, such as Peb4 and CadF, which are involved in the adhesion of *C. jejuni* to abiotic surfaces (Asakura et al., 2007; Sulaeman et al., 2012). Increased oxidative stress under aerobic conditions is associated with biofilm stimulation in *C. jejuni* (Oh et al., 2016). In addition, biofilm formation is also affected by nutritional factors. For instance, nutrient-rich culture media and high salt concentrations reduce biofilm formation in *C. jejuni* (Reeser et al., 2007). Iron is an essential nutrient required for all organisms (Chandrangsu et al., 2017) and is associated with biofilm formation in some bacteria, such as *Streptococcus mutans* (Berlutti et al., 2004). *Staphylococcus aureus* (Lin et al., 2012), and *Pseudomonas aeruginosa* (Banin et al., 2005). Since iron affects various biological processes in *C. jejuni*, such as gene expression regulation (e.g., Fur regulon) and protein glycosylation (e.g., *pglA*, *pglC*, and *pglH*) (Palyada et al., 2004), we hypothesized that iron may be involved in biofilm formation in *C. jejuni*. To prove this hypothesis, in this study, we investigated the effect of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron on biofilm formation in *C. jejuni* NCTC 11168 and 70 *C. jejuni* strains isolated from raw chicken.

RESULTS

Stimulation of Biofilm Formation by Iron

To examine the effect of iron on biofilm formation, biofilm assays were performed with minimal essential medium alpha (MEMα), which does not contain iron, with/without iron supplementation.

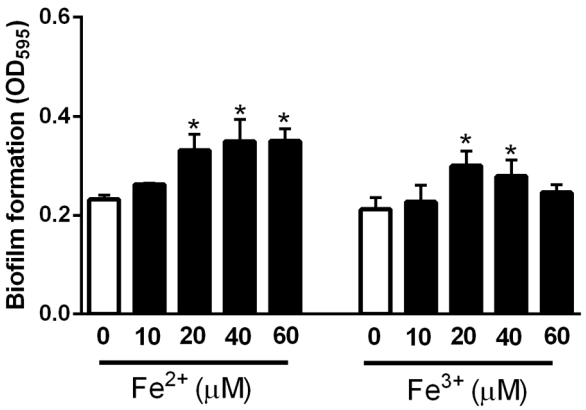


FIGURE 1 | Stimulation of biofilm formation by iron in *C. jejuni* NCTC 11168. The data show the means and standard deviations of three samples in a representative experiment. The experiments were repeated three times, and similar results were obtained in all repeated experiments. The statistical analysis was performed with Student's *t*-test in comparison with the non-treated sample. $^*P < 0.05$.

Interestingly, biofilm formation in *C. jejuni* was significantly enhanced by iron (**Figure 1**). Although both Fe^{2+} and Fe^{3+} affected biofilm formation in *C. jejuni*, Fe^{2+} and Fe^{3+} stimulated biofilm formation at different concentration ranges (**Figure 1**). Although the averages of bacterial counts in biofilms were slightly reduced at iron concentrations ≥20 μM, the reduction was not statistically significant, and the viability of *C. jejuni* in biofilms was not altered at the iron concentrations tested in the study (Supplementary Figure S1A). These results showed that iron, both Fe^{2+} and Fe^{3+}, enhanced biofilm formation in *C. jejuni*.

Inhibition of Iron-Mediated Biofilm Promotion by a Chelator and an Antioxidant

To confirm the effect of iron on biofilm stimulation, biofilm assays were performed in the supplementation with an iron chelator. The treatment of biofilms with an iron chelator significantly inhibited the iron-mediated enhancement of biofilm formation in *C. jejuni* (**Figure 2A**). While iron is an essential nutrient, it may generate reactive oxygen species (ROS) through the Fenton/Haber–Weiss reaction (Cornelis et al., 2011). Since oxidative stress affects biofilm formation in *C. jejuni* (Oh and Jeon, 2014; Oh et al., 2016), we hypothesized that the iron-mediated biofilm promotion may be related to oxidative stress. To examine this hypothesis, we investigated the effect of antioxidant treatment on biofilm formation in the presence of iron. The levels of total ROS were increased by iron and reduced by an iron chelator and an antioxidant (**Figure 2B**). The intracellular levels of iron were increased by iron supplementation and reduced by an iron chelator (**Figure 2C**). Antioxidant treatment inhibited the iron-mediated promotion of biofilm formation, although the intracellular iron level of iron- and antioxidant-treated biofilms was comparable to that in the biofilms treated with only iron (**Figure 2C**). Interestingly, biofilm

formation was enhanced in proportion to the level of total ROS (**Figures 2A,B**), not that of intracellular iron (**Figures 2A,C**). The viability of *C. jejuni* in biofilms was not affected by the treatment conditions used in the study (Supplementary Figure S1B). These results suggested that biofilm promotion by iron is associated with oxidative stress in *C. jejuni*.

Increased Production of EPS by Iron

The formation of biofilms was observed in the presence and absence of iron using fluorescence microscopy. Iron supplementation significantly enhanced the establishment of microcolonies at the early stage (12 h) of biofilm formation and also increased the development of matured biofilm structures at 24 h (**Figure 3**), suggesting that iron may affect the early and late stages of biofilm formation in *C. jejuni*. To observe EPS production, biofilms were stained with BOBO3 and calcofluor white (CW) to detect eDNA and extracellular polysaccharides, respectively. BOBO-3 is a DNA-binding red fluorescent dye and cannot penetrate through the membrane and thus is used to detect eDNA. CW is a fluorescent dye that binds to β1–3 and β1–4 carbohydrate linkages and has been used to detect polysaccharides in *C. jejuni* biofilms (McLennan et al., 2008). Both Fe^{2+} and Fe^{3+} substantially increased the production of eDNA and extracellular polysaccharides in biofilms; however, an iron chelator and an antioxidant reduced the levels of eDNA and extracellular polysaccharides (**Figure 3**). These findings demonstrated that iron promoted biofilm formation in *C. jejuni* by stimulating the production of eDNA and extracellular polysaccharides.

Effect of Iron on Biofilm Formation in 70 Strains of *C. jejuni* From Retail Raw Chicken

Using the iron concentrations determined with *C. jejuni* NCTC 11168 (40 μM Fe^{2+} and 20 μM Fe^{3+}; **Figure 1**), the effect of iron on biofilm formation was evaluated in 70 *C. jejuni* strains that were isolated from retail raw chicken in our previous study (Oh et al., 2015). The levels of biofilm formation in the tested strains varied significantly in the absence of iron, ranging from low (**Figure 4A**), medium (**Figure 4B**), to high levels (**Figure 4C**), and iron significantly stimulated biofilm formation in the tested strains with strain-dependent variations (**Figure 4** and Supplementary Figure S2). Similar to *C. jejuni* NCTC 11168 (**Figure 1**), 51 (72.9%) of the 70 tested strains exhibited biofilm promotion by both 40 μM Fe^{2+} and 20 μM Fe^{3+} (**Figure 5A**). However, biofilm formation in 14 (20%) strains was enhanced by either only 40 μM Fe^{2+} or 20 μM Fe^{3+}, not by both (**Figure 5A**), and biofilm formation in five strains (7.1%) was promoted by neither 40 μM Fe^{2+} nor 20 μM Fe^{3+} (**Figure 5A**). The intrinsic level of biofilm formation was not correlated to the multilocus sequence typing (MLST) clonal complexes (CCs) of the strains. Overall, MLST CCs 21 and 45 were distributed in weak-, medium-, and strong-biofilm formers; however, minor MLST CCs, such as 353, 354, and 362, were not found in strong-biofilm formers (**Figure 5B**).

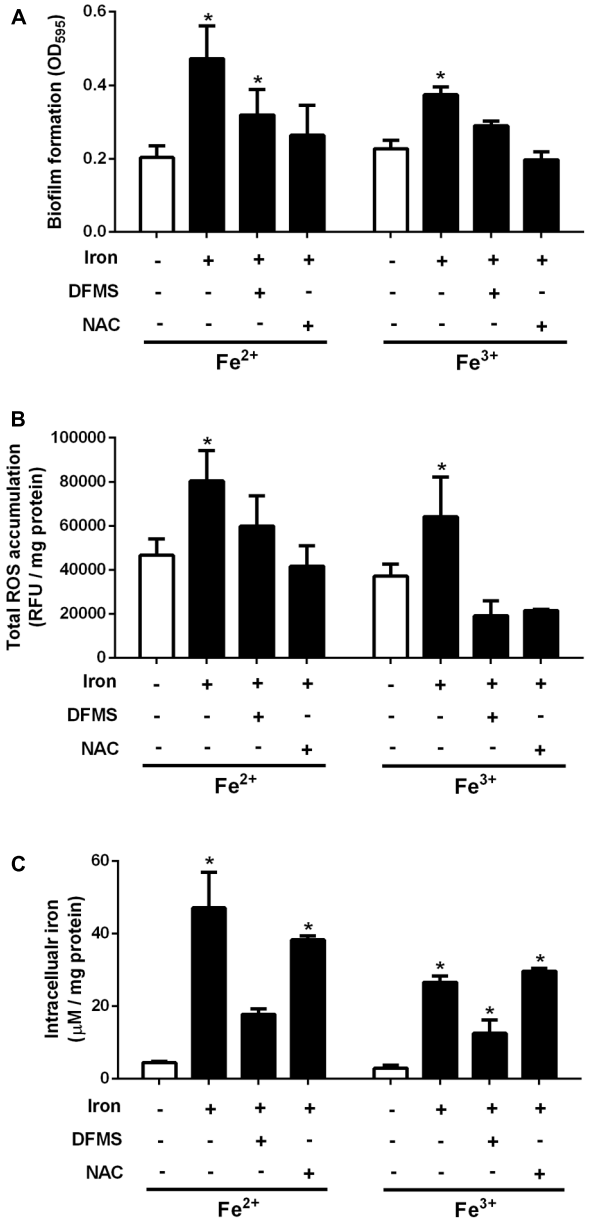


FIGURE 2 | Effects of iron, a chelator, and an antioxidant on the levels of biofilm formation **(A)**, ROS production **(B)**, and intracellular iron **(C)** in *C. jejuni* NCTC 11168. The concentrations of Fe^{2+} and Fe^{3+} were 40 μM and 20 μM, respectively, which were determined based on the results of the biofilm assay (**Figure 1**). Twenty micromolar DFMS and 1 μM N-acetylcysteine (NAC) were used as an iron chelator and an antioxidant, respectively. The results are the means and standard deviations of three samples in a representative experiment. The experiments were repeated three times, and similar results were observed in all repeated experiments. The statistical analysis was performed with Student's *t*-test compared to the non-treated sample. $^*P < 0.05$.

Assuming strain variations in iron uptake and/or oxidative stress defense, we conducted biofilm assays at different iron concentrations with the 19 strains whose biofilm formation was not enhanced by either 40 μM Fe^{2+} or 20 μM Fe^{3+} or

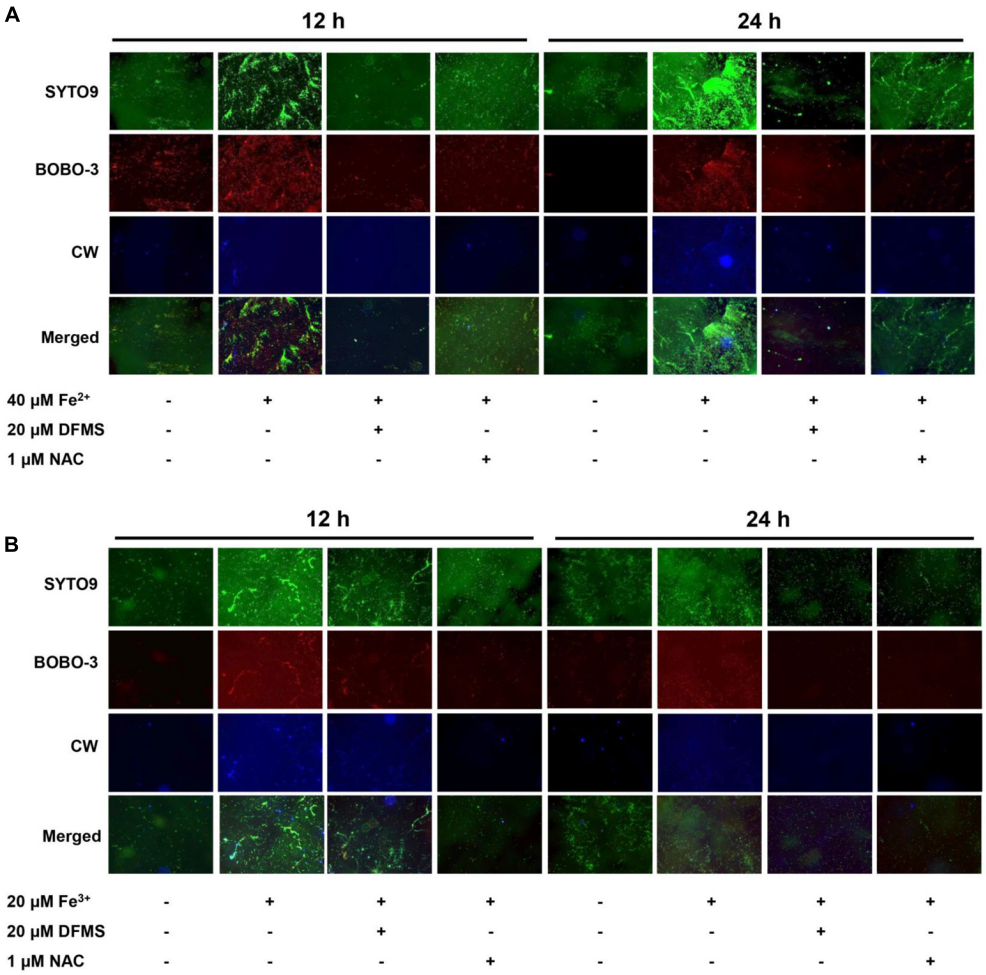


FIGURE 3 | Stimulatory effects of Fe^{2+} **(A)** and Fe^{3+} **(B)** on biofilm formation in *C. jejuni* NCTC 11168. Biofilms were observed with fluorescence microscopy after staining with SYTO9, BOBO3, and calcofluor white (CW) to detect total DNA (i.e., biofilm structures), extracellular DNA, and extracellular polysaccharides, respectively. The experiments were repeated three times and generated similar results. DFMS and N-acetylcysteine (NAC) were used as an iron chelator and an antioxidant, respectively.

both (**Figures 4**, **5A**). Interestingly, biofilm formation in all tested strains was enhanced by both Fe^{2+} and Fe^{3+} at different concentrations with substantial strain variations (**Figure 6**). These results show that most *C. jejuni* strains increased biofilm formation in similar concentration ranges (ca. 40 μM Fe^{2+} and 20 μM Fe^{3+}). However, different iron concentrations were required for biofilm stimulation in some *C. jejuni* strains.

DISCUSSION

As an essential nutrient, iron is involved in various biological processes in *C. jejuni* (Palyada et al., 2004). Fe^{2+} may diffuse through outer-membrane porins and then pass through FeoB in the cytoplasmic membrane in *C. jejuni* (Naikare et al., 2006; Miller et al., 2009). Fe^{3+} uptake is mediated by multiple membrane transporters, whose expression is regulated by the ferric uptake regulator Fur (Miller et al., 2009). The findings in this study demonstrate that both Fe^{2+} and Fe^{3+} stimulate biofilm formation in *C. jejuni*. Effects of iron on biofilm formation have also been reported in other bacteria. Iron-depleted saliva increases aggregation and biofilm formation of *S. mutans*, an important pathogen causing dental caries (Berlutti et al., 2004). Iron enhances biofilm formation in *S. aureus* ATCC 35556 and a few clinical strains of *S. aureus* (Lin et al., 2012). However, the effect of iron on biofilm formation appears to be strain-dependent in *S. aureus* since iron reduces biofilm formation in *S. aureus* Newman (Johnson et al., 2005). Biofilm formation in most of the strains tested in this study was stimulated 40 μM Fe^{2+} and 20 μM Fe^{3+} (**Figures 4**, **5A**), which were effective at biofilm promotion in *C. jejuni* NCTC 11168 (**Figure 1**). In some other *C. jejuni* strains, 40 μM Fe^{2+} and 20 μM Fe^{3+} did not enhance biofilm formation, and different iron concentrations were required to promote biofilm formation in these strains (**Figure 6**). Variations in the iron concentrations impacting biofilm formation may be associated with the strain variations in iron-associated genes in

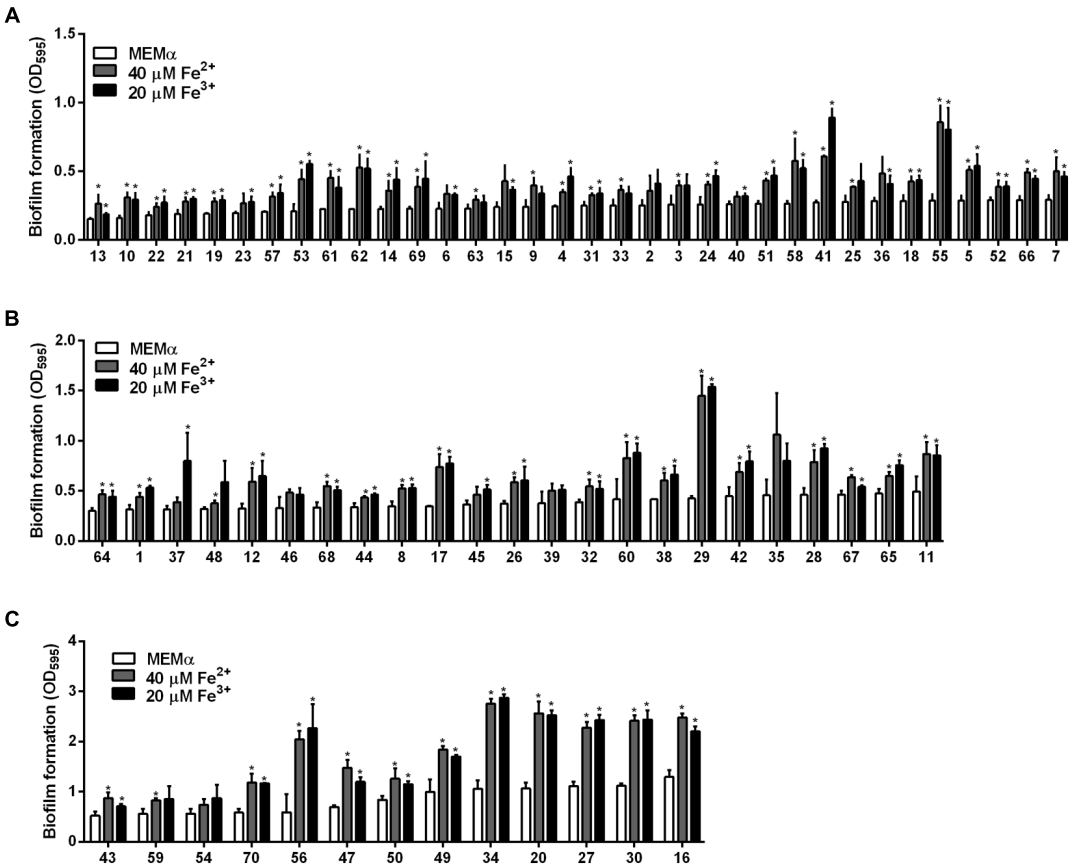


FIGURE 4 | Effects of iron (40 μM Fe^{2+} and 20 μM Fe^{3+}) on biofilm formation in 70 chicken isolates of *C. jejuni* that inherently form biofilms at low **(A)**, medium **(B)**, and high **(C)** levels. The inherent capability of biofilm formation was determined based on the OD_{595} in the biofilm assay. The low-, medium-, and high-level biofilm formers were those that generated OD_{595} of less than 0.3, between 0.3 and 0.5, and greater than 0.5, respectively, in the absence of iron. The results show the means and standard deviations of three samples in a single experiment. The experiments were repeated three times, and similar results were observed in all repeated experiments. The statistical analysis was performed with Student's *t*-test compared to the control without iron treatment. $^*P < 0.05$.

C. jejuni. Genes encoding iron transporters, which are found in the genome of some *C. jejuni* strains, have been found to be non-functional or absent in other strains (Miller et al., 2009). Presumably, the diversities in the genes involved in iron uptake might be associated with the strain-dependent variations in iron concentrations affecting biofilm stimulation. We could not observe any correlation between the MLST CCs and the levels of biofilm formation (**Figure 5B**), presumably because the MLST scheme is based on the polymorphisms of the seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt,* and *uncA*) (Dingle et al., 2001). However, biofilm formation is complicated and involves proteins of various biological processes, such as motility, chemotaxis, oxidative stress response, heat shock response, and energy generation (Kalmokoff et al., 2006). This might be the reason why the MLST sequence types were not well correlated with biofilm formation in this study.

Biofilm formation in *P. aeruginosa* is inhibited by lactoferrin, a mammalian iron chelator (Singh et al., 2002). The sequestration of iron by lactoferrin reduces the intracellular levels of iron and inhibits biofilm formation (Banin et al., 2005). The anti-biofilm effect of lactoferrin in *P. aeruginosa* involves incessant twitching motility that affects bacterial attachment to a surface and microcolony formation during biofilm development (Singh et al., 2002). Twitching motility is mediated by type IV pili (Mattick, 2002). Similarly, iron stimulates biofilm formation in *E. coli* by controlling the expression of type I fimbriae (Wu and Outten, 2009). However, *C. jejuni* does not produce pili (Gaynor et al., 2001). This indicates that a different mechanism may be involved in iron-mediated biofilm promotion in *C. jejuni*.

It has been reported that oxidative stress affects biofilm formation in *C. jejuni* (Kim et al., 2015). A mutation of *ahpC* leads to the accumulation of total ROS and lipid hydroperoxides and enhances biofilm formation in *C. jejuni*, and antioxidant treatment inhibits the biofilm promotion by an *ahpC* mutation (Oh and Jeon, 2014). PerR and CosR, key oxidative stress defense regulators in *C. jejuni*, also consistently affect biofilm formation in *C. jejuni* (Oh and Jeon, 2014; Turonova et al., 2015). Although *C. jejuni* is microaerophilic, interestingly, aerobic exposure facilitates biofilm formation in *C. jejuni* (Reuter et al., 2010). Our previous study has shown that oxidative stress plays a role in biofilm stimulation in *C. jejuni* under aerobic conditions (Oh et al., 2016). Oxidative

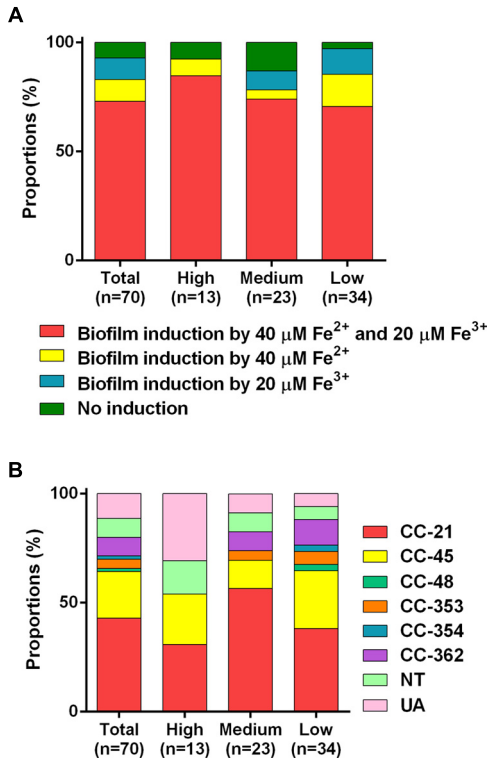


FIGURE 5 | MLST clonal complexes (CCs) of 70 tested strains of *C. jejuni* with different biofilm formation capabilities **(A)**, and the proportions of the *C. jejuni* strains whose biofilm formation was enhanced by 40 μM Fe^{2+} and/or 20 μM Fe^{3+} **(B)**. The low-, medium-, and high-level biofilm formers are the strains that generated OD_{595} of less than 0.3, between 0.3 and 0.5, and greater than 0.5, respectively, in the absence of iron. The MLST CCs of the 70 *C. jejuni* strains tested in the study were determined in our previous report (Oh et al., 2015).

stress affects biofilm formation in some other bacteria. Biofilm formation in *Mycobacterium avium* increases by the autoinducer-2 (AI-2) signaling molecules by the induction of oxidative stress response involving the upregulation of genes encoding alkyl hydroperoxidases (e.g., *ahpC* and *ahpD*), not through quorum sensing (Geier et al., 2008). An *ahpC* mutation in *Acinetobacter oleivorans* DR1 increases the accumulation of H_2O_2 in the cell, which enhances biofilm formation by the induction of exopolysaccharide production in biofilms (Jang et al., 2016). In *C. jejuni*, iron supplementation increased the accumulation of total ROS (**Figure 2B**) and the production of eDNA and extracellular polysaccharides in *C. jejuni* biofilms (**Figure 3**). Aerobic exposure and iron supplementation commonly result in the increase in oxidative stress. Based on the findings of this study, biofilm stimulation by iron through oxidative stress in *C. jejuni* may involve EPS production (**Figure 3**). EPS constitutes over 90% of the dry mass of biofilms (Flemming and Wingender, 2010) and contributes to the nutrient acquisition and desiccation tolerance (Flemming et al., 2016). Similarly, biofilm stimulation by iron in *S. aureus* is mediated by the increased production of polysaccharide intercellular adhesin (i.e., β-1,6-linked N-acetyl glucosamine polymer) involved in biofilm formation (Lin et al., 2012). Exposure to increased iron concentrations augments the accumulation of ROS in *C. jejuni* (**Figure 2B**) and the production of EPS in biofilms (**Figure 3**). Presumably, the enhanced production of EPS by iron may help *C. jejuni* to reduce exposure to oxygen and other stress conditions by facilitating the formation of biofilm matrices encasing *C. jejuni*.

Cationic metal ions can be toxic for planktonic bacterial cells at high concentrations; however, the absorption and accumulation of metal ions in biofilms stabilizes biofilms and prevents their erosion by shear forces in *B. subtilis* (Grumbein et al., 2014). EDTA disrupts *P. aeruginosa* biofilms and enhances the dispersal of bacterial cells from biofilms (Banin et al., 2006). However, Mg^{2+}, Ca^{2+}, and Fe^{2+} inhibit the effect of EDTA on biofilm disruption, suggesting that divalent cations are important components that stabilize biofilms in *P. aeruginosa* (Banin et al., 2006). In addition to the effect of iron on oxidative stress, we cannot exclude the possibility that iron may also be involved in the stabilization of biofilm structure in *C. jejuni*. Future studies are required to elucidate the molecular mechanisms underlying the interplay between iron and oxidative stress in biofilm formation in *C. jejuni*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Campylobacter jejuni NCTC 11168, the first genome-sequenced strain, was primarily used in this study. Seventy strains of *C. jejuni* were isolated from raw chicken in our previous study (Oh et al., 2015). The *C. jejuni* strains were routinely maintained at 37°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) on either Mueller-Hinton agar plates or MEMα (Gibco, #41061-029), which does not contain iron. The microaerobic conditions were generated using a cylinder containing the premixed gas.

Biofilm Assays

Biofilm assays were carried out according to a protocol described in our previous study using MEMα (Oh and Jeon, 2014). Briefly, bacterial suspension was prepared from an overnight culture and then diluted with fresh MEMα to an OD_{600} of 0.07 and placed into a 96-well plate (Corning, #3595) in the presence of iron (Fe^{2+} or Fe^{3+}), iron chelator (Deferoxamine mesylate, DFMS), or antioxidant (N-acetyl-L-cysteine, NAC). After 24 h, biofilms were washed twice with PBS (pH 7.4) and stained with 1% crystal violet. The dye eluted with the elution buffer (10% acetic acid and 30% methanol) was measured with a plate reader (FLUOstar Omega; BMG Labtech, Germany) at 595 nm. For bacterial counting of biofilms, *C. jejuni* biofilm samples were washed twice with PBS and resuspended in fresh MH broth. The resuspended biofilm samples were serially diluted in MH broth and spread on MH agar for enumeration. The experiment was conducted with triplicate samples and independently repeated at least three times.

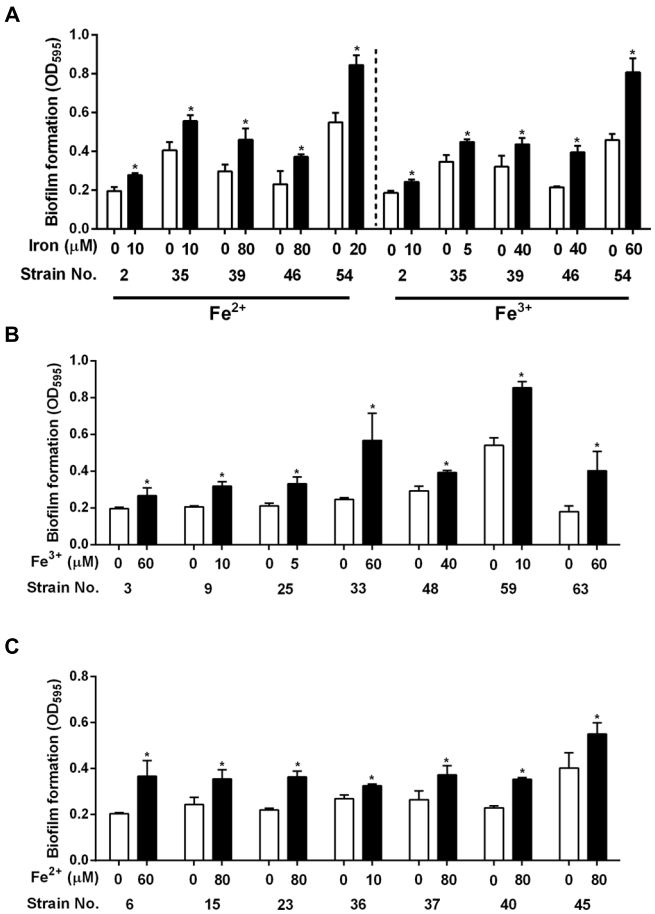


FIGURE 6 | Biofilm stimulation at different iron concentrations in the *C. jejuni* strains whose biofilm formation was enhanced by neither 40 μM Fe^{2+} nor 20 μM Fe^{3+} **(A)**, by 40 μM Fe^{2+} but not by 20 μM Fe^{3+} **(B)**, and by 20 μM Fe^{3+} but not by 40 μM Fe^{2+} **(C)**. The results show the means and standard deviations of three samples in a single experiment. Similar results were observed in all experiments repeated three times independently. The statistical analysis was performed with Student's *t*-test in comparison with the control without iron treatment. $^*P < 0.05$.

Measurement of Total ROS

The total ROS level in biofilms was determined with CM-H_2DCFDA (Thermo Fisher, United States), a general oxidative stress indicator, according to our previous study (Oh and Jeon, 2014). Briefly, biofilms were washed twice with PBS and re-suspended in PBS (pH 7.4). After addition of 10 μM CM-H_2DCFDA, fluorescence was measured with a fluorometer (FLUOstar Omega) at ex 485 nm/em 520 nm. The total ROS levels were normalized with the total protein amounts that were determined using a Bradford assay. The experiment was conducted with triplicate samples and independently repeated three times.

Fluorescence Microscopic Analysis of Biofilms

Biofilm formation was also analyzed with fluorescence microscopy. Biofilms were developed on a circle cover glass in a 24-well plate for 24 h at 37°C under microaerobic conditions. Biofilm samples were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The biofilms were then washed with PBS and stained with SYTO9, BOBO3, and CW to detect total (both intracellular and extracellular) DNA, intracellular DNA, and extracellular polysaccharides, respectively. CW binds β1–3 and β1–4 carbohydrate linkages and was previously used to detect extracellular polysaccharides in biofilms (McLennan et al., 2008). After washing, the biofilms were analyzed with a fluorescence microscope (Carl Zeiss, Axio Imager A1). The experiment was repeated three times.

Measurement of eDNA in Biofilms

The isolation of eDNA from biofilms was performed as described previously (Wu and Xi, 2009). After washing twice with PBS, biofilms were harvested with 2% EDTA and incubated at 4°C for 3 h with shaking (250 rpm). An equal volume of 2% cetyltrimethyl ammonium bromide (CTAB) was added, and the suspension was incubated on ice for 1 h. After centrifugation at 10,000 × *g* for 10 min, the pellet was re-suspended in TE buffer, and an

equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) solution was added. After centrifugation, the top phase of each sample was transferred to a new tube, and 2× volume of ice-cold ethanol and 1/10× volume of 3 M sodium acetate were added. After incubation at −20°C for 1 h, the pellets were washed twice with 70% ethanol. After dissolving with water, DNA concentrations were measured with a spectrophotometer and normalized with the amount of total proteins in biofilms that was determined with a Bradford assay. The experiment was conducted with triplicate samples and repeated three times.

Measurement of Intracellular Iron Levels

The intracellular iron concentration was measured as described previously with slight modifications (Riemer et al., 2004). Briefly, biofilms were washed twice with PBS and disrupted with a sonicator (BioRuptor Plus; Diagenode, United States). The biofilm samples were mixed with an iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1 M ascorbic acid) and incubated at room temperature for 30 min. The absorbance at 550 nm was measured with a plate reader (FLUOstar Omega). Intracellular iron levels were normalized with protein concentrations that were determined with a Bradford assay. The experiment was conducted with triplicate samples and independently repeated three times.

Statistical Analysis

The statistical analysis was performed with Student's *t*-test in comparison with the control without iron treatment using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, United States).

AUTHOR CONTRIBUTIONS

EO and BJ designed the project. EO and KA performed the experiments. EO and BJ data analysis. EO, KA, and BJ wrote the manuscript.

FUNDING

This study was supported by NSERC Discovery Grant (401843-2012-RGPIN) and the Canada Foundation for Innovation (CFI) to BJ.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01204/full#supplementary-material

REFERENCES

Al-Haideri, H., White, M. A., and Kelly, D. J. (2016). Major contribution of the type II beta carbonic anhydrase CanB (Cj0237) to the capnophilic growth phenotype of *Campylobacter jejuni*. *Environ. Microbiol.* 18, 721–735. doi: 10.1111/1462-2920.13092

Asakura, H. I, Yamasaki, M., Yamamoto, S., and Igimi, S. (2007). Deletion of peb4 gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 275, 278–285. doi: 10.1111/j.1574-6968.2007.00893.x

Banin, E., Brady, K. M., and Greenberg, E. P. (2006). Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl. Environ. Microbiol.* 72, 2064–2069. doi: 10.1128/AEM.72.3.2064-2069.2006

Banin, E., Vasil, M. L., and Greenberg, E. P. (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11076–11081. doi: 10.1073/pnas.0504266102

Berlutti, F., Ajello, M., Bosso, P., Morea, C., Petrucca, A., Antonini, G., et al. (2004). Both lactoferrin and iron influence aggregation and biofilm formation in *Streptococcus* mutans. *Biometals* 17, 271–278. doi: 10.1023/B:BIOM.0000027704.53859.d3

Branda, S. S., Vik, S., Friedman, L., and Kolter, R. (2005). Biofilms: the matrix revisited. *Trends Microbiol.* 13, 20–26. doi: 10.1016/j.tim.2004.11.006

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., Mcguiggan, J. T., Marsh, P. D., Keevil, C. W., et al. (1998). Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. *Appl. Environ. Microbiol.* 64, 733–741.

Chandrangsu, P., Rensing, C., and Helmann, J. D. (2017). Metal homeostasis and resistance in bacteria. *Nat. Rev. Microbiol.* 15, 338–350. doi: 10.1038/nrmicro.2017.15

Cornelis, P., Wei, Q., Andrews, S. C., and Vinckx, T. (2011). Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics* 3, 540–549. doi: 10.1039/c1mt00022e

Davis, L., and DiRita, V. (2008). Growth and laboratory maintenance of *Campylobacter jejuni*. *Curr. Protoc. Microbiol.* Chapter 8:Unit 8A.1.1-8A.1.7. doi: 10.1002/9780471729259.mc08a01s10

Dingle, K., Colles, F., Wareing, D., Ure, R., Fox, A., Bolton, F., et al. (2001). Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* 39, 14–23. doi: 10.1128/JCM.39.1.14-23.2001

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* 14, 563–575. doi: 10.1038/nrmicro.2016.94

Gaynor, E. C., Ghori, N., and Falkow, S. (2001). Bile-induced 'pili' in *Campylobacter jejuni* are bacteria-independent artifacts of the culture medium. *Mol. Microbiol.* 39, 1546–1549. doi: 10.1046/j.1365-2958.2001.02341.x

Geier, H., Mostowy, S., Cangelosi, G. A., Behr, M. A., and Ford, T. E. (2008). Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium*, leading to biofilm formation. *Appl. Environ. Microbiol.* 74, 1798–1804. doi: 10.1128/AEM.02066-07

Grumbein, S., Opitz, M., and Lieleg, O. (2014). Selected metal ions protect *Bacillus subtilis* biofilms from erosion. *Metallomics* 6, 1441–1450. doi: 10.1039/c4mt00049h

Hermans, D., Pasmans, F., Messens, W., Martel, A., Van Immerseel, F., Rasschaert, G., et al. (2012). Poultry as a host for the zoonotic pathogen *Campylobacter jejuni*. *Vector Borne Zoonotic Dis.* 12, 89–98. doi: 10.1089/vbz.2011.0676

Huang, H., Brooks, B. W., Lowman, R., and Carrillo, C. D. (2015). Campylobacter species in animal, food, and environmental sources, and relevant testing programs in Canada. *Can. J. Microbiol.* 61, 701–721. doi: 10.1139/cjm-2014-0770

Jang, I. A., Kim, J., and Park, W. (2016). Endogenous hydrogen peroxide increases biofilm formation by inducing exopolysaccharide production in *Acinetobacter oleivorans* DR1. *Sci. Rep.* 6:21121. doi: 10.1038/srep21121

Johnson, M., Cockayne, A., Williams, P. H., and Morrissey, J. A. (2005). Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves fur-dependent and fur-independent mechanisms. *J. Bacteriol.* 187, 8211–8215. doi: 10.1128/JB.187.23.8211-8215.2005

Jokinen, C., Edge, T. A., Ho, S., Koning, W., Laing, C., Mauro, W., et al. (2011). Molecular subtypes of *Campylobacter* spp., *Salmonella enterica*, and *Escherichia*

coli O157:H7 isolated from faecal and surface water samples in the Oldman River watershed, Alberta, Canada. *Water Res.* 45, 1247–1257. doi: 10.1016/j.watres.2010.10.001

Kalmokoff, M., Lanthier, P., Tremblay, T.-L., Foss, M., Lau, P. C., Sanders, G., et al. (2006). Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188, 4312–4320. doi: 10.1128/JB.01975-05

Kemp, R., Leatherbarrow, A., Williams, N., Hart, C., Clough, H., Turner, J., et al. (2005). Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. *Appl. Environ. Microbiol.* 71, 1876–1882. doi: 10.1128/AEM.71.4.1876-1882.2005

Kim, J.-C., Oh, E., Kim, J., and Jeon, B. (2015). Regulation of oxidative stress resistance in *Campylobacter jejuni*, a microaerophilic foodborne pathogen. *Front. Microbiol.* 6:751. doi: 10.3389/fmicb.2015.00751

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Lin, M. H., Shu, J. C., Huang, H. Y., and Cheng, Y. C. (2012). Involvement of iron in biofilm formation by *Staphylococcus aureus*. *PLoS One* 7:e34388. doi: 10.1371/journal.pone.0034388

Maal-Bared, R., Bartlett, K. H., Bowie, W. R., and Hall, E. R. (2012). *Campylobacter* spp. distribution in biofilms on different surfaces in an agricultural watershed (Elk Creek, British Columbia): using biofilms to monitor for *Campylobacter*. *Int. J. Hyg. Environ. Health* 215, 270–278. doi: 10.1016/j.ijheh.2011.12.004

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu. Rev. Microbiol.* 56, 289–314. doi: 10.1146/annurev.micro.56.012302.160938

McLennan, M. K., Ringoir, D. D., Frirdich, E., Svensson, S. L., Wells, D. H., Jarrell, H., et al. (2008). *Campylobacter jejuni* biofilms up-regulated in the absence of the stringent response utilize a calcofluor white-reactive polysaccharide. *J. Bacteriol.* 190, 1097–1107. doi: 10.1128/JB.00516-07

Miller, C. E., Williams, P. H., and Ketley, J. M. (2009). Pumping iron: mechanisms for iron uptake by *Campylobacter*. *Microbiology* 155, 3157–3165. doi: 10.1099/mic.0.032425-0

Murphy, C., Carroll, C., and Jordan, K. N. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Naikare, H., Palyada, K., Panciera, R., Marlow, D., and Stintzi, A. (2006). Major role for FeoB in *Campylobacter jejuni* ferrous iron acquisition, gut colonization, and intracellular survival. *Infect. Immun.* 74, 5433–5444. doi: 10.1128/IAI.00052-06

Oh, E., and Jeon, B. (2014). Role of alkyl hydroperoxide reductase (AhpC) in the biofilm formation of *Campylobacter jejuni*. *PLoS One* 9:e87312. doi: 10.1371/journal.pone.0087312

Oh, E., Kim, J. C., and Jeon, B. (2016). Stimulation of biofilm formation by oxidative stress in *Campylobacter jejuni* under aerobic conditions. *Virulence* 7, 846–851. doi: 10.1080/21505594.2016.1197471

Oh, E., Mcmullen, L., and Jeon, B. (2015). High prevalence of hyper-aerotolerant *Campylobacter jejuni* in retail poultry with potential implication in human infection. *Front. Microbiol.* 6:1263. doi: 10.3389/fmicb.2015.01263

Palyada, K., Threadgill, D., and Stintzi, A. (2004). Iron acquisition and regulation in *Campylobacter jejuni*. *J. Bacteriol.* 186, 4714–4729. doi: 10.1128/JB.186.14.4714-4729.2004

Reeser, R. J., Medler, R. T., Billington, S. J., Jost, B. H., and Joens, L. A. (2007). Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73, 1908–1913. doi: 10.1128/AEM.00740-06

Reuter, M., Mallett, A., Pearson, B. M., and Van Vliet, A. H. (2010). Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76, 2122–2128. doi: 10.1128/AEM.01878-09

Riemer, J., Hoepken, H. H., Czerwinska, H., Robinson, S. R., and Dringen, R. (2004). Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal. Biochem.* 331, 370–375. doi: 10.1016/j.ab.2004.03.049

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). Campylobacter spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Singh, P. K., Parsek, M. R., Greenberg, E. P., and Welsh, M. J. (2002). A component of innate immunity prevents bacterial biofilm development. *Nature* 417, 552–555. doi: 10.1038/417552a

Sulaeman, S., Hernould, M., Schaumann, A., Coquet, L., Bolla, J. M., Dé, E., et al. (2012). Enhanced adhesion of *Campylobacter jejuni* to abiotic surfaces is mediated by membrane proteins in oxygen-enriched conditions. *PLoS One* 7:e46402. doi: 10.1371/journal.pone.0046402

Turonova, H., Briandet, R., Rodrigues, R., Hernould, M., Hayek, N., Stintzi, A., et al. (2015). Biofilm spatial organization by the emerging pathogen *Campylobacter jejuni*: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6:709. doi: 10.3389/fmicb.2015.00709

Willison, H. J., Jacobs, B. C., and Van Doorn, P. A. (2016). Guillain-Barre syndrome. *Lancet* 388, 717–727. doi: 10.1016/S0140-6736(16)00339-1

Wu, J., and Xi, C. (2009). Evaluation of different methods for extracting extracellular DNA from the biofilm matrix. *Appl. Environ. Microbiol.* 75, 5390–5395. doi: 10.1128/AEM.00400-09

Wu, Y., and Outten, F. W. (2009). IscR controls iron-dependent biofilm formation in *Escherichia coli* by regulating type I fimbria expression. *J. Bacteriol.* 191, 1248–1257. doi: 10.1128/JB.01086-08

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GP and handling Editor declared their shared affiliation.

Copyright © 2018 Oh, Andrews and Jeon. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

REVIEW
published: 12 June 2018
doi: 10.3389/fmicb.2018.01236

Detection of Foodborne Pathogens by Surface Enhanced Raman Spectroscopy

Xihong Zhao[1], Mei Li[1] and Zhenbo Xu[2]**

[1] Research Center for Environmental Ecology and Engineering, Key Laboratory for Green Chemical Process of Ministry of Education, Key Laboratory for Hubei Novel Reactor and Green Chemical Technology, School of Environmental Ecology and Biological Engineering, Wuhan Institute of Technology, Wuhan, China, [2] School of Food Science and Engineering, South China University of Technology, Guangzhou, China

Food safety has become an important public health issue in both developed and developing countries. However, as the foodborne illnesses caused by the pollution of foodborne pathogens occurred frequently, which seriously endangered the safety and health of human beings. More importantly, the traditional techniques, such as PCR and enzyme-linked immunosorbent assay, are accurate and effective, but their pretreatments are complex and time-consuming. Therefore, how to detect foodborne pathogens quickly and sensitively has become the key to control food safety. Because of its sensitivity, rapidity, and non-destructive damage to the sample, the surface enhanced Raman scattering (SERS) is considered to be a powerful testing technology that is widely used to different fields. This review aims to give a systematic and comprehensive understanding of SERS for rapid detection of pathogen bacteria. First, the related concepts of SERS are stated, such as its work principal, active substrate, and biochemical origins of the detection of bacteria by SERS. Then the latest progress and applications in food safety, from detection and characterization of targets in label-free method to label method, is summarized. The advantages and limitations of different SERS substrates and methods are discussed. Finally, there are still several hurdles for the further development of SERS techniques into real-world applications. This review comes up with the perspectives on the future trends of the SERS technique in the field of foodborne pathogens detection and some problems to be solved urgently. Therefore, the purpose is mainly to understand the detection of foodborne pathogens and to make further emphasis on the importance of SERS techniques.

Keywords: SERS, foodborne pathogens, rapid detection, nanoparticles, food safety

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Soner Soylu,
Mustafa Kemal University, Turkey
Lingxin Chen,
Yantai Institute of Coastal Zone Research (CAS), China

****Correspondence:***
Zhenbo Xu
zhenbo.xu@hotmail.com
Xihong Zhao
xhzhao2006@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *10 February 2018*
Accepted: *22 May 2018*
Published: *12 June 2018*

Citation:
Zhao X, Li M and Xu Z (2018) Detection of Foodborne Pathogens by Surface Enhanced Raman Spectroscopy. Front. Microbiol. 9:1236. doi: 10.3389/fmicb.2018.01236

INTRODUCTION

Foodborne illnesses caused by foodborne pathogens, such as *Salmonella*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Escherichia coli* O157: H7, and *Shigella*, led to a serious public health problem in food safety throughout the world. People are infected with foodborne illnesses that may cause a symptom of diarrhea or even death (Havelaar et al., 2015). In recent years, the incidence rate of microorganism is 61.92%, which mainly refers to *V. parahaemolyticus*, *Salmonella*, *E. coli* O157:H7, and so on. In 2015, the national food safety risk assessment center received 176

reports of foodborne outbreaks including food poisoning through the foodborne disease outbreak monitoring report system, affecting 2,065 people, including 11 deaths. Of these incidents, 97 were caused by food production, processing, and business operations, affecting 1,533 people, of whom 1 was killed; 79 of them were caused by non-food production and processing operations and 532 were sick, of whom 10 were killed (Yang et al., 2017). Additionally, according to the Centers for Disease Control and Prevention (CDC) 2016 estimates, foodborne diseases active surveillance network (FoodNet) identified 24,029 cases, 5,512 hospitalizations, and 98 deaths caused by confirmed or culture-independent diagnostic tests (CIDTs) positive-only infections. The largest number of confirmed or CIDT positive-only infections in 2016 was reported for *Campylobacter* (8,547), followed by *Salmonella* (8,172), *Shigella* (2,913), *E. coli* (1,845), *Cryptosporidium* (1,816), *Yersinia* (302), *Vibrio* (252), *Listeria* (127), and *Cyclospora* (55). The proportion of infections that were CIDT positive without culture confirmation in 2016 was largest for *Campylobacter* (32%) and *Yersinia* (32%), followed by *E. coli* (24%), *Shigella* (23%), *Vibrio* (13%), and *Salmonella* (8%). The overall increase in CIDT positive-only infections for these six pathogens in 2016 was 114% (range = 85–1,432%) compared with 2013–2015. Among infections with a positive CIDT result in 2016, a reflex culture was attempted on approximately 60% at either a clinical or state public health laboratory. The proportion of infections that were positive was highest for *Salmonella* (88%) and *E. coli* (87%), followed by *Shigella* (64%), *Yersinia* (59%), *Campylobacter* (52%), and *Vibrio* (46%) (Kapaya et al., 2018). Among them, *Salmonella* is the main cause of the global infection, and the risk of children is much higher than that of the adult (Ao et al., 2015). From the above analysis and summary, a rapid and effective method of the detection of foodborne pathogens should be urgently needed to guarantee the food safety and human health.

The traditional methods to detect foodborne pathogens are different according to the different requirements. The common and widespread method is to inoculate micro-organisms on the agar plate. First of all, we should cultivate microbes for several days, and then the microorganisms can be differentiated according to the corresponding physiological and biochemical features when they are cultured. From the above, it is these tedious processes that limit the extensive application of the method in reality. On the one hand, it will take a lot of time to identify bacterial pathogens and operate the complicated experiment. On the other hand, it is difficult to accurately tell some pathogenic bacteria with extremely similar physical and chemical features (Du et al., 2003). At present, with the rapid development of detection technology of foodborne pathogens, the technique is moving rapidly from traditional cell culture to modern detection technology, such as immunoassays technique (Chemburu et al., 2005), biosensor (Pedrero et al., 2009), PCR (Guan et al., 2013), gene probe (Shigemura et al., 2005), as well as impedance method (Cady et al., 1978), and so on. We have summarized their advantages and disadvantages in detail in **Table 1**. It can be seen from the table that although these techniques with good rapidity and effectiveness, these techniques are unable to detect bacterial pathogens at low concentrations (Zhao et al., 2014). Obviously, different detection methods have their own advantages and disadvantages, as reported by Cho and Ku (2017). Therefore, there is an urgent need for a detection method that has the advantages of minimum sample pretreatment, low cost, high sensitivity, good repetitiveness, and good on-site interpretation. The most important thing is that it can detect the lower concentration of the sample as easily as possible.

Spectroscopy techniques have been applied to the detection of foodborne pathogens in food, such as Raman spectrum, infrared spectroscopy, fluorescence spectroscopy, and other spectral detection techniques. One of them is the infrared spectroscopy that identifies the pathogenic bacteria from the food in order to obtain "fingerprints map." And then the map will be compared and analyzed to get the composition of the bacterial cell wall and the information of target from the molecular characteristics of specific peaks. Ultimately, we can base on the above message to distinguish between different foodborne pathogens. So it is of great significance to study the mechanism of the infrared spectrum to establish a rapid detection method (Alvarezordóñez and Prieto, 2012). However, infrared spectrum is unsuitable to detect the samples in solution conditions, which causes it not to give full play to its detection advantages (Lu et al., 2011). Similarly, Raman spectroscopy also relies on the inelastic scattering of excitation light and molecular resonance to generate "fingerprints map," which can be used to get the information of specific biomolecules. Raman spectroscopy is a real-time detection method that has the ability to quickly and effectively detect a variety of chemical structures and material composition (Craig et al., 2013). Compared with Raman spectroscopy, the detection range of infrared spectroscopy is narrower. Raman spectroscopy can provide more specific and readable biological or chemical information than the infrared spectrum over a wide range of laser wavelengths. But Raman spectroscopy has been less widely used than infrared spectroscopy due to its relative weak signals, fluorescence interference, and high cost of equipment. Hence, there is an urgent need for a better way to detect foodborne pathogens.

As a result, the surface enhanced Raman spectroscopy is developed during more widespread and broader applications in different fields. This technology combining the advantages of infrared spectroscopy and Raman spectrum can not only detect samples in solution, but also it provides higher discrimination and sensitivity by using noble metal nanostructures to enhance the low-concentration single molecule Raman signal to several orders of magnitude (typically 10^7 to 10^{14}) (Sengupta et al., 2006). It is found that weak Raman scattering signals are greatly enhanced using noble-metal nanoparticles, while fluorescence is suppressed, as shown in **Figure 1**. Therefore, the identification and detection of targets by SERS has drawn much attention recently due to its high efficiency, sensitivity, and stability in aqueous solution. Because SERS can let some molecules to be

TABLE 1 | Emerging technologies for the detection of pathogens.

Technique	Method	Advantages	Disadvantages
Immunological methods	Enzyme-linked immunosorbent assay (ELISA)	High specificity and sensitivity	Complex and narrow detection span, some cross-reaction
	Immunomagnetic separation assay (IMS)	Efficient and good specificity	Higher cost
	Immunoblot technique (IBT)	High resolution and high sensitivity	Complex operation
Metabolic technology	Microcalorimetry	Strong versatility and applicability	Long cycle and weak heat signal
	ATP bioluminescence technology	Fast, easy, and high sensitivity	It is difficult to distinguish between microbial and non-microbial ATP
	DNA probe	Specificity and fast, accurate	The added markers are hard to solve
	PCR, Multiplex, PCR	Reliable and efficient, linearity	Error due to non-target DNA amplification
Molecular biology technology	Quantitative PCR	Automated and real-time, high accuracy	Demanding equipment and expensive fluorescent probes, photo bleaching
	Isothermal amplification	High sensitivity and specificity	Difficult primer design
	microarray technique	High throughput and high efficiency, automated	Gene chip preparation and testing costs are high
	Electrochemical biosensors	Simple and good repeatability	Homogeneous sample necessary
Biosensor-based methods	Optical biosensors	Fast and real-time, label free	High equipment requirements
	Piezoelectric biosensors	Automated and high sensitivity	High equipment requirements

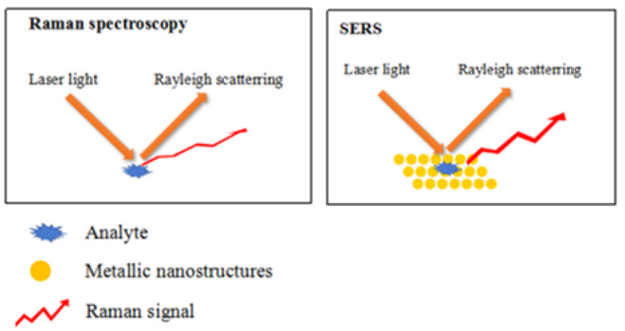


FIGURE 1 | Schematic illustration of the difference between Raman spectroscopy and SERS.

adsorbed to the surface of some rough metal (such as gold and silver), which make them interact between each other to greatly enhance the signal of the Raman spectroscopy (Najafi et al., 2014). This special phenomenon is called surface enhanced Raman spectroscopy. It is because of its good and specific characteristics that the SERS was widely applied into the food industry (Zheng and He, 2014), pesticide residue (Liu et al., 2013), medical field (Han H.W. et al., 2009), environment (Woo et al., 2012), and so on. For example, Xie et al. (2017) combined the hybrid molecularly imprinted polymer with SERS to obtain a novel biosensor to detect chloramphenicol in milk. In addition, Jiang et al. (2018) used Ag-nanorod arrays for the detection of chemical residues in food oil via SERS. From the above-related research papers, SERS is still a hotpot today.

Nevertheless, although many targets combined with nanoparticles are enhanced by SERS, how to clearly specify of these signals of the observed vibrational signatures in the SERS and to determine the cellular mechanisms that explain the specific vibrational characteristics of these targets remains a problem. Zeiri et al. (2004) prepared silver colloid mixed with bacteria to explore the strong and highly specific Raman signal of various chemical components. The results showed that it was related to the biochemical components in bacteria, such as DNA, carboxylates, and perhaps phosphates. Premasiri and Ziegler (2010) equally credited the molecular origin of these signals to molecular components at the outer layer of the cell walls. In addition, some other inquiry experiments have been done, but the specific mechanisms for molecular enhancement have not yet been fully understood.

Consequently, if we want to understand how SERS is able to detect foodborne pathogens more quickly and effectively, we need to look through some relevant concepts in general. For instance, Stöckel et al. (2016) recently reported a review on how to quickly and reliably detect microorganisms under different conditions, including real-life environments and different parts of the body fluids in human. Liu et al. (2017) also summarized the latest trends and developments in the detection of pathogens based on labeled and label-free SERS. But there are few related literatures about the identification and detection of pathogenic bacteria by SERS in food due to the fact that the food components interfere with the signal of the bacteria. As a result, this paper comprehensively reviews the current trends and developments of pathogen detection by SERS and summarizes the achievements. This paper is mainly aimed at talking about the detection of bacteria, which contains related progress of working principle, signal source, and active substrate. The advantages and limitations of SERS in detecting foodborne pathogens are also discussed here. In addition, SERS combined with other technologies will be highlighted. Finally, we will mention some potential future developments of SERS technique in the field of foodborne pathogens and some problems that need to be solved urgently.

THE BACKGROUND AND PRINCIPLE OF SERS

Initially, vibrational spectroscopy methods, such as infrared spectroscopy (Randall, 1927) and Raman spectroscopy (Raman, 1928; Raman and Krishnan, 1928), were introduced in the early 20th century and have quickly developed as rapid and non-destructive tools in different fields. However, due to its weak inelastic scattered signals, so this technique compared to infrared spectroscopy was less applied. It was not until 1974 that Fleischmann and others discovered that pyridine molecules were absorbed onto the roughened metallic surface, causing a significant enhancement of the Raman signals by many orders of magnitude (Fleischmann et al., 1974). But they did not realize that was a new physical phenomenon at that time. Subsequently, Van Duyne and his team found through experimentation and systematic calculations that there are six orders of magnitude increasing relative to common Raman scattering signal of molecular in water environment, which was called SERS (Jeanmaire and Duyne, 1977). Not long after that, it is also due to the discovery of the SERS, which makes it quickly applied to various fields and has become a hot spot of research in all walks of life.

Since the late 1990s, SERS technology has been applied to the food detection, which mainly promoted by the need for fast and sensitive tools to detect food contaminants. In fact, the main application of SERS in food science at that time was to detect chemical and microbiological hazards rather than analyzing food components (Li and Church, 2014). SERS is currently used in food analysis which includes thiram (Guo et al., 2015), melamine (Lang et al., 2015), and ciprofloxacin (Xie et al., 2013a). Compared to traditional HPLC, GC and Raman, SERS is still an emerging technology in food analysis. Although the application of SERS is less relative to them, its application has increased a lot. Several years later, SERS has also been developed as a diagnostic tool for the detection of foodborne microorganisms through Raman fingerprinting, such as *Salmonella* (Chen et al., 2017), *Staphylococcus aureus* (Zhang et al., 2015), and *E. coli* (Pandey et al., 2017).

Despite the Raman signal of various components has been detected through SERS, some explanations of the observed vibration signatures of these signals have not yet been clarified. Thus, how to clearly specify the molecular origin of the vibrational signatures that appear in the SERS spectra of bacterial cells and to determine the cellular mechanisms that explain these species-specific vibrational signatures have been attracted much attention. Efrima and Zeiri (2010) discussed why SERS can be used to detect bacteria and the main reasons causing some differences between the spectra of several bacteria. SERS can be used to detect bacteria mainly due to its good Raman signal and can be used to detect single molecule level. The two main reasons that cause the differences of Raman signal are the internal or external differences of cells, which implied the production of the colloids of cellular interior or the component of cell wall ("external"). Premasiri and Ziegler (2010) discovered the SERS spectra of bacterium mainly related to adenine on the cell wall. Kubryk et al. (2016) combined a stable isotope approach with SERS to study the origin of the band at around 730 cm^{-1} in the SERS spectra of bacteria, which was assigned to adenine-related compounds. Premasiri et al. (2016) again studied the difference of Raman spectra of bacteria at 785 nm related to adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP. Therefore, by judicious selecting the appropriate excitation wavelength and SERS-active media (e.g., gold and silver), and understanding the pre-resonance and non-resonance conditions of various cellular components, the repeatable Raman spectrum of bacteria can be simply and effectively obtained.

There have been several investigations related to the use of SERS for the detection of bacteria. The first report of bacterial SERS spectra by Efrima and Bronk (1998) used silver colloid substrate to detect *E. coli* and explain that the major bands are related to peptides and polysaccharides in cell walls and their membranes. Latest reports have shown that SERS can be used for bacterial identification under different conditions from those reported by Efrima. In addition, because *Bacillus* and *Clostridium* species is the presence of a large proportion of dipicolinic acid (DPA), in order to rapidly detect bacterial spores at low concentrations and to distinguish them better, the DPA can be used as a potential biomarker for the detection of spores. Zhang et al. (2005) used a low-cost SERS biosensor to detect the bacterial spores by targeting the DPA biomarker.

Thus, SERS mainly refers to as SERS substrates, which are necessary for SERS measurements and their SERS activity. SERS mainly refers to the use of the rough metal surfaces or metallic nanoparticles through special preparation, in a certain excitation region, the sample interacts with the special preparation of well-performing SERS substrates to enhance Raman signal of the surface or near molecular (Meheretu et al., 2014), as shown in **Figure 2**. However, the molecular mechanism of the SERS enhancement has not been clearly demonstrated. At present, there are two broad categories of SERS mechanisms commonly accepted: chemical enhancement mechanism (Gersten et al., 1979) and physical enhancement mechanism (Gersten and Nitzan, 1980). The Raman signal of SERS signal is closely related to the material of active substrate. The preparation of a good SERS active substrate is precondition for obtaining a more efficient Raman signal. Different active substrates have different enhancement effects on the samples. For example, the material of active substrate (Kleinman et al., 2013), the shape and size of the nanoparticles, the number of probes adsorbed on the active substrate, and the distance between them all affect the enhancement efficiency of SERS (Zhang et al., 2012).

ACTIVE SUBSTRATE OF SERS

Surface enhanced Raman spectroscopy is a novel technology of detection and analysis with high sensitivity, high testability, and good structural information of molecules through the characteristic peaks of Raman spectrum. Therefore, whether SERS is able to be developed an analysis method applied to practical application in the future, the key to the study of SERS

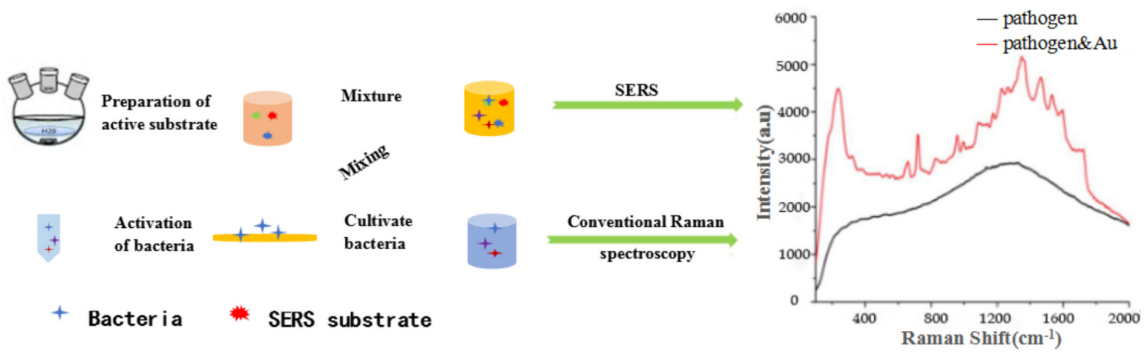


FIGURE 2 | Schematic illustration of the overall procedure for SERS detection of pathogens combined with active substrates.

is to prepare active substrates with high sensitivity, good stability, good reproducibility, and high selectivity.

At present, the most all-pervading and traditional active substrates are noble metal colloids (Rycenga et al., 2010), roughened noble metal surfaces (Moskovits, 1978), nanosphere self-assembly (Haynes and Duyne, 2016), template-directed deposition (Hyunhyub and Tsukruk, 2008), core-shell nanomaterial (Shen et al., 2013), and so on. Among them, the noble metal colloids in suspensions or aggregation are used mostly for SERS detection because of their low cost, easy preparation, and wide range of materials. However, on the one hand, the storage of noble metal colloids compared with nanostructured metal surfaces is complex, but also the nanoparticles were easy to gather and precipitates are unstable. The particle size of nanoparticles is poorly controlled. The method of template-directed deposition is easy to control the distance and improve the reproducible of nanoparticles, but it is not easy to save and its manufacture of roughness is troublesome. The signal to noise ratio of the film substrate is low. The spherical nanomaterial prepared by self-assembly is easily affected by the array and the diameter and spacing of nanomaterial (Huang et al., 2015; Li et al., 2017). The advantages of rough metal surfaces are that they are easy fast to operate, but they are easily absorbed by other substances, affecting results accuracy of SERS detection (Zheng et al., 2016). In a word, various SERS active substrates have their benefits and their shortcomings. We should be based on the requirement of a different environment to choose an optimal active substrate for SERS measurement. Here, we take colloid-based substrates as an example.

There are some advances in colloid-based substrates including the preparation of multi-component nanoparticles, such as Au-coated ZnO nanorods (Sinha et al., 2011), AgNP-coated amino-modified polystyrene microspheres (Zhao et al., 2013), Au-core shell silica nanoparticles (Au@SiO_2 nanoparticles) (Quyen et al., 2013), silver-coated gold nanoparticles (Au@AgNPs) (Murshid et al., 2013), β-cyclodextrin coated SiO_2@Au@Ag core/shell nanoparticles (Lu et al., 2015), porous Au–Ag alloy nanoparticles (Liu et al., 2016), and rhodamine derivatives (RhD) grafted Au@Ag core–shell nanocubes (CSNs) (Li et al., 2018). Nanoparticles with extremely high SERS activity and uniform surface morphology have been reported. For example, Au@SiO_2 nanoparticles are synthesized with various silica shell thicknesses to form core/shell nanoparticles, then it was applied to detect Rhodamine 6G, which was found to show a good enhancement of SERS signal (Quyen et al., 2013). In the same way, in order to improve the stability of SERS active substrate in various environments, Au@AgNPs are synthesized to exhibit different Raman activities and stability by controlling the uniform deposition of gold and minimizing galvanic replacement. The Raman enhancement of Au@AgNPs is stronger than single nanoparticles (Murshid et al., 2013).

On the one hand, different reporter molecules are selected to have different effects on the activity of SERS active substrates, such as organic dye. On the other hand, in order to obtain a stable and reproducible active substrate, different surface coatings need to choose, which will affect the activity of SERS active substrate (Wang et al., 2013c). Besides, with some special methods or materials, the activity of the active substrate can also be greatly improved. For example, with the high efficiency and accuracy of the microfluidic system, Li and Zhang prepared a low-cost and feasible SERS active substrate by depositing nanoparticles on a paper substrate and successfully detecting Rhodamine 6G at a low concentration (Li et al., 2013; Zhang et al., 2014). According to its characteristics, there are mainly two ways: label method and label-free method, as shown in **Figure 3**.

SERS FOR PATHOGEN BACTERIA DETECTION

Label Method

In recent years, the differential detection of bacteria based on SERS requires many labeled elements, such as peptides, antibodies, and carbohydrates (Wang G. et al., 2010). This method that nanoparticles are modified by specific Raman reporting molecules and target recognition elements has a high Raman activity in sensitivity. This can also be called an indirect detection of target molecules by SERS. The preparation of SERS tags for bacterial detection requires multiple steps, including the design of SERS active substrates, the attachment of reporter, and the design of surface coatings and surface ligands, as shown in **Figure 4**.

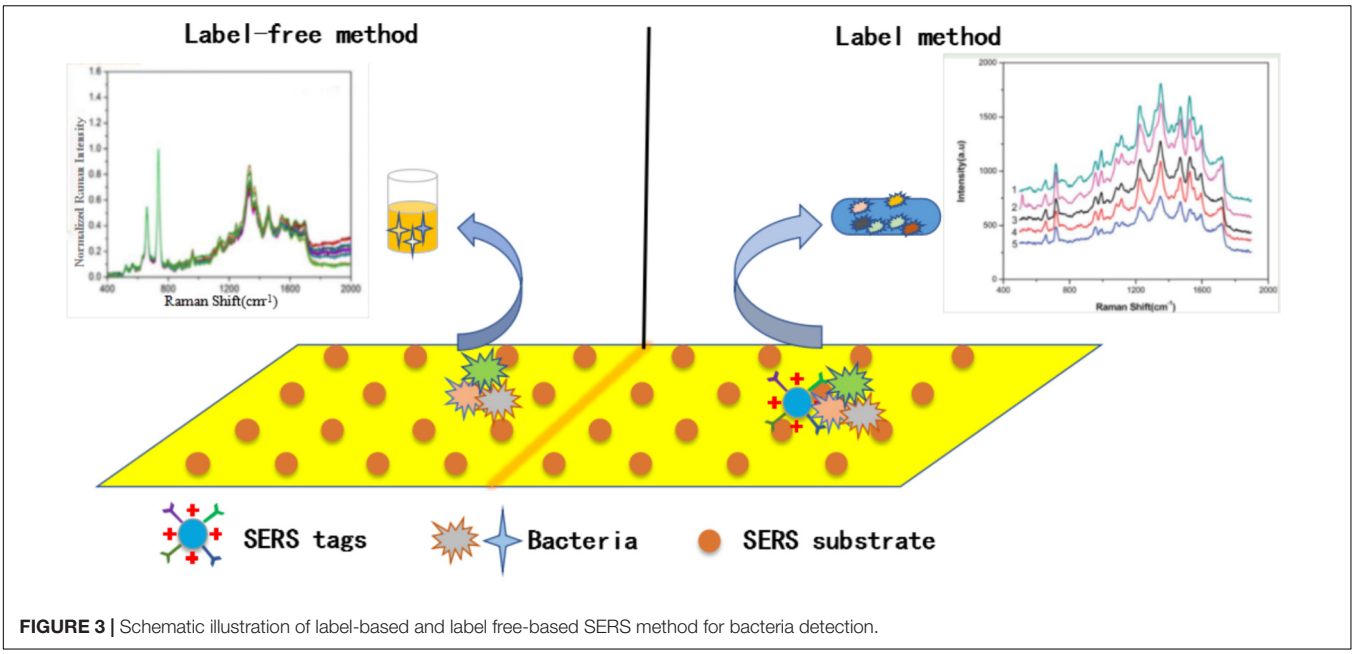


FIGURE 3 | Schematic illustration of label-based and label free-based SERS method for bacteria detection.

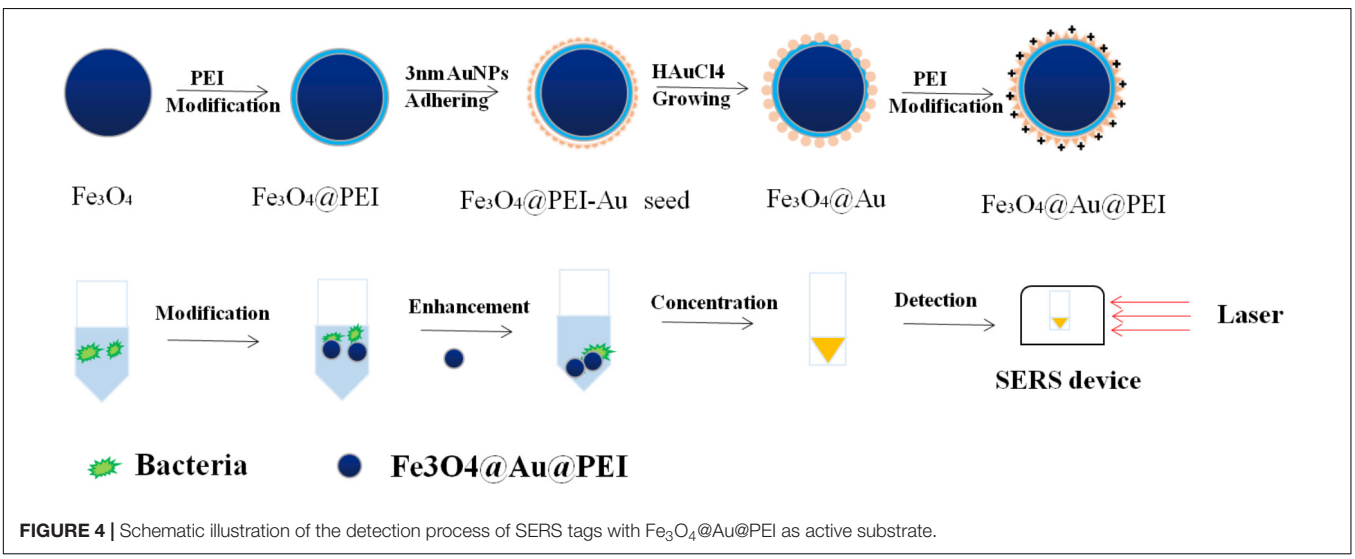


FIGURE 4 | Schematic illustration of the detection process of SERS tags with Fe_3O_4@Au@PEI as active substrate.

Lin and Hamme (2014) modified gold nanoparticles with the help of Rhodamine 6G to obtain specific active SERS substrates. It was the first time that this method was applied to detect multiple drug-resistant *Salmonella* DT104 by SERS, and then the active substrate was characterized through electron microscopy to acquire the TEM image of *Salmonella*. The results showed that it was better to compare photo thermal response of the hybrid nanomaterial than single-walled carbon nanotubes (SWCNTs) and gold nanoparticles. Through this research approach, it can increase its great potential for rapid detection and photo-thermal therapy of clinical samples. Zhang H. et al. (2016) fabricated ordered hierarchical micro/nanostructured arrays with monolayer colloidal crystals as masks. They controlled the experimental conditions to synthesize different the morphology of hierarchical micro/nanostructured arrays, and then 4-aminothiophenol was added into active SERS substrate to measure the signal of surface Raman spectrum. By comparing different morphologies of nanostructured arrays and optimizing them, nanostructured arrays with better Raman signals were obtained. This provides a new direction for the preparation of active substrates and gets a higher Raman signal.

Penn et al. (2013) developed a kind of antibody-modified membrane which was used to act a function of SERS immunoassay of nanoparticle. The thin layer of gold was deposited on a polycarbonate track etched film, the capture antibody was immobilized on the surface of the gold plating film by coupling chemistry to serve as a capture substrate. And the target and Raman reporter were transported to the capture substrate via a syringe. The membrane was used to identify the

feasibility of SERS-based immunoassay. In this article, an SERS-based immunoassay assay conducted on a membrane filter that implements flow was the first time to be applied to enhance Raman signal and significantly shorten the time of experiment.

In order to better capture the target and maintain the stability of the nanoparticles, Wang J. et al. (2016) used label-free method for the detection of bacterial pathogens by SERS. Based on the combination of polyethylenimine-modified Au-coated magnetic microspheres (Fe_3O_4@Au@PEI) and concentrated Au@Ag nanoparticles, which were called the capture-enrichment-enhancement (CEE) three-step method. Subsequently, this active substrate based on SERS was used to detect microorganisms in tap water and milk samples as well as to measure the minimum limits of *E. coli* and *S. aureus*. On the one hand, the results show that this method is not only suitable for general detection, but the Raman signal of detection by SERS is higher than before. On the other hand, the detection of foodborne pathogens by three-step method shows that the method has the advantages of shorter measuring time, simple operation steps, and higher sensitivity than the previously reported method based on SERS for the detection of bacteria. Wang J. et al. (2016) synthesized an Au-coated magnetic nanoparticles core/shell nanocomposites with nanoscale rough surfaces, which was characterized by its highly uniform in size and shape. When used for the detection of *S. aureus*, this active substrate exhibits excellent and good SERS activity.

However, in order to further improve the detection sensitivity, there are many studies using a "sandwich-type" assay combined with SERS technology to detect pathogens. At first, the antibody is immobilized on the surface of the substrate (i.e., the immunological substrate). And then the bacteria to be detected in the solution were specifically captured by the immunological substrate. Finally, the nano-metal sols labeled with the antibody and the Raman probe carried out immune recognition (Dong et al., 2014; Hou et al., 2014). The Raman signal of the testing molecules is obtained through the SERS.

Wang et al. (2011) have done a lot of research on immunoassay and obtained progress. In 2011, they used silica-coated magnetic nanoparticles as a magnetic probe to form a sandwich assay, which was applied into the detection of foodborne pathogens by SERS, such as *Salmonella* and *S. aureus*. What is more, the limit of peanut and spinach which were included in multiple pathogen detection were detected by SERS. Wang et al. (2013b) designed a specially reduced the antibody half-fragment. The signal of SERS was greatly improved by the interactions of sandwiched antibody–antigen to provide an efficient and convenient means for SERS immunoassay platform and expand the application for SERS. Based on SERS, Han X.X. et al. (2009) established a specific method using a sandwich-based immunoassay to set up the gold–protein–gold (Au/Au) and gold–protein–silver (Au/Ag) sandwiches assay, which resulted in high reproducibility of SERS signal. It was also found that the latter signal of SERS was stronger than the former about seven times, due to the different contributions of the two metal layers to the SERS. This kind of sandwiched structure provided higher sensitivity and repeatability for the detection by SERS.

On the one hand, though the label-based SERS has the advantage of higher sensitivity and repeatability and can be applied to the detection of multiplex pathogenic bacteria, organic dye may bring about the message of microbes lost. On the other hand, the addition of organic dyes may increase the experimental analysis time. In addition, the preparation of SERS tags is complex and costly. Therefore, there is an urgent need to develop rapid, simple, and low-cost SERS methods.

Label-Free Method

The label-free SERS method is a direct detection method. Because there is no special need to add into active substrate during the detection of targets by SRES, such as dye molecules. So Raman's signal will not be disturbed by other components and the information obtained is more accurate and reliable. In addition, this kind of detection method is widely used because of its simple, rapid, low cost, and rich information of molecules detected by SERS. Here we primarily discuss two types of SERS substrates including their advantages and limitations of applications in detecting pathogenic bacteria, because they are the most common, the simplest active substrates.

Metallic Colloid-Based SERS Method

The metallic colloid (such as gold and silver) is most widely used and studied for the SERS measurements. The common and effective synthesis method used for this type of nanoparticles is the chemical reduction, as shown in **Figure 5**. The method mainly depends on the interaction between the bacterial liquid and the SERS active substrate in solution to directly obtain the Raman signal of molecules. On the one hand, they are inexpensive and easily prepared. On the other hand, this method operated on the material from the molecular level, so that relatively uniform nanoparticles can be obtained. In addition, the reaction in the solution can better control the size and shape of nanoparticles. The rough preparation process is shown in **Figure 3**. Noble metal nanoparticles mainly including gold (Au) and silver (Ag) colloids in diameters between 10 and 200 nm are widely used to detect variable analytes.

Based on SERS, Cowcher et al. (2013) mixed silver nanoparticle colloids with the bacterial liquid for rapid detection of *Bacillus* and other pathogens. SERS is used to detect *Bacillus* in food because of its fast analysis speed and high sensitivity. Compared with the microscopy, the results showed that the SERS can more rapidly and efficiently detect DPA biomarker, which served as a marker of bacillus *in vivo*. The method has the advantage of being convenient, readable, and cheap. However, the limitation of this method is the extraction of the DPA from the spores. Mungroo et al. (2016) developed a microfluidic platform for the detection of pathogenic bacteria by SERS based on silver nanoparticle active substrates. SERS was combined with stoichiometry, principal component analysis, and linear discriminant analysis to effectively and quickly identify eight common species of foodborne pathogens. And then each species of pathogen was created a series of peak assignments which established a unique identification of each species, but it was still a bit difficult to identify Gram-negative and Gram-positive bacteria. However, this study provides a good reference

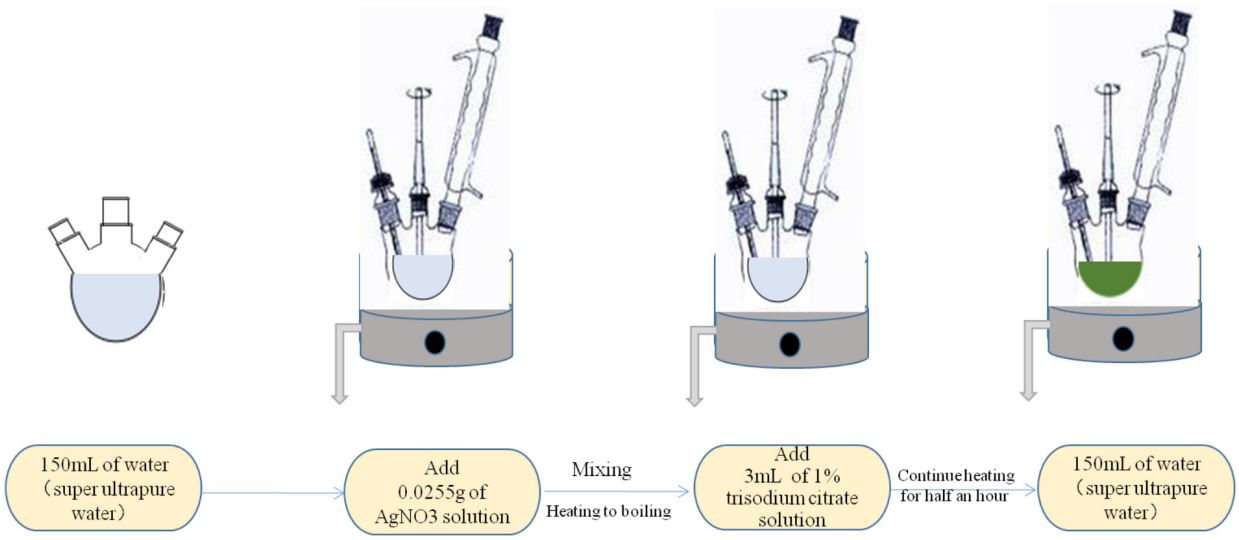


FIGURE 5 | Schematic illustration of the method of preparation silver colloidal nanoparticles by chemical reduction.

for the detection of multiple pathogens in the food industry. Xie et al. (2013b) mixed different batch gold colloid with seven different bacteria to distinguish the foodborne pathogens. From the results, the gold colloid is beneficial to rapidly and sensitively detect foodborne pathogens through the SERS.

According to the above description, most of studies focus on the SERS effect form single metal nanoparticles, so there are some shortcomings; for example, the poor enhancement effect of single gold nanoparticles and the poor stability and homogeneity of silver. In order to better integrate their advantages, there are also metal nanocomposite for the measurement of target by SERS. For instance, many nanolayer shells on Ag cores (such as Ag/carbon, Ag/Au, and Ag/SiO2) were fabricated to improve the Ag nanostructure's time-stability. Khlebtsov et al. (2015) prepared uniform Au@Ag core/shell cuboids and dumbbells with controllable Ag shells of 1–25 nm in thickness, which provided a comparation of new active substrates for targets in low concentrations with high SERS response of internal molecules in core/shell metal nanostructure. Stated thus, metallic colloids are widely used for the detection of foodborne pathogens by changing its preparation conditions, shape, and size. Although this particle is low in cost and easy to prepare, the stability of this particle is not good, which may have a certain impact on the results of the detection.

Nanostructured Metal Surface-Based SERS Method

As is well known to us, the metal nanoparticles with a good and stable Raman signal are cheap and easy to prepare. However, due to its poor repeatability, the application and development of metal nanoparticles is limited. Therefore, in order to improve the repeatability of active substrate for pathogenic bacteria detection, many researchers turned to the pathogens detection by SERS based on the nanostructured metal surface.

Wang Y. et al. (2010) assembled silver nanoclusters (AgNCs) to form spheres, which was used as SERS-active substrate with the diameter of 60–80 nm. As a result, it was found that not only the Raman signal of SERS can be enhanced to 10^8, but also the sensitivity of the SERS detection was improved and outstanding repeatability was achieved. What is more, three kinds of pathogens, as well as live and dead bacteria, were also detected and classified by SERS based on the special active substrate. With in-depth study of the active substrate, it provides the possibility the detection of single cell. Kee et al. (2013) used a plasmonic nanohole sensor to quickly, efficiently, and quantitatively monitor the bacterial growth and antibiotic sensitivity. The plasmonic nanohole arrays were fabricated by a mask-based deep ultraviolet lithography method and were measured the characteristic of bulk refractive index sensitivity and surface mass sensitivity. With *E. coli* as an experimental object, the effect of the plasmonic nanohole sensor was tested. According to analysis, *E. coli* was specifically captured and rapidly grew on its surface. In addition, the sensor was found to be able to detect antibiotic sensitivity rapidly and efficiently within 2 h. These studies are beneficial to the clinical application of bacterial infection.

In 2009, by self-assembly technique for preparing solid substrate of SERS, Yan et al. (2009) developed in identification of three types of foodborne pathogens, which included *E. coli*, *Bacillus cereus*, and *S. aureus*. From the result, there was a strong reproducible SERS signal, and the signal not only came from small molecules, but also from the bacterial cells. A few years later, the researchers also studied the reasons for the SERS enhancement signals by using the self-assembly technique guided based on the template (Reinhard and Dal Negro, 2011).

Based on the SERS, Wu et al. (2013) prepared silver nanorod array substrates that were applied to detect the pathogenic bacteria in food, such as mung bean sprouts, spinacia oleracea, and romaine lettuce. By optimizing the length of silver nanorod array, the minimum value of Raman signal of pathogenic bacteria detected by SERS was obtained. At the same time,

the mathematical analysis method was used to reduce the experimental operation cycle. These provide a powerful platform for SERS to detect foodborne pathogens at low concentrations and expand the prospect of practical applications. It also plays a solid foundation for the popularization and application of SERS technology.

Overall, variable active substrates have the weaknesses and strengths of different functions. Just like we need to choose different methods to detect bacteria, we should reasonably select the suitable active substrates for our test object to avoid unnecessary errors (Law et al., 2015).

OTHER

The SERS is not only used for the detection of foodborne pathogens in foods, but also it has many applications in other aspects. Besides, there are also many improved methods to enhance the Raman signal of SERS active substrates, so as to realize the effective and rapid detection of different components by SERS.

In order to extend the application of SERS tags, recently several SERS-related multimodal probes had been developed. Park et al. (2009) combined functionalized gold nanorods with the biomarker molecules of the analyte to allow this SERS substrate to target and image the targets. For instance, SERS tags were integrated with fluorescence, which was a more novel study. Lin et al. (2014) used a novel graphene oxide to encapsulate gold nanoparticles and then functionalized it to obtain a good Raman signal. This active substrate was used to rapidly detect foodborne pathogens and sensitive Raman imaging of *S. aureus* and *E. coli*. In the same year, the institute had developed a fluorescent SERS dual-mode tag that combines the lanthanide-based upconversion nanoparticles with near-infrared lasers to achieve the first biological imaging of living cells and *in vivo*, which provides infinite possibility for medical applications in future (Niu et al., 2014; Zhang et al., 2014).

As for other applications, Wang et al. (2013a) based on 4-mercaptopyridine (4-MPY) to obtain functionalized silver nanoparticles, which were used for the detection of heparin through SERS. The limit of heparin was acquired in terms of sensitivity, selectivity, and linearity. The method exhibited satisfying results, which indicated a great practicality for application in real analysis and monitor other related fields. Based on lateral flow assay biosensor, Fu et al. (2016) developed a novel low-concentration quantitative analysis of a specific biomarker with SERS. The specific biomarker of selected human immunodeficiency virus type 1 DNA was sensitively and quantitatively measured successfully by labeled gold nanoparticles. Similarly, Wang J. et al. (2016) and Wang X. et al. (2017) used labeled active substrate to simultaneously detect dual DNA markers, which were related to Kaposi's sarcoma-associated herpesvirus and bacillary angiomatosis. The result showed that Raman signal of target became more sensitive than previous studies. In addition, for medical research, it is very important for us to pay more attention to accurate analysis of specific biomarkers in clinical aspects. Cheng et al. (2017) combined magnetic beads with SERS tags to achieve the detection of the early diagnosis and treatment of cancer. The result demonstrated that SERS is promising for application in the accurate diagnosis of cancer.

In a word, SERS may become a powerful tool in any field in the future. What is more, this technology will continue to be developed and improved to apply to more aspects.

SERS COMBINED WITH OTHER TECHNOLOGIES

At present, SERS has been applied in various fields; however, the molecular mechanism of the interaction between the active substrate and the sample to be tested is still unclear. In the past, the molecular enhancement mechanisms of SERS have been studied only by normal detection by SERS and theoretical modeling. Nowadays, Zhang M. et al. (2016) got a good result by combining SERS with isotope tracing to demonstrate the mechanism of surface chemical reactions. On the other hand, in order to further apply SERS, Xie et al. (2013b) used SERS to detect seven kinds of pathogenic bacteria to acquire the characteristic peaks of these pathogens, and combined with principal component analysis and cluster analysis to classify these pathogens to better distinguish the categories of pathogens.

It is well known that enzyme-linked immunosorbent assay (ELISA) is also a very universal method widely used to detect foodborne pathogens, because the main characteristic of ELISA is its high sensitivity. But one important and key aspect of this approach is that the method has a preprocessing process. If the pretreatment is not properly done, due to the complex diversity of the sample, the samples containing salt, acid, metal ions, and so on will affect the results of the detection by ELISA. SERS has also been widely used in biological systems with good selectivity and sensitivity. Therefore, Ko et al. (2015) combined the ELISA with the SERS to achieve direct detection of these samples without preprocessing and improve the sensitivity of detection. What is more, Wang W. et al. (2016) obtained the SERS activity of EC-SERS by combining the ultra-microelectrode with SERS in order to obtain real-time transient Raman information, and the combination has a more stable and high signal of SERS. Similarly, in order to detect the amount of residues in samples, Wang L. et al. (2017) combined molecular imprinting with SRES detection technology to finally obtain a good specificity and sensitivity of Raman spectroscopy signals, besides the time of detection by SERS is greatly shortened. Wang W. et al. (2016) studied the materials of SERS active substrate by using the electrochemical method to improve the stability and sensitivity of SERS.

In addition, there are various techniques associated with the SERS, such as high performance liquid chromatography (Hassanain et al., 2016), infrared spectroscopy (Chen et al., 2015), and thin-layer chromatography (Lv et al., 2015). It is because of this combination techniques, making the application of SERS greatly expanded and widely used in various fields. In addition,

due to the poor reproducibility of the active substrate of the SERS technology, which limits its application to the detection of foodborne pathogens in foods for on site, while the microfluidic system called "Lap on a chip" has the advantages of low dosage, high efficiency, accuracy, and so on. The SERS-microfluidic system can overcome this shortcoming (Jung et al., 2007; Pu et al., 2017; Wang C. et al., 2017). The above shows that the purpose of the combined technology is to learn from each other and to achieve the optimization of the method, thus expanding the prospect of the application of the detection of SERS technology in various fields.

OUTLOOK

The current research has shown that SERS technology has been widely applied to the detection and identification of foodborne pathogens in food, but there are still several aspects to be further improved here. First of all, how should we pretreat the food sample before we are going to detect foodborne pathogens by SERS in food. Because the failure of the preprocessing process will affect the effect of the detection; secondly, for different types of active SERS substrate, there are many influencing factors, such as the size and shape of active substrate. How should we better control these factors to improve Raman signal? In addition, how can we better combine the SERS with other technologies? Finally, there is no researcher to collect and summarize the fingerprint and analysis information of pathogenic bacteria of SERS, so as to create a shared network platform and database for search and operation. If the "Raman fingerprinting" database and network sharing platform for many kinds of microorganisms are established, it will be able to cross the boundaries of time and region to realize the sharing of resources among multiple research institutions and improve the efficiency of scientific research. The researchers will be able to directly obtain the fingerprint of the known microorganism and match it with the microbial information identified by the traditional method, which can achieve fast and effective detection of foodborne pathogenic bacteria by SERS and solve a series of related food safety issues caused by foodborne pathogens. Therefore, how to solve these problems quickly and effectively is one of the future directions for SERS development.

CONCLUSION

After two decades of development, SERS has been widely used in various fields. With the progress of the times, this trend of development will not be weakened. For the detection of foodborne pathogens based on SERS technology is universally paid attention to. Therefore, the application of this technology to the detection of foodborne pathogens will still be a hot spot. What is more, SERS technology can achieve stable, reliable, rapid, and sensitive identification of pathogenic bacteria in food. People can set up fast, simple, specific, sensitive, and low-consumption detection by SERS according to the theory and application practice of SERS technology. Although the detection technology by SERS has not been totally found their way into products, but with the improvement of micro-fabrication technology and nanotechnology, Raman spectroscopy has been also continuously miniaturized. The emergence of a variety of portable and hand-held Raman spectrometers has made it possible to detect foodborne pathogens directly and rapidly on site. Therefore, the prospects of SERS development are unlimited.

AUTHOR CONTRIBUTIONS

XZ and ML wrote the manuscript. XZ, ML, and ZX revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

FUNDING

This work has been supported by the National Natural Science Foundation of China (31501582), Hubei Provincial Natural Science Foundation of China (2018CFB514), National Key Research and Development Program of China (2016YFD04012021), Guangdong Special Support Program (2016TQ03N682), Pearl River S&T Nova Program of Guangzhou (201710010061), and Open Project Program of Key Laboratory for Green Chemical Process of Ministry of Education in Wuhan Institute of Technology (2017007).

REFERENCES

Alvarezordóñez, A., and Prieto, M. (2012). Technical and methodological aspects of fourier transform infrared spectroscopy in food microbiology research. *Springerbriefs Food Health Nutr.* 96, 1–18.

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J., and Crump, J. A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerg. Infect. Dis.* 21, 941–949. doi: 10.3201/eid2106.140999

Cady, P., Dufour, S. W., Shaw, J., and Kraeger, S. J. (1978). Electrical impedance measurements: rapid method for detecting and monitoring microorganisms. *J. Clin. Microbiol.* 7, 265–272.

Chemburu, S., Wilkins, E., and Abdel-Hamid, I. (2005). Detection of pathogenic bacteria in food samples using highly-dispersed carbon particles. *Biosens. Bioelectron.* 21, 491–499. doi: 10.1016/j.bios.2004.11.025

Chen, J., Park, B., Huang, Y. W., Zhao, Y., and Kwon, Y. (2017). Label-free SERS detection of *Salmonella* Typhimurium on DNA aptamer modified AgNR substrates. *J. Food Meas. Charact.* 11, 1773–1779. doi: 10.1007/s11694-017-9558-6

Chen, L., Mungroo, N., Daikuara, L., and Neethirajan, S. (2015). Label-free NIR-SERS discrimination and detection of foodborne bacteria by in situ synthesis of Ag colloids. *J. Nanobiotechnol.* 13, 1–9. doi: 10.1186/s12951-015-0106-4

Cheng, Z., Choi, N., Wang, R., Lee, S., Moon, K. C., Yoon, S. Y., et al. (2017). Simultaneous detection of dual prostate specific antigens using surface-enhanced raman scattering-based immunoassay for accurate diagnosis of prostate cancer. *ACS Nano* 11, 4926–4933. doi: 10.1021/acsnano.7b01536

Cho, I. H., and Ku, S. (2017). Current technical approaches for the early detection of foodborne pathogens: challenges and opportunities. *Int. J. Mol. Sci.* 18:E2078. doi: 10.3390/ijms18102078

Cowcher, D. P., Xu, Y., and Goodacre, R. (2013). Portable, quantitative detection of *Bacillus* bacterial spores using surface-enhanced Raman scattering. *Anal. Chem.* 85, 3297–3302. doi: 10.1021/ac303657k

Craig, A. P., Franca, A. S., and Irudayaraj, J. (2013). Surface-enhanced Raman spectroscopy applied to food safety. *Annu. Rev. Food Sci. Technol.* 4, 369–380. doi: 10.1146/annurev-food-022811-101227

Dong, S., Zhou, J., Hui, D., Pang, X., Wang, Q., Zhang, S., et al. (2014). Interaction between edge dislocations and amorphous interphase in carbon nanotubes reinforced metal matrix nanocomposites incorporating interface effect. *Int. J. Solids Struct.* 51, 1149–1163. doi: 10.1016/j.ijsolstr.2013.12.011

Du, G., Geng, J., Chen, J., and Lun, S. (2003). Mixed culture of nitrifying bacteria and denitrifying bacteria for simultaneous nitrification and denitrification. *World J. Microbiol. Biotechnol.* 19, 433–437. doi: 10.1023/A:1023985229493

Efrima, S., and Bronk, B. V. (1998). Silver colloids impregnating or coating bacteria. *J. Phys. Chem. B* 102, 5947–5950. doi: 10.1021/jp9813903

Efrima, S., and Zeiri, L. (2010). Understanding SERS of bacteria. *J. Raman Spectrosc.* 40, 277–288. doi: 10.1002/jrs.2121

Fleischmann, M., Hendra, P. J., and Mcquillan, A. J. (1974). Raman spectra of pyridine adsorbed at a silver electrode. *Chem. Phys. Lett.* 26, 163–166. doi: 10.1016/0009-2614(74)85388-1

Fu, X., Cheng, Z., Yu, J., Choo, P., Chen, L., and Choo, J. (2016). A SERS-based lateral flow assay biosensor for highly sensitive detection of HIV-1 DNA. *Biosens. Bioelectron.* 78, 530–537. doi: 10.1016/j.bios.2015.11.099

Gersten, J., and Nitzan, A. (1980). Electromagnetic theory of enhanced Raman scattering by molecules adsorbed on rough surfaces. *J. Chem. Phys.* 73, 3023–3037.

Gersten, J. I., Birke, R. L., and Lombardi, J. R. (1979). Theory of enhance i light scattering from molecules adsorbed at the metal-solution interface. *Phys. Rev. Lett.* 43, 147–150. doi: 10.1103/PhysRevLett.43.147

Guan, Z. P., Jiang, Y., Gao, F., Zhang, L., Zhou, G. H., and Guan, Z. J. (2013). Rapid and simultaneous analysis of five foodborne pathogenic bacteria using multiplex PCR. *Eur. Food Res. Technol.* 237, 627–637. doi: 10.1007/s00217-013-2039-1

Guo, P., Sikdar, D., Huang, X., Si, K. J., Xiong, W., Gong, S., et al. (2015). Plasmonic core-shell nanoparticles for SERS detection of the pesticide thiram: size- and shape-dependent Raman enhancement. *Nanoscale* 7, 2862–2868. doi: 10.1039/c4nr06429a

Han, H. W., Yan, X. L., Dong, R. X., Ban, G., and Li, K. (2009). Analysis of serum from type II diabetes mellitus and diabetic complication using surface-enhanced Raman spectra (SERS). *Appl. Phys. B* 94, 667–672. doi: 10.1007/s00340-008-3299-5

Han, X. X., Kitahama, Y., Itoh, T., Wang, C. X., Zhao, B., and Ozaki, Y. (2009). Protein-mediated sandwich strategy for surface-enhanced Raman scattering: application to versatile protein detection. *Anal. Chem.* 81, 3350–3355. doi: 10.1021/ac802553a

Hassanain, W. A., Izake, E. L., Sivanesan, A., and Ayoko, G. A. (2016). Towards interference free HPLC-SERS for the trace analysis of drug metabolites in biological fluids. *J. Pharm. Biomed. Anal.* 136, 38–43. doi: 10.1016/j.jpba.2016.12.019

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World health organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Med.* 12:e1001923. doi: 10.1371/journal.pmed.1001923

Haynes, C. L., and Duyne, R. P. V. (2016). Nanosphere lithography: a versatile nanofabrication tool for studies of size-dependent nanoparticle optics. *J. Phys. Chem. B* 105, 5599–5611. doi: 10.1021/jp010657m

Hou, M., Wang, Y., Liu, S., Guo, J., Li, Z., and Lu, P. (2014). Sensitivity-enhanced pressure sensor with hollow-core photonic crystal fiber. *J. Lightwave Technol.* 32, 4637–4641. doi: 10.1109/JLT.2014.2358713

Huang, Z. Q., He, X. Q., and Liew, K. M. (2015). A sensitive interval of imperfect interface parameters based on the analysis of general solution for anisotropic matrix containing an elliptic inhomogeneity. *Int. J. Solids Struct.* 73–74, 67–77. doi: 10.1016/j.ijsolstr.2015.07.009

Hyunhyub, K., and Tsukruk, V. V. (2008). Nanoparticle-decorated nanocanals for surface-enhanced Raman scattering. *Small* 4, 1980–1984. doi: 10.1002/smll.200800301

Jeanmaire, D. L., and Duyne, R. P. V. (1977). Surface Raman spectroelectrochemistry: part I. Heterocyclic, aromatic, and aliphatic amines adsorbed on the anodized silver electrode. *J. Electroanal. Chem. Interfacial Electrochem.* 84, 1–20. doi: 10.1016/S0022-0728(77)80224-6

Jiang, Y., Sun, D. W., Pu, H., and Wei, Q. (2018). Surface enhanced Raman spectroscopy (SERS): a novel reliable technique for rapid detection of common harmful chemical residues. *Trends Food Sci. Technol.* 75, 10–22. doi: 10.1016/j.tifs.2018.02.020

Jung, J., Chen, L., Lee, S., Kim, S., Seong, G. H., Choo, J., et al. (2007). Fast and sensitive DNA analysis using changes in the FRET signals of molecular beacons in a PDMS microfluidic channel. *Anal. Bioanal. Chem.* 387, 2609–2615. doi: 10.1007/s00216-007-1158-6

Kapaya, F., Mwansa, F. D., Sakubita, P., Gama, A., Langa, N., Chewe, O., et al. (2018). A foodborne disease outbreak investigation experience in a College in Lusaka, Zambia, 2017. *Pan Afr. Med. J.* 29:100. doi: 10.11604/pamj.2018.29.100.14737

Kee, J. S., Lim, S. Y., Perera, A. P., Zhang, Y., and Mi, K. P. (2013). Plasmonic nanohole arrays for monitoring growth of bacteria and antibiotic susceptibility test. *Sens. Actuators B Chem.* 182, 576–583. doi: 10.1016/j.snb.2013.03.053

Khlebtsov, B. N., Liu, Z., Ye, J., and Khlebtsov, N. G. (2015). Au@Ag core/shell cuboids and dumbbells: optical properties and SERS response. *J. Quant. Spectrosc. Radiat. Transf.* 167, 64–75. doi: 10.1016/j.jqsrt.2015.07.024

Kleinman, S. L., Frontiera, R. R., Henry, A. I., Dieringer, J. A., and Van Duyne, R. P. (2013). Creating, characterizing, and controlling chemistry with SERS hot spots. *Phys. Chem. Chem. Phys.* 15, 21–36. doi: 10.1039/C2CP42598J

Ko, J., Lee, C., and Choo, J. (2015). Highly sensitive SERS-based immunoassay of aflatoxin B1 using silica-encapsulated hollow gold nanoparticles. *J. Hazard. Mater.* 285, 11–17. doi: 10.1016/j.jhazmat.2014.11.018

Kubryk, P., Niessner, R., and Ivleva, N. P. (2016). The origin of the band at around 730 cm(-1) in the SERS spectra of bacteria: a stable isotope approach. *Analyst* 141, 2874–2878. doi: 10.1039/c6an00306k

Lang, T., Pang, S., and He, L. (2015). Integration of colorimetric and SERS detection for rapid screening and validation of melamine in milk. *Anal. Methods* 7, 6426–6431. doi: 10.1039/C5AY00955C

Law, W. F., Mutalib, N. S., Chan, K. G., and Lee, L. H. (2015). Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Front. Microbiol.* 5:770. doi: 10.3389/fmicb.2014.00770

Li, B., Zhang, W., Chen, L., and Lin, B. (2013). A fast and low-cost spray method for prototyping and depositing surface-enhanced Raman scattering arrays on microfluidic paper based device. *Electrophoresis* 34, 2162–2168. doi: 10.1002/elps.201300138

Li, H., Chen, Q., Hassan, M. M., Ouyang, Q., Jiao, T., Xu, Y., et al. (2018). AuNS@Ag core-shell nanocubes grafted with rhodamine for concurrent metal-enhanced fluorescence and surfaced enhanced Raman determination of mercury ions. *Anal. Chim. Acta* 1018, 94–103. doi: 10.1016/j.aca.2018.01.050

Li, W.-L., Wu, A., Li, Z.-C., Zhang, G., and Yu, W.-Y. (2017). A new calibration method between an optical sensor and a rotating platform in turbine blade inspection. *Meas. Sci. Technol.* 28:035009. doi: 10.1088/1361-6501/aa50df

Li, Y. S., and Church, J. S. (2014). Raman spectroscopy in the analysis of food and pharmaceutical nanomaterials. *J. Food Drug Anal.* 22, 29–48. doi: 10.1016/j.jfda.2014.01.003

Lin, D., Qin, T., Wang, Y., Sun, X., and Chen, L. (2014). Graphene oxide wrapped SERS tags: multifunctional platforms toward optical labeling, photothermal ablation of bacteria, and the monitoring of killing effect. *ACS Appl. Mater. Interfaces* 6, 1320–1329. doi: 10.1021/am405396k

Lin, Y., and Hamme, A. T. II (2014). Targeted highly sensitive detection/eradication of multi-drug resistant *Salmonella* DT104 through gold nanoparticle-SWCNT bioconjugated nanohybrids. *Analyst* 139, 3702–3705. doi: 10.1039/C4AN00744A

Liu, B., Zhou, P., Liu, X., Sun, X., Li, H., and Lin, M. (2013). Detection of pesticides in fruits by surface-enhanced raman spectroscopy coupled with gold nanostructures. *Food Bioproc. Technol.* 6, 710–718. doi: 10.1007/s11947-011-0774-5

Liu, K., Bai, Y., Zhang, L., Yang, Z., Fan, Q., Zheng, H., et al. (2016). Porous Au-Ag nanospheres with high-density and highly accessible hotspots for SERS analysis. *Nano Lett.* 16, 3675–3681. doi: 10.1021/acs.nanolett.6b00868

Liu, Y., Zhou, H., Hu, Z., Yu, G., Yang, D., and Zhao, J. (2017). Label and label-free based surface-enhanced Raman scattering for pathogen bacteria detection: a review. *Biosens. Bioelectron.* 94, 131–140. doi: 10.1016/j.bios.2017.02.032

Lu, X., Al-Qadiri, H. M., Lin, M., and Rasco, B. A. (2011). Application of mid-infrared and Raman spectroscopy to the study of bacteria. *Food Bioproc. Technol.* 4, 919–935. doi: 10.1007/s11947-011-0516-8

Lu, Y., Yao, G., Sun, K., and Huang, Q. (2015). β-Cyclodextrin coated SiO2@Au@Ag core-shell nanoparticles for SERS detection of PCBs. *Phys. Chem. Chem. Phys.* 17, 21149–21157. doi: 10.1039/c4cp04904g

Lv, D., Cao, Y., Lou, Z., Li, S., Chen, X., Chai, Y., et al. (2015). Rapid on-site detection of ephedrine and its analogues used as adulterants in slimming dietary supplements by TLC-SERS. *Anal. Bioanal. Chem.* 407, 1313–1325. doi: 10.1007/s00216-014-8380-9

Meheretu, G. M., Cialla, D., and Popp, J. (2014). Surface enhanced raman spectroscopy on silver nanoparticles. *Int. J. Biochem. Biophy.* 2, 63–67.

Moskovits, M. (1978). Surface roughness and the enhanced intensity of Raman scattering by molecules adsorbed on metals. *J. Chem. Phys.* 69, 4159–4161. doi: 10.1063/1.437095

Mungroo, N. A., Oliveira, G., and Neethirajan, S. (2016). SERS based point-of-care detection of food-borne pathogens. *Microchim. Acta* 183, 697–707. doi: 10.1007/s00604-015-1698-y

Murshid, N., Gourevich, I., Coombs, N., and Kitaev, V. (2013). Gold plating of silver nanoparticles for superior stability and preserved plasmonic and sensing properties. *Chem. Commun.* 49, 11355–11357. doi: 10.1039/c3cc46075d

Najafi, R., Mukherjee, S., Hudson, J. Jr., Sharma, A., and Banerjee, P. (2014). Development of a rapid capture-cum-detection method for *Escherichia coli* O157 from apple juice comprising nano-immunomagnetic separation in tandem with surface enhanced Raman scattering. *Int. J. Food Microbiol.* 189, 89–97. doi: 10.1016/j.ijfoodmicro.2014.07.036

Niu, X., Chen, H., Wang, Y., Wang, W., Sun, X., and Chen, L. (2014). Upconversion fluorescence-SERS dual-mode tags for cellular and in vivo imaging. *ACS Appl. Mater. Interfaces* 6, 5152–5160. doi: 10.1021/am500411m

Pandey, A., Gurbuz, Y., Ozguz, V., Niazi, J. H., and Qureshi, A. (2017). Graphene-interfaced electrical biosensor for label-free and sensitive detection of foodborne pathogenic *E. coli* O157:H7. *Biosens. Bioelectron.* 91, 225–231. doi: 10.1016/j.bios.2016.12.041

Park, H., Lee, S., Chen, L., Lee, E. K., Shin, S. Y., Lee, Y. H., et al. (2009). SERS imaging of HER2-overexpressed MCF7 cells using antibody-conjugated gold nanorods. *Phys. Chem. Chem. Phy.* 11, 7444–7449. doi: 10.1039/b904592a

Pedrero, M., Campuzano, S., and Pingarrón, J. M. (2009). Electroanalytical sensors and devices for multiplexed detection of foodborne pathogen microorganisms. *Sensors* 9, 5503–5520. doi: 10.3390/s90705503

Penn, M. A., Drake, D. M., and Driskell, J. D. (2013). Accelerated surface-enhanced Raman spectroscopy (SERS)-based immunoassay on a gold-plated membrane. *Anal. Chem.* 85, 8609–8617. doi: 10.1021/ac402101r

Premasiri, W. R., Lee, J. C., Sauer-Budge, A., Théberge, R., Costello, C. E., and Ziegler, L. D. (2016). The biochemical origins of the surface-enhanced Raman spectra of bacteria: a metabolomics profiling by SERS. *Anal. Bioanal. Chem.* 408, 4631–4647. doi: 10.1007/s00216-016-9540-x

Premasiri, W. R., and Ziegler, L. D. (2010). On the molecular origin of bacterial SERS spectra. *AIP Conf. Proc.* 1267, 1087–1088. doi: 10.1063/1.3482311

Pu, H., Xiao, W., and Sun, D. W. (2017). SERS-microfluidic systems: a potential platform for rapid analysis of food contaminants. *Trends Food Sci. Technol.* 70:114. doi: 10.1016/j.tifs.2017.10.001

Quyen, T. T. B., Su, W. N., Chen, K. J., Pan, C. J., Rick, J., Chang, C. C., et al. (2013). Au@SiO2 core/shell nanoparticle assemblage used for highly sensitive SERS-based determination of glucose and uric acid. *J. Raman Spectrosc.* 44, 1671–1677. doi: 10.1002/jrs.4400

Raman, C. V. (1928). A change of wave-length in light scattering. *Nature* 121:619.

Raman, C. V., and Krishnan, K. S. (1928). A new type of secondary radiation. *Nature* 121, 501–502.

Randall, H. M. (1927). Infra-red spectroscopy. *Science* 65, 167–173. doi: 10.1126/science.65.1677.167

Reinhard, B. M., and Dal Negro, L. (2011). *Engineered SERS Substrates Employing Nanoparticle Cluster Arrays with Multiscale Signal Enhancement*. Boston, MA: Trustees Of Boston University.

Rycenga, M., Camargo, P. H., Li, W., Moran, C. H., and Xia, Y. (2010). Understanding the SERS effects of single silver nanoparticles and their dimers, one at a time. *J. Phys. Chem. Lett.* 1, 696–703. doi: 10.1021/jz900286a

Sengupta, A., Mujacic, M., and Davis, E. J. (2006). Detection of bacteria by surface-enhanced Raman spectroscopy. *Anal. Bioanal. Chem.* 386, 1379–1386. doi: 10.1007/s00216-006-0711-z

Shen, J., Zhu, Y., Yang, X., Zong, J., and Li, C. (2013). Multifunctional Fe3O4@Ag/SiO2/Au core-shell microspheres as a novel SERS-activity label via long-range plasmon coupling. *Langmuir* 29, 690–695. doi: 10.1021/la304048v

Shigemura, K., Shirakawa, T., Okada, H., Tanaka, K., Kamidono, S., Arakawa, S., et al. (2005). Rapid detection and differentiation of Gram-negative and Gram-positive pathogenic bacteria in urine using TaqMan probe. *Clin. Exp. Med.* 4, 196–201. doi: 10.1007/s10238-004-0056-x

Sinha, G., Depero, L. E., and Alessandri, I. (2011). Recyclable SERS substrates based on Au-coated ZnO nanorods. *ACS Appl. Mater. Interfaces* 3, 2557–2563. doi: 10.1021/am200396n

Stöckel, S., Kirchhoff, J., Neugebauer, U., Rösch, P., and Popp, J. (2016). The application of Raman spectroscopy for the detection and identification of microorganisms. *J. Raman Spectrosc.* 47, 89–109. doi: 10.1002/jrs.4844

Wang, C., Madiyar, F., Yu, C., and Li, J. (2017). Detection of extremely low concentration waterborne pathogen using a multiplexing self-referencing SERS microfluidic biosensor. *J. Biol. Eng.* 11:9. doi: 10.1186/s13036-017-0051-x

Wang, G., Wang, Y., Chen, L., and Choo, J. (2010). Nanomaterial-assisted aptamers for optical sensing. *Biosens. Bioelectron.* 25, 1859–1868. doi: 10.1016/j.bios.2009.11.012

Wang, J., Wu, X., Wang, C., Rong, Z., Ding, H., Li, H., et al. (2016). Facile synthesis of au-coated magnetic nanoparticles and their application in bacteria detection via a SERS method. *ACS Appl. Mater. Interfaces* 8, 19958–19967. doi: 10.1021/acsami.6b07528

Wang, L., Xu, Y., Tan, X., Tapas, S., and Zhang, J. (2017). Aim and shoot: molecule-imprinting polymer coated MoO3 for selective SERS detection and photocatalytic destruction of low-level organic contaminants. *RSC Adv.* 7, 36201–36207. doi: 10.1039/C7RA05547A

Wang, W., Huang, Y. F., Liu, D. Y., Wang, F. F., Tian, Z. Q., and Zhan, D. (2016). Electrochemically roughened gold microelectrode for surface-enhanced Raman spectroscopy. *J. Electroanal. Chem.* 779, 126–130. doi: 10.1016/j.jelechem.2016.04.008

Wang, X., Chen, L., Fu, X., Chen, L., and Ding, Y. (2013a). Highly sensitive surface-enhanced raman scattering sensing of heparin based on anti-aggregation of functionalized silver nanoparticles. *Appl. Mater. Interfaces* 5, 11059–11065. doi: 10.1021/am404142e

Wang, X., Choi, N., Cheng, Z., Ko, J., Chen, L., and Choo, J. (2017). Simultaneous detection of dual nucleic acids using a SERS-based lateral flow assay biosensor. *Anal. Chem.* 89, 1163–1169. doi: 10.1021/acs.analchem.6b03536

Wang, Y., Lee, K., and Irudayaraj, J. (2010). Silver nanosphere SERS probes for sensitive identification of pathogens. *J. Phys. Chem. C* 114, 16122–16128. doi: 10.1021/jp1015406

Wang, Y., Ravindranath, S., and Irudayaraj, J. (2011). Separation and detection of multiple pathogens in a food matrix by magnetic SERS nanoprobes. *Anal. Bioanal. Chem.* 399, 1271–1278. doi: 10.1007/s00216-010-4453-6

Wang, Y., Tang, L. J., and Jiang, J. H. (2013b). Surface-enhanced Raman spectroscopy-based, homogeneous, multiplexed immunoassay with antibody-fragments-decorated gold nanoparticles. *Anal. Chem.* 85, 9213–9220. doi: 10.1021/ac4019439

Wang, Y., Yan, B., and Chen, L. (2013c). SERS tags: novel optical nanoprobes for bioanalysis. *Chem. Rev.* 113, 1391–1428. doi: 10.1021/cr300120g

Woo, C., Yoon, K. Y., Byeon, J. H., Kim, K., and Hwang, J. (2012). Development of rapid assessment method to determine bacterial viability based on ultraviolet and visible (UV-Vis) spectroscopy analysis including application to bioaerosols. *Aerosol Air Quality Research* 12, 399–408.

Wu, X., Xu, C., Tripp, R. A., Huang, Y. W., and Zhao, Y. (2013). Detection and differentiation of foodborne pathogenic bacteria in mung bean sprouts using field deployable label-free SERS devices. *Analyst* 138, 3005–3012. doi: 10.1039/c3an00186e

Xie, Y., Li, P., Zhang, J., Wang, H., Qian, H., and Yao, W. (2013a). Comparative studies by IR, Raman, and surface-enhanced Raman spectroscopy of azodicarbonamide, biurea and semicarbazide hydrochloride. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 114, 80–84. doi: 10.1016/j.saa.2013.05.055

Xie, Y., Xu, L., Wang, Y., Shao, J., Wang, L., Wang, H., et al. (2013b). Label-free detection of the foodborne pathogens of *Enterobacteriaceae* by surface-enhanced Raman spectroscopy. *Anal. Methods* 5, 946–952. doi: 10.1039/C2AY26107C

Xie, Y., Zhao, M., Hu, Q., Cheng, Y., Guo, Y., Qian, H., et al. (2017). Selective detection of chloramphenicol in milk based on a molecularly imprinted polymer-surface-enhanced Raman spectroscopic nanosensor. *J. Raman Spectrosc.* 48, 204–210. doi: 10.1002/jrs.5034

Yan, B., Thubagere, A., Premasiri, W. R., Ziegler, L. D., Negro, L. D., and Reinhard, B. M. (2009). Engineered SERS substrates with multiscale signal enhancement: nanoparticle cluster arrays. *ACS Nano* 3, 1190–1202. doi: 10.1021/nn800836f

Yang, S. C., Lin, C. H., Aljuffali, I. A., and Fang, J. Y. (2017). Current pathogenic *Escherichia coli* foodborne outbreak cases and therapy development. *Arch. Microbiol.* 199, 811–825. doi: 10.1007/s00203-017-1393-y

Zeiri, L., Bronk, B. V., Shabtai, Y., Eichler, J., and Efrima, S. (2004). Surface-Enhanced Raman spectroscopy as a tool for probing specific biochemical components in bacteria. *Appl. Spectrosc.* 58, 33–40. doi: 10.1366/000370204322729441

Zhang, H., Liu, D., Hang, L., Li, X., Liu, G., Cai, W., et al. (2016). Effective SERS-active substrates composed of hierarchical micro/nanostructured arrays based on reactive ion etching and colloidal masks. *Nanotechnology* 27:395304. doi: 10.1088/0957-4484/27/39/395304

Zhang, H., Lu, H., Ho, H. P., Zhou, Y., Yu, X., and Luan, F. (2012). Diffraction resonance with strong optical-field enhancement from gain-assisted hybrid plasmonic structure. *Appl. Phys. Lett.* 100, 338A–346A.

Zhang, H., Ma, X., Liu, Y., Duan, N., Wu, S., Wang, Z., et al. (2015). Gold nanoparticles enhanced SERS aptasensor for the simultaneous detection of *Salmonella* typhimurium and *Staphylococcus aureus*. *Biosens. Bioelectron.* 74, 872–877. doi: 10.1016/j.bios.2015.07.033

Zhang, M., Zhao, L. B., Luo, W. L., Pang, R., Zong, C., Zhou, J. Z., et al. (2016). Experimental and theoretical study on isotopic surface-enhanced raman spectroscopy for the surface catalytic coupling reaction on silver electrodes. *J. Phys. Chem. C* 120, 11956–11965. doi: 10.1021/acs.jpcc.6b02252

Zhang, W., Li, B., Chen, L., Wang, Y., Gao, D., Ma, X., et al. (2014). Brushing, a simple way to fabricate SERS active paper substrates. *Anal. Methods* 6, 2066–2071. doi: 10.1039/C4AY00046C

Zhang, X., Young, M. A., Lyandres, O., and Van Duyne, R. P. (2005). Rapid detection of an anthrax biomarker by surface-enhanced Raman spectroscopy. *J. Am. Chem. Soc.* 127, 4484–4489. doi: 10.1021/ja043623b

Zhao, X., Lin, C. W., Wang, J., and Oh, D. H. (2014). Advances in rapid detection methods for foodborne pathogens. *J. Microbiol. Biotechnol.* 24, 297–312. doi: 10.4014/jmb.1310.10013

Zhao, Y., Luo, W., Kanda, P., Cheng, H., Chen, Y., Wang, S., et al. (2013). Silver deposited polystyrene (PS) microspheres for surface-enhanced Raman spectroscopic-encoding and rapid label-free detection of melamine in milk powder. *Talanta* 113, 7–13. doi: 10.1016/j.talanta.2013.03.075

Zheng, J., and He, L. (2014). Surface-enhanced raman spectroscopy for the chemical analysis of food. *Compr. Rev. Food Sci. Food Saf.* 13, 317–328. doi: 10.1111/1541-4337.12062

Zheng, Y., Mao, S., Liu, S., Wong, D. S.-H., and Wang, Y.-W. (2016). Normalized relative RBC-based minimum risk bayesian decision approach for fault diagnosis of industrial process. *IEEE Trans. Ind. Electron.* 63, 7723–7732. doi: 10.1109/TIE.2016.2591902

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Zhao, Li and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 June 2018
doi: 10.3389/fmicb.2018.01123

Prevalence and Characterization of *Staphylococcus aureus* Cultured From Raw Milk Taken From Dairy Cows With Mastitis in Beijing, China

Wei Wang[1†], *Xiaohui Lin*[2†], *Tao Jiang*[1], *Zixin Peng*[1], *Jin Xu*[1], *Lingxian Yi*[3], *Fengqin Li*[1*], *Séamus Fanning*[1,4,5*] *and Zulqarnain Baloch*[3*]

[1] *Key Laboratory of Food Safety Risk Assessment, Ministry of Health, China National Center for Food Safety Risk Assessment, Beijing, China,* [2] *Physics and Chemical Department, Tianjin Center for Disease Control and Prevention, Tianjin, China,* [3] *College of Veterinary Medicine, South China Agricultural University, Guangzhou, China,* [4] *UCD-Centre for Food Safety, School of Public Health, Physiotherapy and Sports Science, University College Dublin, Dublin, Ireland,* [5] *School of Biological Sciences, Institute for Global Food Security, Queen's University Belfast, Belfast, United Kingdom*

OPEN ACCESS

Edited by:
Maria Schirone,
Università di Teramo, Italy

Reviewed by:
Pierluigi Di Ciccio,
Università degli Studi di Parma, Italy
Jesús Santos,
Universidad de León, Spain

***Correspondence:**
Fengqin Li
lifengqin@cfsa.net.cn
Séamus Fanning
sfanning@ucd.ie
Zulqarnain Baloch
znbalooch@yahoo.com

[†]*These authors have contributed equally to this work and co-first authors*

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *07 March 2018*
Accepted: *14 May 2018*
Published: *22 June 2018*

Citation:
Wang W, Lin X, Jiang T, Peng Z, Xu J, Yi L, Li F, Fanning S and Baloch Z (2018) Prevalence and Characterization of Staphylococcus aureus Cultured From Raw Milk Taken From Dairy Cows With Mastitis in Beijing, China. Front. Microbiol. 9:1123. doi: 10.3389/fmicb.2018.01123

The colonization of dairy herds and subsequent contamination of raw milk by *Staphylococcus aureus* (*S. aureus*), especially those expressing a multi-drug resistance (MDR), biofilm and toxins producing ability, remains an important issue for both the dairy producer and public health. In this study, we investigated the prevalence, antimicrobial resistance, virulence, and genetic diversity of *S. aureus* in raw milk taken from 2 dairy farms in Beijing, China. Ninety (46.2%, 90/195) samples were positive for *S. aureus*. Resistant to penicillin (PEN) (31.3%), ciprofloxacin (18.8%) and enrofloxacin (15.6%) were the most often observed. Isolates cultured from farm B showed significantly higher resistance to penicillin (73.9%), ciprofloxacin (34.8%), enrofloxacin (34.8%), tilmicosin (17.4%), and erythromycin (17.4%) than those from farm A ($p < 0.05$). Totally, 94.8% *S. aureus* harbored at least one virulence gene and the *pvl* (93.8%), *sec* (65.6%), and *sea* (60.4%) genes were the most frequently detected. The *pvl* and *sec* genes were more often detected in isolates from farm A (97.3% and 84.9% respectively) than those from farm B ($p < 0.05$). Of all 77 staphylococcus enterotoxin (SE)-positive isolates, more than 90% could produce enterotoxins and 70.1% could produce two types. Biofilm related genes (*icaA/D*, *clf/B*, *can*, and *fnbA*) were detected in all96 isolates. All 96 isolates could produce biofilm with 8.3, 70.8, and 18.8% of the isolates demonstrating weak, moderate and strong biofilm formation, respectively. A total of 5 STs, 7 *spa* types (1 novel *spa* type t17182), 3*agr* types (no *agr*II), and 14 *Sma*I-pulso-types were found in this study. PFGE cluster II-CC1-ST1-t127-*agr* III was the most prevalent clone (56.3%). Isolates of *agr* III (PFGE Cluster I/II-CC1-ST1-t127/2279) had higher detection of virulence genes than those of *agr* I and *agr* IV. TheMSSA-ST398-t1456-*agr* I clone expressed the greatest MDRbut with no virulence genes and weakly biofilm formation. Our finding indicated a relatively high prevalence of *S. aureus* with less antimicrobial resistance but often positive for enterotoxigenicity and biofilm formation. This study could help identify predominant clones and provide surveillance measures to eliminate and decrease the contamination of *S. aureus* in raw milk of dairy cows with mastitis.

Keywords: *Staphylococcus aureus*, raw milk, mastitis, antimicrobial susceptible test, virulence factors, enterotoxin production, biofilm, molecular typing

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the leading sources of intra-mammary infections in dairy cows (Dufour et al., 2012; Zecconi and Scali, 2013). It is reported that 10–40% of the mastitis cases are caused by *S. aureus* in China and other countries (Kateete et al., 2013; Basanisi et al., 2017; Liu et al., 2017). Mastitis is a global challenge that it can result in financial losses for the dairy industry and the economy due to the substandard quality of milk, treatment costs, and causing subsequent new infection of other cows (Schroeder, 2012). Contaminated raw milk at farm level, may lead to subsequent problems further along the food chain giving rise to *S. Aureus* associated food contamination (Jakobsen et al., 2011; Rola et al., 2016).

S. aureus associated food poisoning in humans and similarly mastitis in animal is caused by those isolates possessing virulence factors (Hennekinne et al., 2012). This bacterium produces wide range of factors, for example toxic shock syndrome toxin-1 (TSST-1), staphylococcus enterotoxin (SE), and Panton-Valentine leukocidin (PVL). SEs is regarded as the major cause of *S. aureus* associated food poisoning (Bergdoll et al., 1981; Hennekinne et al., 2012). It is reported that more than 90% of *S. aureus*-associated food poisoning outbreaks were attributed to the classical SEs (denoted as SEA to SEE) encoded by *sea* to *see* genes (Tarekgne et al., 2016). The TSST-1 toxin could result in toxic shock syndrome by reducing the host immune response, while PVL could destruct host leukocyte and cause tissue necrosis (Schlievert et al., 1981).

Antimicrobial therapy is an important strategy for mastitis control as well as human infections (Gomes and Henriques, 2016). However, *S. aureus* often exhibit resistance to multiple classes of antimicrobial agents as a response to the selective pressure of antimicrobials, which will narrow the treatment options for clinicians and veterinarians (Gomes and Henriques, 2016). It is reported that many *S. aureus*-associated food poisoning outbreaks were due to multi-drug resistant (MDR) *S. aureus* including methicillin-resistant *S. aureus* (MRSA) (Johler et al., 2015; Jans et al., 2017). Furthermore, formation of biofilms, highly organized multicellular complexes, is often associated with both epithelial adhesion and evasion of host immune defenses (Melchior et al., 2009). Biofilm associated protein (Bap) plays an important role in primary attachment and recruitment of *S. aureus* (Khoramian et al., 2015; Felipe et al., 2017). The *icaA* and *icaD* genes that form part of the *icaABCD* gene cluster (intracellular adhesion locus) are essential for biofilm formation (Khoramian et al., 2015; Felipe et al., 2017). Additionally, the collagen binding proteins (Cna), clumping factors (ClfA and ClfB) and fibronectin binding proteins (FnbA and FnbB) also have associations with biofilm production according to previous studies (Khoramian et al., 2015; Pereyra et al., 2016).

Molecular epidemiology-based methods are essential tools for the study of clonal relatedness, genetic diversity, and also tracking the dissemination of *S. aureus* infections. It was reported that certain *S. aureus* lineages were specifically associated with milk, such as CC97 (Clonal complex), and particular clonal lineages may be prevalent geographically, and have specific antimicrobial resistance and virulence patterns (Hata et al., 2010). This study aimed to estimate the prevalence of *S. aureus* among raw milk from dairy cows with clinical mastitis from two dairy farms during August to December in 2016 in Beijing, China, and to describe the characteristics of the isolates, in order to provide groundwork for further studies on the control and prevention of contamination of *S. aureus* in raw milk of dairy cows with mastitis.

MATERIALS AND METHODS

Sampling and Isolation of *S. aureus*

Recruitment of cows into this study was done in consultation with veterinarians and sampling process was carried on with the agreement of the dairy farms' owners. Raw milk samples were collected from cows presenting with clinical mastitis consistent with poor milk yield, color change and udders inflammation. Milk collection process was performed after cleaning the teats, initial streams of milk discarded and teat tips scrubbed with cotton balls moistened with 75% alcohol. Teat-cleaning before milking and treatment with antibiotics at dry-off were not performed. In total, one milk sample from each cow was collected and 195 individual milk samples of 195 cows were obtained from 2 dairy farms during August to December in 2016 in Beijing, China. These two dairy farms belong to one of the largest dairy production companies in China, which mainly supply consumers in Beijing and other regions in China, and also export internationally. Both farms were managed with an intensive breeding system, with the herd size of about 500 locating cows.

The *S. aureus* contamination was detected in raw milk samples according to National Food Safety Standards of China document GB 4789.10-2016. Briefly, a 25-ml milk sample was taken and mixed thoroughly, and then transferred into 225 mL 10% (w/v%) saline solution (Land Bridge, Beijing, China) and homogenize it and solutions were incubated at 37°C for 24 h. A loopful of the incubated culture were streaked onto Baird-Parker Agar supplemented with 5% egg yolk and tellurite, and Blood Agar with sterile defibrinated sheep blood (Land Bridge, Beijing, China), respectively, then incubated at 37°C for 24–48 h. Putative *S. aureus* isolates were tested for coagulase activity, and were further confirmed using API STAPH test strips (bio-Mérieux, Marcyl'Etoile, France). Finally, all isolates were subjected the detection of 16SrRNA and *nuc* genes by PCR (**Table 1**; Murakami et al., 1991). All confirmed *S. aureus* isolates were stored in BHI with 40% [v/v%] glycerol (Land Bridge, Beijing, China) at −80°C. No more than2 isolates of each sample were chose for subsequent studies.

Antimicrobial Susceptibility Testing (AST)

In this study, broth dilution method was applied to estimate the antimicrobial susceptibility of all tested isolates using the Biofosun® Gram-positive panel (Fosun Diagnostics, Shanghai, China) and interpreted by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). The antimicrobial agents

TABLE 1 | Primers used in this study.

Gene	Oligonucleotide sequence (5′–3′)	Size of product	Annealing temperature	References
nuc-F	GCGATTGATGGTGATACGGTT	798	55	Murakami et al., 1991
nuc-R	AGCCAAGCCTTGACGAACTAAAGC			
16S rRNA-F	AGAGTTTGATCATGGCTCAG	270	55	
16S rRNA-R	GGACTACCAGGGTATCTAAT			
mecA-F	AAAATCGATGGTAAAGGTTGGC	533	55	
mecA-R	AGTTCTGCAGTACCGGATTTGC			
sea-F	ACGATCAATTTTTACAGC	544	44.5	Rosec and Gigaud, 2002
sea-R	TGCATGTTTTCAGAGTTAATC			
seb-F	ATTCTATTAAGGACACTAAGTTAGGGGA	404	44.5	Jarraud et al., 2002
seb-R	ATCCCGTTTCATAAGGCGAGT			
sec-F	GACATAAAAGCTAGGAATTT	257	46.2	Rosec and Gigaud, 2002
sec-R	AAATCGGATTAACATTATCCA			
sed-F	CAAATATATTGATATAATGA	330	44.5	Khoramrooz et al., 2016
sed-R	AGTAAAAAAGAGTAATGCAA			
see-F	CAAAGAAATGCTTTAAGCAATCTTAGGC	482	44.5	Jarraud et al., 2002
see-R	CACCTTACCGCCAAAGCTG			
tst-F	ACCCCTGTTCCCTTATCATC	326	54	Khoramrooz et al., 2016
tst-R	TTTTCAGTATTTGTAACGCC			
lukS/F-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	55	McClure et al., 2006
lukS/F-R	GCATCAAGTGTATTGGATAGCAAAAGC			
pan-agr	ATGCACATGGTGCACATGC	–	55	Shopsin et al., 2003
agrI	GTCACAAGTACTATAAGCTGCGAT	440	55	
agrII	GTATTACTAATTGAAAAGTGCCATAGC	573	55	
agrIII	CTGTTGAAAAAGTCAACTAAAAGCTC	406	55	
agrIV	CGATAATGCCGTAATAC CCG	588	55	
fnbA-F	GATACAAACCCAGGTGGTGG	191	52	Zmantar et al., 2008
fnbA-R	TGTGCTTGACCATGCTCTTC			
fnbB-F	ACGCTCAAGGCGACGGCAAAG	197	62	Pereyra et al., 2016
fnbB-R	ACCTTCTGCATGACCTTCTGCACCT			
clfA-F	CCGGATCCGTAGCTGCAGATGCACC	1000	60	Zmantar et al., 2008
clfA-R	GCTCTAGATCACTCATCAGGTTGTTCAGG			
clfB-F	TGCAAGTGCAGATTCCGAAAAAAAC	194	62	Klein et al., 2012
clfB-R	CCGTCGGTTGAGGTGTTTCATTTG			
cna-F	AAAGCGTTGCCTAGTGGAGAC	192	54	Zmantar et al., 2008
cna-R	AGTGCCTTCCCAAACCTTTT			
bap-F	CCCTATATCGAAGGTGTAGAATTG	971	60	Darwish and Asfour, 2013
bap-R	GCTGTTGAAGTTAATACTGTACCTGC			
icaA-F	CCTAACTAACGAAAGGTAG	1351	49	
icaA-R	AAGATATAGCGATAAGTGC			
icaD-F	AAACGTAAGAGAGGTGG	381	49	Pereyra et al., 2016
icaD-R	GGCAATATGATCAAGATAC			

included Ceftiofur (EFT) (0.25–64 μg/mL), Chloramphenicol (CHL) (0.5–128 μg/mL), Ciprofloxacin (CIP) (0.125–16 μg/mL), Daptomycin (DAP) (0.06–16 μg/mL), Enrofloxacin (ENO) (0.125–32 μg/mL), Erythromycin (ERY) (0.125–16 μg/mL), Fosfomycin (FOS) (0.5–256 μg/mL), Gentamycin (GEN) (0.5–64 μg/mL), Penicillin (PEN) (0.06–32 μg/mL), Tetracycline (TET) (0.25–64 μg/mL), Tilmicosin (TIL) (0.5–64 μg/mL), and Vancomycin (VAN) (0.06–128 μg/mL). *S. aureus* ATCC^{TM}29213 was used as the reference strain for the AST.

Detection of MRSA, Virulence and Biofilm Related Genes

Frozen isolates were cultured overnight at 37°C in BHI (Land Bridge, Beijing, China). The genomic DNA was then extracted with TIANamp Bacterial DNA extraction kit (TianGenDNA Kit DP302, Beijing, China), and the quality of DNA was evaluated by a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, NH, USA). Sterile deionized water was used to dilute the extracted DNA to 50 mg/L, which was suitable for real-time PCR assays. The genes encoding the methicillin resistance gene

(*mecA*), SEs (*sea* to *see*), toxic-shock syndrome toxin (*tst*), Panton-Valentine leukocidin (*lukF*), biofilm related genes (*bap*, *icaA*, and *icaD*), and adhesion related genes (*fnbA*, *fnbB*, *clfA*, *clfB*, and *can*) were detected by PCR. The primers were supplied by Thermo Fisher Scientific (Waltham, MA, USA; **Table 1**). Positive and negative controls were included in all PCRs.

Detection of SEs Production

SEs (SEA to SEE)production was detected by immuno-colloidal gold chromatographic test strips (Longrunbio, Beijing, China). In brief, the supernatant of 24 h cultures of *S. aureus* (1×10^9 CFU/mL) positive with SEs genes grown at 37°C in a shake-tube (Xuzhou Yanjia Glass Products, Xuzhou, China) containing 5 mL BHI (Land Bridge, Beijing, China) was separated from cells by centrifugation at 8,000 × g for 20 min. The supernatant was heated at 100°C for 10 min. Then 200 μL of the heated supernatant were tested for the presence of the SEs by the strip test assay. The samples 100 ng/mL of SEA to SEE were used as a positive control and phosphate buffer was used as negative control.

Biofilm Formation

Biofilm production was assessed by a 96-well microtiter plate assay using minimal medium M9 (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 2 mM $MgSO_4$, 0.1% glucose, and 0.1 mM $CaCl_2$; Müsken et al., 2010). After overnight growth in tryptone soy broth medium (TSB; Oxoid Ltd., Basingstoke, UK), 200 μL of cell suspension diluted to 1:100 was transferred into each microtiter plate well, and the later was incubated at 37°C for 72 h. After three brief washes with 200 μL phosphate-buffered saline (PBS) solution and a 20-min fixation step with 200 μL methanol, all plates were stained with 200 μL 0.4% (wt/vol) crystal violet (CV) for 15 min and washed with 200 μL PBS for another 15 min. The formed biofilm was then dissolved with 200 μL 33% (wt/vol) acetic acid for 30 min. The biofilm formation was measured at 570 nm optical density (OD) in a micro-titer plate reader (Tecan, Mannedorf, Switzerland). *Salmonella* Typhimurium ATCC14028, a strong biofilm-forming strain, was selected as the positive control and sterile TSB was used as negative control for the biofilm production assays (Yan et al., 2015). These biofilm assays were performed in triplicate that included biological duplicates. An OD_{570nm} value of 0.6 was applied as the cutoff point to distinguish between biofilm producer from non-biofilm producer [cut-off (ODc) = average OD plus 3 standard deviation (SD) of negative control]. The biofilm formation was classified as strong+++ ($OD_{570nm} > 1.8$), moderate++ ($1.8 > OD_{570nm} > 1.2$), weak+ ($1.2 > OD_{570nm} > 0.6$), and negative − ($OD_{570nm} < 0.6$).

Multilocus Sequence Typing (MLST)

All *S. aureus* isolates were examined by MLST, based on the sequencing of 7 housekeeping genes described previously (Enright et al., 2000). Alleles and the sequence type (ST) were assigned according to the *S. aureus* MLST database (http://www.mlst.net/). The STs were then clustered in to clonal complexes (CC) by eBURST v.3 software (http://eburst.mlst.net; Feil et al., 2004).

spa Typing

The *spa* typing for all *S. aureus* isolates was performed as described previously (Harmsen et al., 2003). The *spa* repeats and types were assigned by the Bio Numerics software v.7.5 (Applied Math, Belgium). If a *spa* repeat did not match any *spa* types, the sequence of this *spa* was then upload to the Ridom *Spa* Server database (http://spa.ridom.de) to assign a new type.

agr Genotyping

The *agr* type of all *S. aureus* isolates was determined using the *agr*-group specific primers (*agr* allele types I–V) and *agr* multiplex PCR as described previously (**Table 1**).

Pulsed-Field Gel Electrophoresis (PFGE)

The genetic relationships of all *S. aureus* isolates were established by PFGE (Murchan et al., 2003; Ribot et al., 2006). In brief, the tested isolates were cultured and plugs were prepared. Chromosomal DNA was digested with the endonuclease *Sma*I (20 units/μL, New England Biolabs) at 30°C for 3 h. The electrophoresis was performed in 1% agarose SeaKem Gold gel in the CHEF DR III apparatus (Bio-Rad, Hercules, California z) at 14°C for 19 h. Macro restriction patterns were interpreted by Bio Numerics software v.7.5 (Applied Math, Belgium) by the un weighted pair group method with arithmetic averages (UPGMA). *Salmonella* Braenderup H9812 was used as a standard size marker.

Simpson's Index of Diversity Calculation

The Simpson's index of diversity (diversity index, DI) was used to evaluate the genetic diversity and discriminatory ability of different typing methods. The formula is as follows:

$$DI = 1 - \frac{1}{[N(N-1)]} \sum_{j-1}^{s} n_j(n_j - 1) \quad (1)$$

n_j is the number of isolates belonging to the *j*th type, and *N* is the total number of tested isolates.

Statistical Analysis

The Chi-square test was calculated using SPSS 20.0 (SPSS, Chicago, USA), in order to analyze the differences in the prevalent rates, the proportion of isolates resistant to antimicrobial agents, and the distribution of virulence genes, biofilm related genes, enterotoxin production, and biofilm production ability between two farms. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Isolation and Identification of *S. aureus*

Of the 195 raw milk samples, 90 (46.2%, 90/195) were confirmed with *S. aureus*, and in all 96 isolates were obtained in this study (**Table 2**). Twelve isolates cultured from six samples (2 isolates were cultured per samples), respectively, were included in this study, as both strains of each sample were subsequently found to have different genetic patterns and/or phenotypes (**Table 3** and **Figure 1**). Of the 90 *S. aureus*-positive samples, 71 of 147 (48.2%)

TABLE 2 | Prevalence of *S. aureus* in raw milk in Beijing.

Farm	No. of samples	No. (%) of samples with confirmed *S. aureus*	No. of *S. aureus* isolates	No. (%) of MRSA isolates
A	147	71 (48.2%)	73	1 (1.4%)
B	48	19 (39.6%)	23	ND*
Total	195	90 (46.2%)	96	1 (1%)

*ND means no detection.

and 19 of 48 (39.6%) raw milk samples collected from farm A and farm B respectively, were positive for *S. aureus*. Meanwhile, 73 and 23 *S. aureus* isolates were obtained from samples collected from farm A and farm B, respectively. Additionally, one *S. aureus* isolate (1%, 1/96) cultured from farm A was then identified to harbor the *mecA* gene, thereby classifying it as a MRSA isolate (**Table 2** and **Figure 1**).

Antimicrobial Susceptibility

Table 4 shows the antimicrobial susceptibility results for the tested isolates. Of the 96 *S. aureus* isolates tested, resistance was most frequently observed to penicillin (31.3%, 30/96), followed by ciprofloxacin (18.8%, 18/96) and enrofloxacin (15.6%, 15/96), and to a lesser extent tilmicosin (6.3%, 6/96), erythromycin (5.2%, 5/96), gentamycin (1.0%, 1/96), chloramphenicol (1.0%, 1/96), and tetracycline (1.0%, 1/96). Isolates from farm B showed significantly higher resistance to penicillin (73.9%), ciprofloxacin (34.8%), enrofloxacin (34.8%), tilmicosin (17.4%), and erythromycin (17.4%) than those from farm A ($p < 0.05$; **Table 4**). All *S. aureus* isolates were susceptible to ceftiofur, daptomycin, and vancomycin. Notably, 52 (54.2%, 52/96) and seven (7.3%, 7/96) isolates, all of which were cultured from farm A, expressed an intermediate phenotype to ciprofloxacin and enrofloxacin, respectively. Meanwhile, for the top three resistant phenotypes to penicillin, ciprofloxacin, enrofloxacin, the MIC_{50} and MIC_{90} were measured at 0.06- and 8-μg/mL, 2- and 8-μg/mL, 0.5 and 4-μg/mL, respectively. Additionally, thirty-seven isolates (38.5%, 37/96) showed resistant to at least one antimicrobial and 6 isolates (6.3%, 6/96) showed resistant to ≥3 classes (MDR) (**Tables 4**, **5** and **Figure 1**). Totally, nine resistance patterns were identified, wherein PEN (16.7%, 16/96), PEN-CIP-ENO-ERY-TIL (5.2%, 5/96) and PEN-CIP-ENO (5.2%, 5/96) were the top three frequently identified patterns. Greater diversity among the resistance patterns from farm A (8 patterns) than those from farm B (3 patterns), were noted (**Table 5** and **Figure 1**). PEN-CIP-ENO-ERY-TIL, and PEN were more frequently detected from farm B than from farm A ($p < 0.05$), while PEN-CHL-GEN-TIL, PEN-CIP-ENO, PEN-CIP, CIP, ENO, and TET were only identified in farm A and CIP-ENO only in farm B.

Presence of Virulence and Biofilm Related Genes

Of the 96 *S. aureus* isolates tested, 91 (94.8%) were detected to have one or more virulence genes, and 6 virulence genes (*tst*, *pvl*,

TABLE 3 | Characteristics of isolates cultured from the same samples[a].

Sample ID	Isolates	Genotype patterns	Virulence genes	Biofilm related genes	Antimicrobial resistance	Enterotoxin production	Biofilm formation	Farms
M11	M11-1	PFGE cluster II-CC1-ST1-t127-*agr* III	*sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	-	SEA-SEC	++	A
	M11-2	PFGE cluster II-CC1-ST1-t127-*agr* III	*pvl-sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	-	SEA-SEC	++	A
M17	M17-1	PFGE cluster II-CC1-ST1-t127-*agr* III	*pvl-sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	-	SEA-SEC	+++	A
	M17-2	PFGE cluster II-CC1-ST1-t127-*agr* III	*pvl-sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA*	-	SEA-SEC	++	A
M23	M23-1	PFGE cluster V-CC50-ST50-t518-*agr* IV	*pvl*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	-	-	+++	A
	M23-2	PFGE cluster V-CC50-ST50-t518-*agr* IV	*pvl-sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	TET	SEC	++	A
M34	M34-1	PFGE cluster II-CC1-ST1-t127-*agr* III	*pvl-sea-sec*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	-	SEA-SEC	++	A
	M34-2	PFGE cluster III-CC97-ST97-t730-*agr* I	*pvl-sec-sed*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	PEN-CIP-ENO	SEC-SED	++	A
M87	M87-1	PFGE cluster I-CC1-ST1-t2279-*agr* III	*pvl-seb*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	PEN	SEB	+++	B
	M87-2	PFGE cluster I-CC1-ST1-t2279-*agr* III	*pvl*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	PEN	-	++	B
M91	M91-1	PFGE cluster I-CC1-ST1-t2279-*agr* III	*pvl-seb*	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	PEN	SEB	++	B
	M91-2	PFGE cluster VI-CC398-ST398-t14156-*agr* I	-	*icaA-icaD-clfA-clfB-can-fnbA-fnbB*	PEN-CIP-ENO-ERY-TIL	-	+	B

[a]"-"means that isolates did not have this genotype or phenotype.

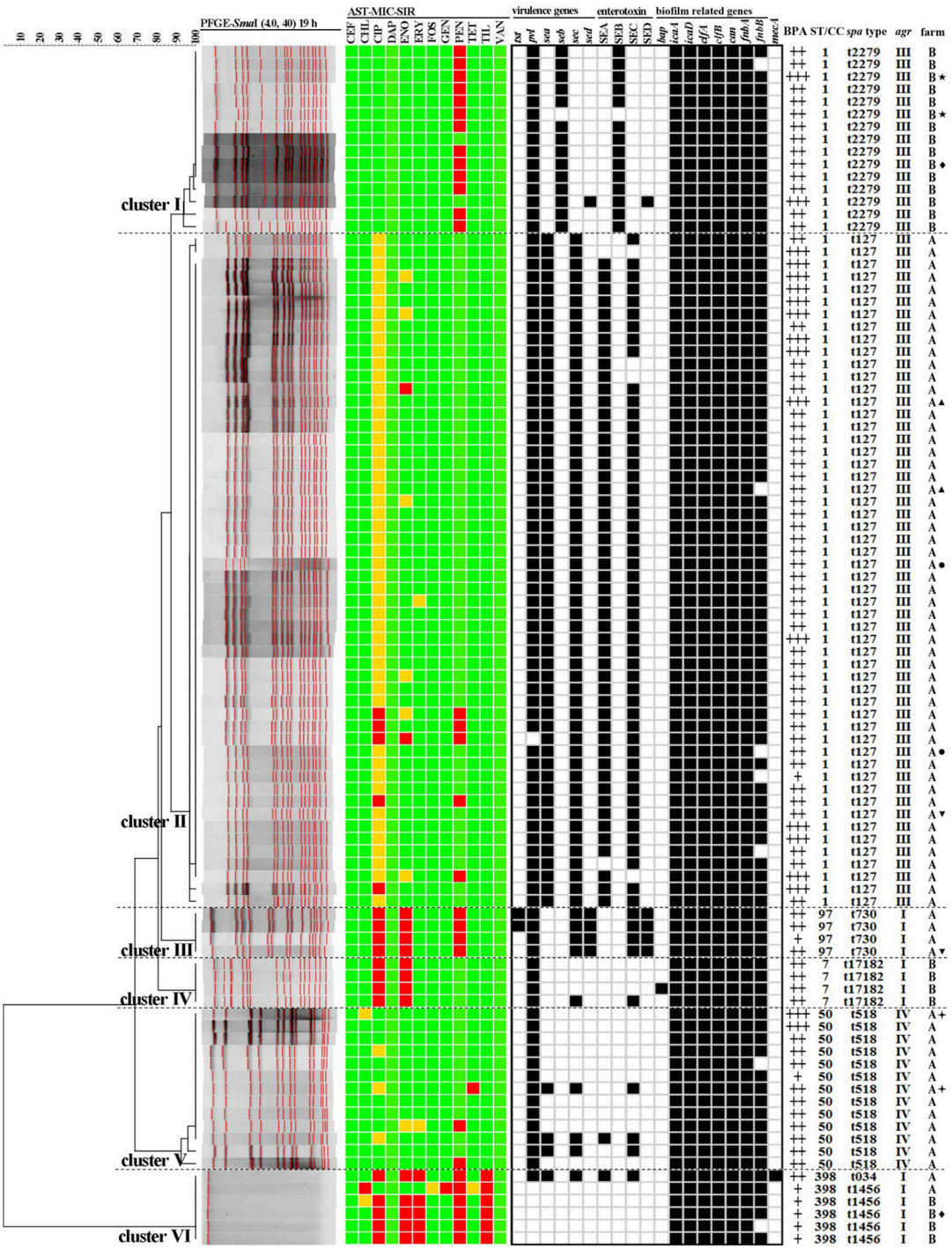


FIGURE 1 | Dendrogram of PFGE patterns and antimicrobial susceptibility testing (AST), virulence genes, enterotoxin production, biofilm and adhesion related genes, *mecA* gene, and molecular characterization of 96 *S. aureus* isolates cultured from raw milk in Beijing China. Ninety-six isolates were grouped into 6 clusters (cluster I-VI) by PFGE patterns and all had more than 92% similarity. The results of AST were showed in different colors according to the MIC values of isolates to different antimicrobial agents. Green squares indicate susceptibility; yellow squares indicate intermediate; and red squares indicate resistance. The detection of virulence genes, enterotoxin production, biofilm and adhesion related genes, and *mecA* gene were summarized by a heat map. Black squares denote that the studied genes were detected in those isolates, or those isolates could produce those types of enterotoxins. White squares denote that those isolates lack these studied genes or could not produce those types of enterotoxins. BPA represents biofilm production ability. ST/CC represents sequence type of MLST and the clone complex (CC) of this ST. *agr* represents *agr* types. Antimicrobial compounds used are abbreviated as follows: TIO, Ceftiofur; CHL, chloramphenicol; CIP, ciprofloxacin; DAP, daptomycin; ENO, enrofloxacin; ERY, erythromycin; FOS, fosfomycin; GEN, gentamycin; PEN, penicillin; TET, tetracycline; TIM, tilmicosin; VAN, vancomycin. The same symbols beside farm number of ●, ▲, ✦, ▼, ★, and ♦ represent isolates cultured from M11, M17, M23, M34, M87, and M91, respectively.

TABLE 4 | Antimicrobial susceptibility of the study isolates to eight of the 12 antimicrobial agents tested.

Antimicrobials	MIC_{50}	MIC_{90}	Range	Resistant, no. of isolates (%)			Intermediate, no. of isolates (%)			Susceptible, no. of isolates (%)		
				Farm A	Farm B	Total	Farm A	Farm B	Total	Farm A	Farm B	Total
Penicillin	0.06	8	0.06—32	13(17.8)	17(73.9)*	30(31.3)	0(0)	0(0)	0(0)	60(82.2)	6(26.1)	66(68.8)
Ciprofloxacin	2	8	0.125—16	10(13.7)	8(34.8)*	18(18.8)	52(71.2)	0(0)	52(54.2)	11(15.1)	15(65.2)	26(27.1)
Enrofloxacin	0.5	4	0.125—32	7(9.6)	8(34.8)*	15(15.6)	7(9.6)	0(0)	7(7.3)	59(80.1)	15(65.2)	74(77.1)
Tilmicosin	2	2	0.5—64	2(2.7)	4(17.4)*	6(6.3)	0(0)	0(0)	0(0)	71(97.3)	19(82.6)	90(93.8)
Erythromycin	0.25	0.25	0.125—16	1(1.4)	4(17.4)*	5(5.2)	2(2.7)	0(0)	2(2.1)	70(95.9)	19(82.6)	89(92.7)
Gentamycin	1	1	0.5—64	1(1.4)	0(0)	1(1)	0(0)	0(0)	0(0)	72(98.6)	23(100)	95(99)
Chloramphenicol	8	8	0.5—128	1(1.4)	0(0)	1(1)	1(1.4)	1(4.3)	2(2.1)	71(97.3)	22(95.7)	93(96.9)
Tetracycline	0.5	0.5	0.25—64	1(1.4)	0(0)	1(1)	1(1.4)	0(0)	1(1)	71(97.3)	23(100)	94(97.9)
Fosfomycin	32	64	0.5—256	0(0)	0(0)	0(0)	1(1.4)	0(0)	1(1)	72(98.6)	23(100)	95(99)
Ceftiofur	0.5	2	0.25—64	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	73(100)	23(100)	96(100)
Daptomycin	0.5	1	0.06—16	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	73(100)	23(100)	96(100)
Vancomycin	0.5	1	0.06—128	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	73(100)	23(100)	96(100)

*$p < 0.05$.

sea to *sed*) were identified with no *see* genes amplified, by PCR in this study (**Table 5** and **Figure 1**). The 4 SEs genes were detected in 80.2% (77/96) of all 96 isolates. The three most frequently detected virulence genes were *pvl* (93.8%, 70/96), *sec* (65.6%, 63/96), and *sea* (60.4%, 58/96), followed by *seb* (14.6%, 14/96), *sed* (5.2%, 6/96), and *tst* (2.1%, 2/96). Prevalence rates of the *pvl* and *sec* genes from farm A (97.3% and 84.9% respectively) were higher than those from farm B (82.6 and 4.3% respectively) ($p < 0.05$). While, the *ts*t and *sea* genes were only identified in farm A, and the *seb* gene was only identified in farm B (**Table 5**). In total, eight different virulence gene patterns were observed. Among all patterns, the *pvl-sea-sec* (59.4%, 57/96) was common, followed by *pvl* (14.6%, 14/96), *pvl-seb* (13.5%, 13/96). The *pvl-sec-sed* and *tst-pvl-sec-sed* patterns were found in 2.1% (2/96 each) of all 96 isolates, respectively, while *pvl-sec*, *sea-sec*, and *pvl-seb-sed* were found in 1% (1/96 each) of all 96 isolates, respectively (**Table 5**).

Table 5 lists the biofilm and adhesion related genes of the 96 *S. aureus* isolates recovered from farm A and farm B. The results show that the *icaA*, *icaD*, *clfA*, *clfB*, *can*, and *fnbA* genes were detected in all of the 96 isolates, while 7 isolates (5 from farm A and 2 from farm B) did not carry the *fnbB* gene and the *bap* gene was only detected in one isolate from farm B.

Determination of Enterotoxin Production, and Biofilm Production Ability

In total, 77 isolates were detected by PCR to have enterotoxin genes, while 53 (55.2%, 53/96), 14 (14.6%, 14/96), 59(61.5%, 59/96), and 5 (5.2%, 5/96) could produce SEA, SEB, SEC, and SED, respectively (**Table 5**). More than 90% of the SEs genes harboring *S. aureus* isolates could produce enterotoxins. Additionally, 54 (70.1%, 54/77) isolates simultaneously produced two types of enterotoxins (**Table 5** and **Figure 1**). Moreover, the MRSA isolates harboring *sea* and *sec* genes also have the ability to producing both enterotoxins, SEA and SEC.

The microtiter plate assay showed that all 96 *S. aureus* from the two farms could produce biofilm, although at different intensities (**Table 5** and **Figure 1**). Eight isolates (8.3%, 8/96), including 4 from farm A and farm B, were able to produce biofilm weakly; 68 strains (70.8%, 68/96), including 53 isolates from farm A and 17 isolates from farm B respectively, showed moderate biofilm formation; 18 strains (18.8%, 18/96), including 16 isolates from farm A and 2 isolates from farm B respectively, showed strong biofilm formation.

MLST

All 96 isolates were typed by MLST as shown in **Table 5** and **Figures 1**–**3**. A total of 5 sequence types (STs) were identified (ST1, ST7, ST50, ST97, and ST398), which were further grouped into 5 CCs. In this study, CC1 was represented by ST1 (CC1-ST1) alone, being found as the most predominate sequence type (71.9%, 69/96) in both two farms, followed by CC50-ST50 (13.5%, 13/96), CC398-ST398 (6.3%, 6/96), and CC7-ST7 and CC398-ST398 (4.2%, 4/96 each). The clonal lineages of *S. aureus* isolates were further analyzed based on the sampling farms. As shown in **Table 5** and **Figure 1**, four clonal lineages were identified from farm A, including CC1-ST1, CC50-ST50, CC97-ST97, and CC398-ST398. In contrast, three clonal lineages were identified from farm B, including CC1-ST1, CC7-ST7, and CC398-ST398.

spa Typing

A total of 7 *spa* types were obtained in all 96 *S. aureus*, with 1 novel *spa* type (t17182) identified (**Table 5** and **Figure 1**). The most prevalent *spa* type was t127 (56.3%, 54/96) and this was associated with isolates cultured from farm A. In addition to t127, four other *spa* types were also found in isolates from farm A (t518, t730, t034, and t14156). Meanwhile, Isolates from farm B were defined by 3 *spa* types, including t2279, t14156, and t17182. Based on MLST, isolates of the sequence types ST7, ST50, and ST97 had their own identical *spa* types (ST50-t518, ST97-t730, and ST7-t17182) (**Table 5** and **Figures 1**, **3**). However, there were some

TABLE 5 | Phenotypes and genotypes of 96 *S. aureus* isolates tested in this study.

Phenotypes or genotypes tested in this study		No. of isolates (%)		
		Farm A	**Farm B**	**Total**
Antimicrobial resistance patterns	PEN	3(4.1)	13(56.5)*	16(16.7)
	CIP	1(1.4)	0(0)	1(1)
	ENO	1(1.4)	0(0)	1(1)
	TET	1(1.4)	0(0)	1(1)
	PEN-CIP	3(4.1)	0(0)	3(3.1)
	CIP-ENO	0(0)	4(17.4)	4(4.2)
	PEN-CIP-ENO	5(6.8)	0(0)	5(5.2)
	PEN-CHL-GEN-TIL	1(1.4)	0(0)	1(1)
	PEN-CIP-ENO-ERY-TIL	1(1.4)	4(17.4)*	5(5.2)
	ND	57(78.1)	2(8.7)	59(61.5)
Virulence genes	*tst*	2(2.7)	0(0)	2(2.1)
	pvl	71(97.3)*	19(82.6)	90(93.8)
	sea	58(79.5)	0(0)	58(60.4)
	seb	0(0)	14(60.9)	14(14.6)
	sec	62(84.9)*	1(4.3)	63(65.6)
	sed	4(5.5)	1(4.3)	5(5.2)
	see	0(0)	0(0)	0(0)
	ND	1(1.4)	4(17.4)	5(5.2)
Virulence gene patterns	*pvl*	10(13.7)	4(17.4)	14(14.6)
	pvl-seb	0(0)	13(56.5)	13(13.5)
	pvl-sec	0(0)	1(4.3)	1(1)
	sea-sec	1(1.4)	0(0)	1(1)
	pvl-sea-sec	57(78.1)	0(0)	57(59.4)
	pvl-seb-sed	0(0)	1(4.3)	1(1)
	pvl-sec-sed	2(2.7)	0(0)	2(2.1)
	tst-pvl-sec-sed	2(2.7)	0(0)	2(2.1)
	ND	1(1.4)	4(17.4)	5(5.2)
Enterotoxin production	SEA	53(72.6)	0(0)	53(55.2)
	SEB	0(0)	14(60.9)	14(14.6)
	SEC	58(79.5)*	1(4.3)	59(61.5)
	SED	4(5.5)	1(4.3)	5(5.2)
	SEE	0(0)	0(0)	0(0)
	ND	11(15.1)	8(34.8)	19(19.8)
Enterotoxin production patterns	SEA	4(5.5)	0(0)	4(4.2)
	SEB	0(0)	13(56.5)	13(13.5)
	SEC	5(6.8)	1(4.3)	6(6.3)
	SEA-SEC	49(67.1)	0(0)	49(51)
	SEB-SED	0(0)	1(4.3)	1(1)
	SEC-SED	4(5.5)	0(0)	4(4.2)
	ND	11(15.1)	8(34.8)	19(19.8)
Biofilm related genes	*icaA-icaD-clfA-clfB-can-fnbA*	5(6.8)	2(8.6)	7(7.3)
	icaA-icaD-clfA-clfB-can-fnbA-fnbB	68(93.2)	20(86.9)	88(91.7)
	bap-icaA-icaD-clfA-clfB-can-fnbA-fnbB	0(0)	1(4.3)	1(1)

(Continued)

TABLE 5 | Continued

Phenotypes or genotypes tested in this study		No. of isolates (%)		
		Farm A	**Farm B**	**Total**
Biofilm production ability[a]	+ (range of OD: 0.913-1.196)	4(5.5)	4(17.39)	8(8.3)
	++ (range of OD: 1.246-1.797)	53(72.6)	17(73.9)	70(72.9)
	+++ (range of OD: 1.807-2.156)	16(21.9)	2(8.7)	18(18.8)
agr types	I	6(8.2)	8(34.8)	14(14.6)
	II	0(0)	0(0)	0(0)
	III	54(74)	15(65.2)	69(71.9)
	IV	13(17.8)	0(0)	13(13.5)
MLST	CC1-ST1	54(74)	15(65.2)	69(71.9)
	CC7-ST7	0(0)	4(17.4)	4(4.2)
	CC50-ST50	13(17.8)	0(0)	13(13.5)
	CC97-ST97	4(5.5)	0(0)	4(4.2)
	CC398-ST398	2(2.7)	4(17.4)	6(6.3)
spa typing	t034	1(1.4)	0(0)	1(1)
	t127	54(74)	0(0)	54(56.3)
	t518	13(17.8)	0(0)	13(13.5)
	t730	4(5.5)	0(0)	4(4.2)
	t2279	0(0)	15(65.2)	15(15.6)
	t14156	1(1.4)	4(17.4)	5(5.2)
	t17182	0(0)	4(17.4)	4(4.2)
Genotype patterns	PFGE cluster I-CC1-ST1-t2279-*agr* III	0(0)	15(65.2)	15(15.6)
	PFGE cluster II-CC1-ST1-t127-*agr* III	54(74)	0(0)	54(56.3)
	PFGE cluster III-CC97-ST97-t730-*agr* I	4(5.5)	0(0)	4(4.2)
	PFGE cluster IV-CC7-ST7-t17182-*agr* I	0(0)	4(17.4)	4(4.2)
	PFGE cluster V-CC50-ST50-t518-*agr* IV	13(17.8)	0(0)	13(13.5)
	PFGE cluster VI-CC398-ST398-t034-*agr* I	1(1.4)	0(0)	1(1)
	PFGE cluster VI-CC398-ST398-t14156-*agr* I	1(1.4)	4(17.4)	5(5.2)

[a]*Quantification of biofilm formation by optical density (OD) determination: (+ + +): strong biofilm producers (OD570 > 1.8), (++): moderate biofilm producers (1.8 > OD570 > 1.2), (+): weak biofilm producers (1.2 > OD570 > 0.6); *p < 0.05.*

exceptions that several isolates owned the identical sequence type but different *spa* types (ST1-t127/t2279, ST398-t034/t1456) (**Table 5** and **Figures 1**, **3**).

agr Genotyping

The distribution of *agr* alleles among the 96 isolates is provided in **Table 5**. Using a multiplex-based PCR, *agr* alleles were successfully identified in 96 isolates. The *agr* III genotype was predominant, representing 71.9% (69/96) of the isolates and was the prevailing *agr* type regardless of the sampling farms of *S. aureus* isolates, followed by *agr* I (14.6%, 14/96) and *agr* VI (13.5%, 13/96). No *agr* II type was detected among all 96 isolates. Furthermore, all 14 isolates with *agr* I were discriminated into three STs and four *spa* types (ST7-t17182, ST97-t730, ST398-t034, and ST398-t1456). All 69 isolates with *agr* III with the same sequence type were discriminated into two *spa* types (ST1-t2279 and ST1-t127). However, all 13 isolates with *agr* IV had the identical sequence type and *spa* type (ST50-t518) (**Table 5** and **Figure 1**).

PFGE Sub-typing and Identification of Major Clones

Among 96 isolates subtyped by PFGE, six isolates (belonging to ST398) could not be typed by this method (**Table 5** and **Figure 1**). The other 90 isolates were distinguished into 14 pulso types and then gathered into five PFGE clusters (Cluster I–V) based on more than 92% genetic similarity. The predominant PFGE cluster was cluster II and included 54 isolates all cultured from farm A, and which were differentiated into 4 pulso types. Fifty of these 54 isolates were found to sharing the same PFGE banding patterns. All isolates in cluster II were characterized as PFGE cluster II-CC1-ST1-t127-*agr* III. Cluster I included 15 isolates with 5 pulso types and included PFGE Cluster I-CC1-ST1-t2279-*agr* III. All 15 isolates in cluster-I were cultured from farm B. Four isolates from farm A were characterized as PFGE Cluster III-CC97-ST97-t730-*agr* I, while another 4 isolates from farm A were included in PFGE Cluster III-CC97-ST97-t730-*agr* I characterized as PFGE Cluster IV-CC7-ST7-t17182-*agr* I. Cluster V included 13 isolates with 3 pulso types that were designated

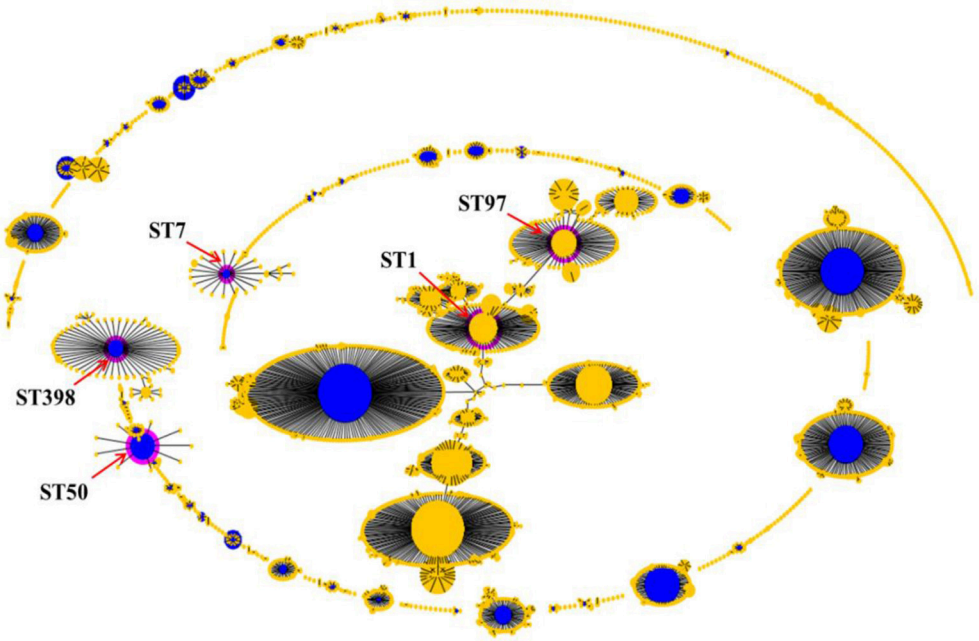


FIGURE 2 | Population snapshot analyses by eBURST on 5779 *S. aureus* strains that belonged to 2766 STs in *S. aureus* MLST database. Pink circles labeled with red arrows and the ST names in black font are used for sequence types identified in this study.

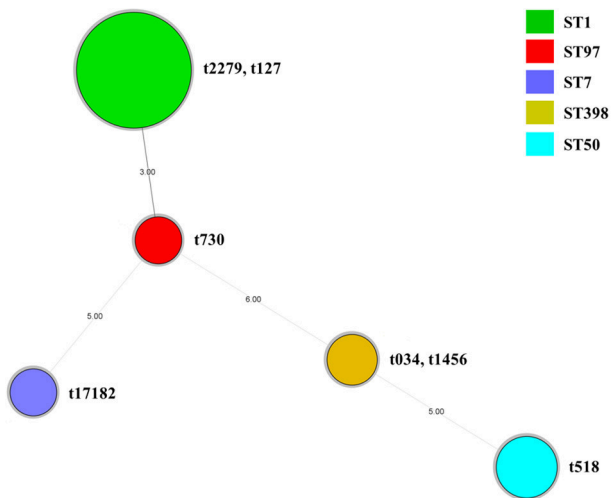


FIGURE 3 | Minimum Spanning Tree of 96 *S. aureus* isolates based on the MLST data. Colors indicate ST of each node. The spa types of 96 *S. aureus* isolates were also showed within each node. Novel spa type identified in this study was t17182.

as Cluster V-CC50-ST50-t518-*agr* IV. Moreover, 6 ST398 isolates that could not be digested with *Sma*I, were grouped as PFGE cluster VI in this study (Cluster VI-CC398-ST398-t034/t1456-*agr* I). The DI values of PFGE, *spa* typing, MLST, and *agr* typing of all 96 isolates were 0.701, 0.641, 0.463, and 0.448, respectively.

Relationship Between Phenotypes and Genotypes

The relationship between antimicrobial resistance, virulence, biofilm and molecular subtypes is shown in **Figure 1**. Each clonal complex had specific antimicrobial resistance, virulence, and biofilm characteristics. Isolates identified as CC1-ST1 clones and contained within PFGE cluster I-t2279-*agr* III were found to be resistance only to PEN with two isolates susceptible to all tested antimicrobial agents tested, followed by three virulence gene patterns denoted as as*pvl-seb*(13/15), *pvl*(1/15), and *pvl-seb-sed* (1/15). Isolates within PFGE cluster II-CC1-ST1-t127-*agr* III exhibited more resistant diversity including PEN-CIP (3/54), PEN-CIP-ENO (1/54), PEN (1/54), CIP (1/54), ENO (1/54),followed by two virulence gene patterns denoted as *pvl-sea-sec* (53/54) and *sea-sec* (1/54). All isolates in this cluster were un-susceptible to CIP. All isolates within PFGE cluster III-CC97-ST97-t730-*agr* I expressed resistance to PEN, CIP, and ENO, followed by two virulence gene patterns, *tst-pvl-sec-sed*(2/4) and *pvl-sec-sed*(2/4). The isolates identified as PFGE cluster IV-CC7-ST7-t17182-*agr* I showed resistant to CIP and ENO, followed by two virulence gene patterns, *pvl*(3/4) and *pvl-sec* (1/4). Only three isolates (3/13) with PFGE cluster V-CC50-ST50-t518-*agr* IV exhibited a resistance phenotype (2 resistant to PEN and 1 resistance to TET) and all 13 isolates in this cluster harbored the *pvl* gene, with three isolates also carrying the *sea* and *sec* genes. In contrast, isolates identified as CC398-ST398 expressed the greatest MDR in this study (5 patterns of PEN-CIP-ENO-ERY-TIL and 1 patterns of PEN-CHL-GEN-TIL). Moreover, the only MRSA isolate with CC398-ST398-t034-*agr* I harbored three virulence genes of *pvl*, *sea* and *sec*,

whereas another 5 CC398-ST398 isolates identified as t1456-*agr* I were found to carry none of the tested virulence genes. Biofilm formation assay showed that this CC398-ST398-t1456-*agr* I clone was only able to produce biofilm weakly in this study.

DISCUSSION

S. aureus has been considered as an important cause of zoonotic disease and the potential transmission of MRSA between livestock and humans through close contact, handling and/or consumption of *S. aureus* infected food of animal origin (Kateete et al., 2013; Song et al., 2015; Pereyra et al., 2016). The infection of dairy herds and contamination of raw milk by *S. aureus*, especially those expressing a MDR phenotype and possessing the ability for produce biofilm and toxins including enterotoxin, TSST-1 and PVL, remains an important public health issue (Cavicchioli et al., 2015; Wang et al., 2016). The public health significance caused by this bacterium is manifested by food-borne poisoning outbreaks caused by dairy products contaminated by *S. aureus*, including one of the largest food-borne outbreaks on record involving 13,420 infected individuals in Japan (Asao et al., 2003; Hennekinne et al., 2012). Of note, food-borne infections attributed to *S. aureus* contaminated dairy foods are also frequently reported in China (Rong et al., 2017). Additionally, the economic cost burden to the dairy farms is considerable; mastitis in dairy cow can result in reductions in milk yield, treatment expense and/or culling in sometimes (Hennekinne et al., 2012). This study investigated the prevalence, genetic diversity, antimicrobial resistance phenotypes, carriage of staphylococcal virulence factors along with testing the capacity of these isolates to produce biofilm and the 5 classical enterotoxins (SEA to SEE). All of these *S. aureus* were isolated from raw milk samples taken in 2 dairy farms in Beijing, China. Acquisition of the prevalence and characteristics of *S. aureus* isolated from raw milk would be helpful to obtain the antimicrobial resistance and virulence markers as well as predominant clones which can help prevent and control the *S. aureus* contamination in dairy herd and protect the end consumer.

In the present study, 46.2% (90/195) of raw milk samples taken from dairy cows with mastitis were positive for *S. aureus*. This prevalence is similar to a recent report in China and other reports in Brazil and Italy (Cavicchioli et al., 2015; Li et al., 2015; Giacinti et al., 2017). However, another recent study reported that the prevalence of *S. aureus* in raw milk of health cows in Beijing was 22.0% (Liu et al., 2017). Overall, our data indicate that *S. aureus* is common and frequently detected in the raw milk of dairy cows with mastitis in Beijing, China. Further research is needed to explore methods of controlling *S. aureus* occurrence in raw milk.

In recent years, the emergence of MDR *S. aureus*, particularly MRSA, leading to animal and human infections, has become a growing public health concern (Li et al., 2015). In the current study, few resistances were detected among all 96 *S. aureus* (38.5% resistant to at least one antimicrobial), which were similar to those in Italy (39.4%) and Poland (23%), but much lower than two previous reports in Chinese (87% and 72.2%, respectively) and those in India (95%) (Li et al., 2015; Rola et al., 2015; Mistry et al., 2016; Giacinti et al., 2017; Liu et al., 2017). Moreover, only 6 isolates (6.3%) showed MDR that was lower than reports in other regions in China (Li et al., 2015; Liu et al., 2017). According to previous studies, penicillin-resistant *S. aureus* are the most prevalence isolates among raw milk and ranged from less than 10% to over 80% (Li et al., 2015; Rola et al., 2015; Liu et al., 2017). In this study, 31.3% of *S. aureus* were resistant to this antimicrobial agent. It was notable that ciprofloxacin- and enrofloxacin-resistant *S. aureus* were found to be the next most frequently detected resistance types in addition to penicillin. Both are fluoroquinolones, wherein ciprofloxacin a third generation fluoroquinolone is used at clinical level while enrofloxacin is specially used for veterinary applications in China (Hoang et al., 2017; Li J. et al., 2017). Once human and/or animals become infected with these resistant isolates, treatment failure using these two antimicrobials, is inevitable. Additionally, 54.2 and 7.3% of the isolates from farm A expressed an intermediate phenotype to ciprofloxacin and enrofloxacin, respectively. Meanwhile, isolates from farm B exhibited significantly higher resistance to a panel of antimicrobial compounds including penicillin, ciprofloxacin, enrofloxacin, tilmicosin, and erythromycin when compared to those from farm A ($p < 0.05$). Moreover, the resistance patterns were different between two farms in that PEN-CIP-ENO-ERY-TIL and PEN were more frequently detected from farm B compared with farm A ($p < 0.05$). These results suggested that the isolates from both farms may have their own resistance characteristics and the resistance patterns from farm A were more diverse than those from farm B ($p < 0.05$). Furthermore, it has been reported that rational management and appropriate usage of antimicrobial compounds in food-producing livestock is very important to control and prevent the spread of drug-resistant isolates (Jessen et al., 2017). All isolates in this study exhibited low-level resistance to other antimicrobial agents tested and similarly the MIC_{50} and MIC_{90} values were relatively low, a situation that is much different to previous reports in China and other countries (Li et al., 2015; Mistry et al., 2016; Liu et al., 2017). The relatively low rate of resistance and MDR isolates observed in this study could be due to the extensive farming systems and the strict management of the use of antimicrobial agents by the company.

MRSA is considered as major cause of hospital-acquired and community-acquired infections (Gopal and Divya, 2017). Additionally, the contaminated animal and associated products have been supposed to be a potential source of community-acquired MRSA (Gopal and Divya, 2017). Recently, the isolation of MRSA from raw milk and dairy products has been reported worldwide (Rola et al., 2016; Tarekgne et al., 2016; Basanisi et al., 2017). In this study, one *S. aureus* isolate (1.4%, 1/96) was identified as MRSA being confirmed by amplifying the *mecA* gene. The current study's prevalence reported for MRSA is lower than those reported previously in China or India (4.8–48.7%) (Li et al., 2015; Mistry et al., 2016; Liu et al., 2017). However, the potential MRSA transmission risk via the food chain, particularly by insufficient pasteurization milk, cannot be ignored.

With regard to the risk of pathogenicity, the presence of virulence genes among all 96 isolates was also assessed in this study. The classic enterotoxin SE determinants, of *S. aureus* are known to cause sporadic food-poisoning incidents or even food-borne outbreaks. It is reported that 89.7% isolates from cow milk related to mastitis carried one or more SEs genes (Song et al., 2015). In the current study, 80.2% of the isolates were positive for SE encoding genes and the *sec* (65.6%) and *sea* (60.4%) genes were the most frequently detected. This finding is similar to those in previous reports from China and Australia, whereas the *sed* gene was mainly detected among isolates from raw milk samples in Poland (Rola et al., 2015; Song et al., 2015; McMillan et al., 2016; Liu et al., 2017). Meanwhile, another Chinese study reported that the *seb* gene was the most commonly detected (Cheng et al., 2016). Additionally, the prevalence rates of the *sec* gene from farm A (84.9%) was higher than from farm B (4.3%) ($p < 0.05$). While, the *sea* gene was only found in farm A, and the *seb* gene was only found in farm B. Therefore, the different prevalence rates observed among all SE genes could be due to the fact that these isolates originated in geographically diverse locations. According to previous reports, the *see* gene was rarely present in raw milk or even retail food in China, and similarly, this marker was not detected in this study. Notably, the *pvl*-encoding gene showed a very high prevalence (93.8%) in the tested isolates, which was similar to previous reports (Esposito et al., 2013; Aires-de-Sousa, 2017). It was reported that the *pvl*-encoding gene were present at a high prevalence among methicillin-sensitive isolates and the Livestock-associated MRSA (LA-MRSA) isolates positive with PVL mostly originated from humans (Price et al., 2012; Wardyn et al., 2012). Two isolates in this study were identified to have the *tst* gene, which could cause severe clinical diseases (Xie et al., 2011). Our data highlight the necessity to identify virulence factors among pathogenic *S. aureus*.

Several studies examined for the presence of SEs genes among *S. aureus* cultured from raw milk and their food products (Asao et al., 2003; Song et al., 2015; Cheng et al., 2016; Liu et al., 2017). However, few reports assessed the enterotoxin producing capacity of these isolates in China. To our best knowledge, this study firstly reported the production of 4 classic SEs in raw dairy milk in China. The results showed that >90% of the SEs (*sea* to *sed*) genes carried *S. Aureus* isolates could produce enterotoxins. Additionally, 54 (70.1%, 54/77) of the SE gene carrying *S. aureus* simultaneously produced two types of enterotoxins, including one MRSA isolate (positive for SEA and SEC). Once enterotoxins were already produced, and these can generally retain their biological activity even after heat treatment (Cavicchioli et al., 2015). Thus, it is necessary to develop measures to eliminate the contamination of this bacterium in dairy products.

The study also investigated the distribution of biofilm and adhesion related genes among all isolate, some of which are also related to bacterial virulence (Rasmussen et al., 2013). In this study, all 96 isolates harbored the *icaAD*, *fnbA*, *clfAB*, and *cna* genes and 92.7% of the isolates harbored the *fnbB* gene. In contrast the *bap* gene was only detected in one isolate. Thus, these isolates have the ability to form biofilm a feature that suggests these bacteria have the potential to persist in this environment. The ability to form biofilms helps *S. aureus* to persist in infections and subclinical and clinical cases of bovine mastitis (Dhanawade et al., 2010). In the present study all 96 *S. aureus* isolates could form biofilms as determined by the microtiter plate assay described above, and these findings agree with a previous report from Argentina but being higher in number than reported in a similar study from Brazil (Lee et al., 2014; Pereyra et al., 2016). The high incidence of biofilm-producing *S. aureus* isolates in this study suggests the necessary for dairy farms to improve the quality assurance systems, in order to decrease and eliminate these isolates.

Our data also highlighted the diverse genetic backgrounds of the *S. aureus* from raw milk by MLST, *spa* typing, *agr* typing and PFGE sub-typing. Since the MLST genotyping for *S. aureus* was first reported, it has been widely used in epidemiological analysis of *S. aureus* infection and associated food poisoning outbreaks (Enright et al., 2000). In this study, five sequence types were obtained by MLST and each was further grouped into a clonal complexes. CC1-ST1 was the predominant clone (71.9%, 69/96), followed by CC50-ST50, CC398-ST398, CC7-ST7, and CC398-ST398, all of which have been reported in raw milk in China, previously (Song et al., 2015). Moreover, the ST1 and ST97 lineages were also detected frequently from bovine milk worldwide, while ST398, the most common livestock-associated MRSA type, has been already found in both food-producing animal and human species (Mistry et al., 2016; Gopal and Divya, 2017). Six isolates were identified as ST398 including the only one detected as a MRSA strain in this study. It was reported that MRSA ST398 is the most prevalent clone in Europe and North America, whereas methicillin-susceptible *S. aureus* (MSSA) ST398 was predominant in Asian regions (Asai et al., 2012; Yan et al., 2014). In total, six known *spa* types (t034, t127, t518, t730, t1456, and t2279) and 1 newly identified *spa* type (t17182) were identified in this study. A previous study also observed *spa* diversity among the STs although some *spa* types corresponded with either an ST or a CC (Chao et al., 2015). The *spa* types, t127 and t2279, have been reported as community-associated clones previously, and these were the top two frequently distributed genotypes among raw milk samples where all isolates of both types were identified as ST1 (Song et al., 2015). Considering the transmission of bacterial species between humans and livestock is increasingly being detected in farm workers in several countries (Huijsdens et al., 2006; Kateete et al., 2013), a recent study showed that the t127 clone could be present in cows, humans and environments (Papadopoulos et al., 2018). Although isolates of this *spa* type exhibited less antimicrobial resistance in this study, the potential of biofilm and enterotoxin producing would lead to persistent existence and subsequent contamination. Therefore, this clone could be important source of contaminations in cow farms, leading to quickly spread and large infections in both dairy herd and human community.

Isolates of ST398 types corresponded to one t034 (MRSA) and 5 to t1456 (MSSA) along with each of the other STs being linked to sole *spa* type. Of note, the ST398-t1456 MSSA was firstly

identified in China, while the ST398-t1456 clone was related to LA-MRSA in Europe (Köck et al., 2013). Furthermore, the newly identified *spa* type t17182 corresponded to ST7, which has been reported to be related to bovine mastitis (Li T. et al., 2017). Moreover, ST50-t518 found in this study was reported to be mainly present in bovines in Denmark (Hasman et al., 2010). The other *spa* type t730, has been less frequently detected then before, and corresponded to the bovine milk-associated sequence type ST97 (Gopal and Divya, 2017). In this study *agr* type III was the most predominant *agr* type (71.9%) among *S. aureus* isolates, which is in accordance with a previous report from Brazil (48.2%) (Silva et al., 2013). However, *agr*I and *agr* II could be predominant types according to previous reports (Fabres-Klein et al., 2015; Khoramrooz et al., 2016; Mistry et al., 2016). Only 14.6 and 13.5% of our isolates were identified as *agr* I and IV respectively, which are lower than previous reports (Fabres-Klein et al., 2015; Mistry et al., 2016). Similar to other studies the *agr* II was not identified in the current study (Fabres-Klein et al., 2015; Khoramrooz et al., 2016; Mistry et al., 2016).

PFGE is generally recognized as the current gold standard method, and it has been widely used in genotyping of various bacteria including bovine mastitis associated *S. aureus* (De Oliveira et al., 2000; McMillan et al., 2016). Previous studies demonstrated that different clonal lineages may exhibit specific patterns of antimicrobial resistance and contain various virulence factors (Hata et al., 2010; Song et al., 2015). In this study, isolates of the PFGE cluster II (56.3%) and cluster I (15.6%) were the most frequently detected. All belonged to ST1 (CC1), t127/2279 along with the *agr* type or *agr* III which were grouped in these two clusters. The *agr* system is related to the regulation of virulence factors and different *agr* groups may have specific virulence patterns (Melchior et al., 2009; Khoramrooz et al., 2016). This study showed that isolates of *agr* III of represented by two clones (PFGE Cluster I/II-CC1-ST1-t127/2279), carried more virulence genes than those of *agr* I and *agr* IV types, suggesting that *agr* profiles may be associated with the virulence potential of *S. aureus*. Furthermore, isolates in PFGE Cluster II-CC1-ST1-t127-*agr* III exhibited the most diversities of antimicrobial resistant, while isolates in PFGE Cluster I-CC1-ST1-t2279-*agr* III was only resistant to PEN. Of note, the 5 MSSA-ST398-t1456-*agr* I isolates expressed the most MDR patterns but with no virulence genes and showed weakly biofilm formation, whereas the MRSA-ST398-t034-*agr* I clone expressed MDR and virulence (*pvl-sea-sec*) as well as showing moderate biofilm formation in this study. All isolates within PFGE cluster III-CC97-ST97-t730-*agr* I clone were resistant to PEN, CIP, and ENO, while all isolates in the PFGE cluster IV-CC7-ST7-t17182-*agr* I showed resistant to CIP and ENO. Geographically, isolates from farm A and farm B were well distinguished phylogenetically in this study. It is interesting that we found different isolates from the same mastitic milk sample that showed different genotypes or phenotypes in this study, which confirmed the fact that different clones could colonize in one host, making it harder to eliminate and control *S. aureus* infections in dairy cows.

CONCLUSIONS

In summary, our research provides detailed epidemiological survey on the prevalence of *S. aureus* in raw milk of dairy cows with mastitis in Beijing, China. This study demonstrated a rather high prevalence of *S. aureus* with enterotoxigenic and biofilm forming abilities that may contribute to *S. aureus* persisting in the dairy farms leading to severe infections and subsequent food poisoning. To the best of our knowledge, this study firstly reported the classic SEs production in raw milk from cows in China. However the percentage of MDR and MRSA isolates was low in this study, their pathogenicity and transmission risk cannot be ignored. Of note, it is necessary to control and eliminate the present of MDR, enterotoxigenic and biofilm formatting *S. aureus* in raw milk. Additionally, our study also demonstrated the genetic diversity these isolates. Results of the present study highlight the dominant genetic lineages of livestock associated found not only in China but also worldwide. Although new *spa* type variants were found, their lineage related sequence type suggested that these strains may also associate with bovine mastitis. Significant differences genetic diversity along with antimicrobial resistance, virulence factors and biofilm formation were observed for *S. aureus* isolates from raw milk. It was shown that *S. aureus* with similar genetic characteristic displayed specific antimicrobial resistance patterns, virulence gene profiles, biofilm formations and geographic features and different clones could colonize in one dairy host. Therefore, monitoring the genotypes of *S. aureus* in dairy cow would give assistance to distinguish prevalent clones, which can help dairy farms develop control measures for mastitis caused by *S. aureus*.

AVAILABILITY OF DATA AND MATERIALS

The aggregate data supporting findings contained within this manuscript will be shared upon request submitted to the corresponding author.

AUTHOR CONTRIBUTIONS

WW, ZB, XL, FL, and SF designed experiments. TJ, ZP, JX, and LY carried out experiments. WW and XL analyzed experimental data. WW, ZB, FL, and SF wrote the manuscript.

FUNDING

This study was funded by the National Key R&D Program of China (2016YFD0401102), and China Food Safety Talent Competency Development Initiative: CFSA 523 Program.

ACKNOWLEDGMENTS

We sincerely thank all the participants who took part in this study.

REFERENCES

Aires-de-Sousa, M. (2017). Methicillin-resistant *Staphylococcus aureus* among animals: current overview. *Clin. Microbiol. Infect.* 23, 373–380. doi: 10.1016/j.cmi.2016.11.002

Asai, T., Hiki, M., Baba, K., Usui, M., Ishihara, K., and Tamura, Y. (2012). Presence of *Staphylococcus aureus* ST398 and ST9 in swine in Japan. *Jpn. J. Infect. Dis.* 65, 551–552. doi: 10.7883/yoken.65.551

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., et al. (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol. Infect.* 130, 33–40. doi: 10.1017/S0950268802007951

Basanisi, M. G., La Bella, G., Nobili, G., Franconieri, I., and La Salandra, G. (2017). Genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk and dairy products in South Italy. *Food Microbiol.* 62, 141–146. doi: 10.1016/j.fm.2016.10.020

Bergdoll, M. S., Crass, B. A., Reiser, R. F., Robbins, R. N., and Davis, J. P. (1981). A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome *Staphylococcus aureus* isolates. *Lancet* 1, 1017–1021 doi: 10.1016/S0140-6736(81)92186-3

Cavicchioli, V. Q., Scatamburlo, T. M., Yamazi, A. K., Pieri, F. A., and Nero, L. A. (2015). Occurrence of *Salmonella*, *Listeria monocytogenes*, and enterotoxigenic *Staphylococcus* in goat milk from small and medium-sized farms located in Minas Gerais State, Brazil. *J. Dairy. Sci.* 98, 8386–8390. doi: 10.3168/jds.2015-9733

Chao, G., Bao, G., Cao, Y., Yan, W., Wang, Y., Zhang, X., et al. (2015). Prevalence and diversity of enterotoxin genes with genetic background of *Staphylococcus aureus* isolates from different origins in China. *Int. J. Food Microbiol.* 211, 142–147. doi: 10.1016/j.ijfoodmicro.2015.07.018

Cheng, J., Wang, Y., Cao, Y., Yan, W., Niu, X., Zhou, L., et al. (2016). The Distribution of 18 enterotoxin and enterotoxin-like genes in *Staphylococcus aureus* strains from different sources in East China. *Foodborne Pathog. Dis.* 13, 171–176. doi: 10.1089/fpd.2015.1963

CLSI (2015). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline.* Wayne; Pennsylvania, PA: Clinical Laboratory Standards Institute.

Darwish, S. F., and Asfour, H. A. (2013). Investigation of biofilm forming ability in Staphylococci causing bovine mastitis using phenotypic and genotypic assays. *Scientific World J.* 2013, 378492. doi: 10.1155/2013/378492

De Oliveira, A. P., Watts, J. L., Salmon, S. A., and Aarestrup, F. M. (2000). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. *J. Dairy Sci.* 83, 855–862. doi: 10.3168/jds.S0022-0302(00)74949-6

Dhanawade, N. B., Kalorey, D. R., Srinivasan, R., Barbuddhe, S. B., and Kurkure, N. V. (2010). Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Vet. Res. Commun.* 34, 81–89. doi: 10.1007/s11259-009-9326-0

Dufour, S., Dohoo, I. R., Barkema, H. W., Descôteaux, L., Devries, T. J., Reyher, K. K., et al. (2012). Manageable risk factors associated with the lactational incidence, elimination, and prevalence of *Staphylococcus aureus*intramammary infections in dairy cows. *J. Dairy Sci.* 95, 1283–1300. doi: 10.3168/jds.2011-4711

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., and Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* 38, 1008–1015. doi: 10.1128/JCM.02421-12

Esposito, S., Purrello, S. M., Bonnet, E., Novelli, A., Tripodi, F., Pascale, R., et al. (2013). Central venous catheter-related biofilm infections: an up-to-date focus on meticillin-resistant *Staphylococcus aureus*. *J. Glob. Antimicrob. Resist.* 1, 71–78. doi: 10.1016/j.jgar.2013.03.002

Fabres-Klein, M. H., Caizer Santos, M. J., Contelli Klein, R., Nunes de Souza, G., and de Oliveira Barros Ribon, A. (2015). An association between milk and slime increases biofilm production by bovine *Staphylococcus aureus*. *BMC Vet. Res.* 11, 3. doi: 10.1186/s12917-015-0319-7

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., and Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* 186, 1518–1530. doi: 10.1128/JB.186.5.1518-1530.2004

Felipe, V., Morgante, C. A., Somale, P. S., Varroni, F., Zingaretti, M. L., Bachetti, R. A., et al. (2017). Evaluation of the biofilm forming ability and its associated genes in *Staphylococcus* species isolates from bovine mastitis in Argentinean dairy farms. *Microb. Pathog.* 104, 278–286. doi: 10.1016/j.micpath.2017.01.047

Giacinti, G., Carfora, V., Caprioli, A., Sagrafoli, D., Marri, N., Giangolini, G., et al. (2017). Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* carrying *mecA* or *mecC* and methicillin-susceptible *Staphylococcus aureus* in dairy sheep farms in central Italy. *J. Dairy Sci.* 100, 7857–7863. doi: 10.3168/jds.2017-12940

Gomes, F., and Henriques, M. (2016). Control of bovine mastitis: old and recent therapeutic approaches. *Curr. Microbiol.* 72, 377–382. doi: 10.1007/s00284-015-0958-8

Gopal, S., and Divya, K. C. (2017). Can methicillin-resistant *Staphylococcus aureus* prevalence from dairy cows in India act as potential risk for community-associated infections? A review. *Vet. World.* 10, 311–318. doi: 10.14202/vetworld.2017.311-318

Harmsen, D., Claus, H., Witte, W., Rothgänger, J., Claus, H., Turnwald, D., et al. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J. Clin. Microbiol.* 41, 5442–5448. doi: 10.1128/JCM.41.12.5442-5448.2003

Hasman, H., Moodley, A., Guardabassi, L., Stegger, M., Skov, R. L., and Aarestrup, F. M. (2010). *Spa* type distribution in *Staphylococcus aureus* originating from pigs, cattle and poultry. *Vet. Microbiol.* 141, 326–331. doi: 10.1016/j.vetmic.2009.09.025

Hata, E., Katsuda, K., Kobayashi, H., Uchida, I., Tanaka, K., and Eguchi, M. (2010). Genetic variation among *Staphylococcus aureus* strains from bovine milk and their relevance to methicillin-resistant isolates from humans. *J. Clin. Microbiol.* 48, 2130–2139. doi: 10.1128/JCM.01940-09

Hennekinne, J. A., De Buyser, M. L., and Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiol. Rev.* 36, 815–836. doi: 10.1111/j.1574-6976.2011.00311.x

Hoang, P. H., Awasthi, S. P., DO Nguyen, P., Nguyen, N. L., Nguyen, D. T., LE, N. H., et al. (2017). Antimicrobial resistance profiles and molecular characterization of *Escherichia coli* strains isolated from healthy adults in Ho Chi Minh City, Vietnam. *J. Vet. Med. Sci.* 79, 479–485. doi: 10.1292/jvms.16-0639

Huijsdens, X. W., van Dijke, B. J., Spalburg, E., van Santen-Verheuvel, M. G., Heck, M. E., Pluister, G. N., et al. (2006). Community-acquired MRSA and pig-farming. *Ann. Clin. Microbiol. Antimicrob.* 5:26. doi: 10.1186/1476-0711-5-26

Jakobsen, R. A., Heggebø, R., Sunde, E. B., and Skjervheim, M. (2011). *Staphylococcus aureus* and Listeria monocytogenes in Norwegian raw milk cheese production. *Food Microbiol.* 28, 492–496. doi: 10.1016/j.fm.2010.10.017

Jans, C., Merz, A., Johler, S., Younan, M., Tanner, S. A., Kaindi, D. W. M., et al. (2017). East and West African milk products are reservoirs for human and livestock-associated *Staphylococcus aureus*. *Food Microbiol.* 65, 64–73. doi: 10.1016/j.fm.2017.01.017

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., et al. (2002). Relationships between *S. aureus* genetic background, virulence factors, agrgroups (Allele), and human disease. *Infect. Immun.* 70, 631–641. doi: 10.1128/IAI.70.2.631-641.2002

Jessen, L. R., Sørensen, T. M., Lilja, Z. L., Kristensen, M., Hald, T., and Damborg, P. (2017). Cross-sectional survey on the use and impact of the Danish national antibiotic use guidelines for companion animal practice. *Acta. Vet. Scand.* 59, 81. doi: 10.1186/s13028-017-0350-8

Johler, S.,Weder, D.,Bridy, C.,Huguenin, M. C., Robert, L., Hummerjohann, J., et al. (2015). Outbreak of staphylococcal food poisoning among children and staff at a Swiss boarding school due to soft cheese made from raw milk. *J. Dairy Sci.* 98, 2944–2948. doi: 10.3168/jds.2014-9123

Kateete, D. P., Kabugo, U., Baluku, H., Nyakarahuka, L., Kyobe, S., Okee, M., et al. (2013). Prevalence and antimicrobial susceptibility patterns of bacteria from milkmen and cows with clinical mastitis in and around Kampala, Uganda. *PLoS ONE* 8:e63413. doi: 10.1371/journal.pone.0063413

Khoramian, B., Jabalameli, F., Niasari-Naslaji, A., Taherikalani, M., and Emaneini, M. (2015). Comparison of virulence factors and biofilm formation among

Staphylococcus aureus strains isolated from human and bovine infections. *Microb. Pathog.* 88, 73–77. doi: 10.1016/j.micpath.2015.08.007

Khoramrooz, S. S., Mansouri, F., Marashifard, M., Malek Hosseini, S. A., Akbarian Chenarestane-Olia, F., Ganavehei, B., et al. (2016). Detection of biofilm related genes, classical enterotoxin genes and *agr* typing among *Staphylococcus aureus* isolated from bovine with subclinical mastitis in southwest of Iran. *Microb. Pathog.* 97, 45–51. doi: 10.1016/j.micpath.2016.05.022

Klein, R. C., Fabres-Klein, M. H., Brito, M. A., Fietto, L. G., and Ribon Ade, O. (2012). *Staphylococcus aureus* of bovine origin: genetic diversity, prevalence and the expression of adhesin-encoding genes. *Vet. Microbiol.* 160, 183–188. doi: 10.1016/j.vetmic.2012.05.025

Köck, R., Schaumburg, F., Mellmann, A., Köksal, M., Jurke, A., Becker, K., et al. (2013). Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) as causes of human infection and colonization in Germany. *PLoS ONE* 8:e55040. doi: 10.1371/journal.pone.0055040

Lee, S. H., Mangolin, B. L., Gonçalves, J. L., Neeff, D. V., Silva, M. P., Cruz, A. G., et al. (2014). Biofilm-producing ability of *Staphylococcus aureus* isolates from Brazilian dairy farms. *J. Dairy Sci.* 97, 1812–1816. doi: 10.3168/jds.2013-7387

Li, J., Hao, H., Cheng, G., Liu, C., Ahmed, S., Shabbir, M. A. B., et al. (2017). Microbial shifts in the intestinal microbiota of *Salmonella* infected chickens in response to enrofloxacin. *Front. Microbiol.* 8:1711. doi: 10.3389/fmicb.2017.01711

Li, L., Zhou, L., Wang, L., Xue, H., and Zhao, X. (2015). Characterization of methicillin-resistant and -susceptible staphylococcal isolates from bovine milk in northwestern China. *PLoS ONE* 10:e0116699. doi: 10.1371/journal.pone.0116699

Li, T., Lu, H., Wang, X., Gao, Q., Dai, Y., Shang, J., et al. (2017). Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. *Front. Cell Infect. Microbiol.* 7:127. doi: 10.3389/fcimb.2017.00127

Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., et al. (2017). Prevalence, antimicrobial susceptibility, and molecular characterization of *Staphylococcus aureus* isolated from dairy herds in northern China. *J. Dairy Sci.* 100, 8796–8803. doi: 10.3168/jds.2017-13370

McClure, J. A., Conly, J. M., Lau, V., Elsayed, S., Louie, T., Hutchins, W., et al. (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. *J ClinMicrobiol.* 44, 1141–1144. doi: 10.1128/JCM.44.3.1141-1144.2006

McMillan, K., Moore, S. C., McAuley, C. M., Fegan, N., and Fox, E. M. (2016). Characterization of *Staphylococcus aureus* isolates from raw milk sources in Victoria, Australia. *BMC Microbiol.* 16, 169. doi: 10.1186/s12866-016-0789-1

Melchior, M. B., van Osch, M. H., Graat, R. M., van Duijkeren, E., Mevius, D. J., Nielen, M., et al. (2009). Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. *Vet. Microbiol.* 137, 83–89. doi: 10.1016/j.vetmic.2008.12.004

Mistry, H., Sharma, P., Mahato, S., Saravanan, R., Kumar, P. A., and Bhandari, V. (2016). Prevalence and characterization of oxacillin susceptible *mecA*-positive clinical isolates of *Staphylococcus aureus* causing bovine mastitis in India. *PLoS ONE* 11:e0162256. doi: 10.1371/journal.pone.0162256

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe, S. (1991). Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* 29, 2240–2244.

Murchan, S., Kaufmann, M. E., Deplano, A., de Ryck, R., Struelens, M., Zinn, C. E., et al. (2003). Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J. Clin. Microbiol.* 41, 1574–1585. doi: 10.1128/JCM.41.4.1574-1585.2003

Müsken, M., Di Fiore, S., Römling, U., and Häussler, S. (2010). A 96-well-plate-based optical method for the quantitative and qualitative evaluation of Pseudomonas aeruginosa biofilm formation and its application to susceptibility testing. *Nat. Protoc.* 5, 1460–1469. doi: 10.1038/nprot.2010.110

Papadopoulos, P., Papadopoulos, T., Angelidis, A. S., Boukouvala, E., Zdragas, A., Papa, A., et al. (2018). Prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. aureus* (MRSA) along the production chain of dairy products in north-western *Greece. Food. Microbiol.* 69, 43–50. doi: 10.1016/j.fm.2017.07.016

Pereyra, E. A., Picech, F., Renna, M. S., Baravalle, C., Andreotti, C. S., Russi, R., et al. (2016). Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. *Vet. Microbiol.* 183, 69–77. doi: 10.1016/j.vetmic.2015.12.002

Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., et al. (2012). *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. *MBio.* 3:e00305-11. doi: 10.1128/mBio.00305-11

Rasmussen, G., Monecke, S., Ehricht, R., and Söderquist, B. (2013). Prevalence of clonal complexes and virulence genes among commensal and invasive *Staphylococcus aureus* isolates in Sweden. *PLoS ONE* 8:e77477. doi: 10.1371/journal.pone.0077477

Ribot, E. M., Fair, M. A., Gautom, R., Cameron, D. N., Hunter, S. B., Swaminathan, B., et al. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog. Dis.* 3, 59–67. doi: 10.1089/fpd.2006.3.59

Rola, J. G., Czubkowska, A., Korpysa-Dzirba, W., and Osek, J. (2016). Occurrence of *Staphylococcus aureus* on farms with small scale production of raw milk cheeses in Poland. *Toxins (Basel).* 8:E62. doi: 10.3390/toxins8030062

Rola, J. G., Korpysa-Dzirba, W., Czubkowska, A., and Osek, J. (2015). Prevalence of enterotoxin genes and antimicrobial resistance of coagulase-positive staphylococci recovered from raw cow milk. *J. Dairy Sci.* 98, 4273–4278. doi: 10.3168/jds.2014-9064

Rong, D., Wu, Q., Xu, M., Zhang, J. and Yu, S. (2017). Prevalence, virulence genes, antimicrobial susceptibility, and genetic diversity of Staphylococcus aureus from retail aquatic products in China. *Front. Microbiol.* 8:714. doi: 10.3389/fmicb.2017.00714

Rosec, J. P., and Gigaud, O. (2002). Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *Int. J. Food Microbiol.* 77, 61–70. doi: 10.1016/S0168-1605(02)00044-2

Schlievert, P. M., Shands, K. N., Dan, B. B., Schmid, G. P., and Nishimura, R. D. (1981). Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. *J. Infect. Dis.* 143, 509–516. doi: 10.1093/infdis/143.4.509

Schroeder, J. W. (2012). *Bovine Mastitis and Milking Management*. North Dakota State University. Available online at: http://www.clemson.edu/extension/scaged/scffa/career-development-events/files/dairy-evaluation/mastitis-control-programs.pdf (Accessed 2018 March).

Shopsin, B., Mathema, B., Alcabes, P., Said-Salim, B., Lina, G., Matsuka, A., et al. (2003). Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J. Clin. Microbiol.* 41, 456–459. doi: 10.1128/JCM.41.1.456-459.2003

Silva, N. C., Guimarães, F. F., Manzi, M. P., Budri, P. E., Gómez-Sanz, E., Benito, D., et al. (2013). Molecular characterization and clonal diversity of methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. *J. Dairy Sci.* 96, 6856–6862. doi: 10.3168/jds.2013-6719

Song, M., Bai, Y., Xu, J., Carter, M. Q., Shi, C., and Shi, X. (2015). Genetic diversity and virulence potential of *Staphylococcus aureus* isolates from raw and processed food commodities in Shanghai. *Int. J. Food Microbiol.* 195, 1–8. doi: 10.1016/j.ijfoodmicro.2014.11.020

Tarekgne, E. K., Skjerdal, T., Skeie, S., Rudi, K., Porcellato, D., Félix, B., et al. (2016). Enterotoxin gene profile and molecular characterization of *Staphylococcus aureus* isolates from bovine bulk milk and milk products of Tigray region, Northern Ethiopia. *J. Food Prot.* 79, 1387–1395. doi: 10.4315/0362-028X.JFP-16-003

Wang, D., Zhang, L., Zhou, X., He, Y., Yong, C., Shen, M., et al. (2016). Antimicrobial susceptibility, virulence genes, and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia, China. *J. Dairy Sci.* 99, 9560–9569. doi: 10.3168/jds.2016-11625

Wardyn, S. E., Forshey, B. M., and Smith, T. C. (2012). High prevalence of Panton-Valentine leukocidin among methicillin-sensitive *Staphylococcus aureus* colonization isolates in rural Iowa. *Microb. Drug. Resist.* 18, 427–433. doi: 10.1089/mdr.2011.0239

Xie, Y., He, Y., Gehring, A., Hu, Y., Li, Q., Tu, S. I., et al. (2011). Genotypes and toxin gene profiles of *Staphylococcus aureus* clinical isolates from China. *PLoS ONE* 6:e28276. doi: 10.1371/journal.pone.0028276

Yan, Q., Wang, J., Gangiredla, J., Cao, Y., Martins, M., Gopinath, G. R., et al. (2015). Comparative genotypic and phenotypic analysis of *Cronobacter* species cultured from four powdered infant formula production facilities: indication of pathoadaptation along the food chain. *Appl. Environ. Microbiol.* 81, 4388–4402. doi: 10.1128/AEM.00359-15

Yan, X., Yu, X., Tao, X., Zhang, J., Zhang, B., Dong, R., et al. (2014). *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. *Int. J. Med. Microbiol.* 304, 379–383. doi: 10.1016/j.ijmm.2013.12.003

Zecconi, A., and Scali, F. (2013). *Staphylococcus aureus* virulence factors in evasion from innate immune defenses in human and animal diseases. *Immunol. Lett.* 150, 12–22. doi: 10.1016/j.imlet.2013.01.004

Zmantar, T., Chaieb, K., Makni, H., Miladi, H., Abdallah, F. B., Mahdouani, K., et al. (2008). Detection by PCR of adhesins genes and slime production in clinical *Staphylococcus aureus*. *J. Basic. Microbiol.* 48, 308–314. doi: 10.1002/jobm.200700289

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Wang, Lin, Jiang, Peng, Xu, Yi, Li, Fanning and Baloch. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 June 2018
doi: 10.3389/fmicb.2018.01259

Optimization of *E. coli* Inactivation by Benzalkonium Chloride Reveals the Importance of Quantifying the Inoculum Effect on Chemical Disinfection

Míriam R. García[1]* *and Marta L. Cabo*[2]

[1] *Bioprocess Engineering Group, IIM-CSIC Spanish National Research Council, Vigo, Spain,* [2] *Microbiology Group, IIM-CSIC Spanish National Research Council, Vigo, Spain*

Optimal disinfection protocols are fundamental to minimize bacterial resistance to the compound applied, or cross-resistance to other antimicrobials such as antibiotics. The objective is twofold: guarantee safe levels of pathogens and minimize the excess of disinfectant after a treatment. In this work, the disinfectant dose is optimized based on a mathematical model. The model explains and predicts the interplay between disinfectant and pathogen at different initial microbial densities (inocula) and dose concentrations. The study focuses on the disinfection of *Escherichia coli* with benzalkonium chloride, the most common quaternary ammonium compound. Interestingly, the specific benzalkonium chloride uptake (mean uptake per cell) decreases exponentially when the inoculum concentration increases. As a consequence, the optimal disinfectant dose increases exponentially with the initial bacterial concentration.

Keywords: benzalkonium chloride (alkyldimethylbenzylammonium chloride), *Escherichia coli*, disinfection, inactivation, kinetic modeling, inoculum effect

OPEN ACCESS

Edited by:
Pierina Visciano,
Università di Teramo, Italy

Reviewed by:
Efstathios D. Giaouris,
University of the Aegean, Greece
Catherine M. Logue,
University of Georgia, United States

***Correspondence:**
Míriam R. García
miriamr@iim.csic.es

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 March 2018*
Accepted: *24 May 2018*
Published: *26 June 2018*

Citation:
García MR and Cabo ML (2018) Optimization of E. coli Inactivation by Benzalkonium Chloride Reveals the Importance of Quantifying the Inoculum Effect on Chemical Disinfection. Front. Microbiol. 9:1259. doi: 10.3389/fmicb.2018.01259

1. INTRODUCTION

Quaternary ammonium compounds (QACs) are chemicals produced at high volumes with low toxicity that may induce resistance to disinfectants or cross-resistance to other antimicrobials (Langsrud et al., 2003; Tezel and Pavlostathis, 2015). They are widely used in medical-related facilities, and in the food and pharmaceutical industries.

QACs differ from common disinfectants in water treatments as they are not chemically transformed during their application and may be released and diluted in the environment. Most QACs are only degraded under aerobic conditions by bacterial species in the genera of *Xanthomonas*, *Aeromonas*, and *Pseudomonas*. The impact of the degraders is still a matter of controversy. Tezel and Pavlostathis (2011) claim that this biodegradation creates sub-inhibitory concentrations in environmental media such as surface water and soil where susceptible species may develop bacterial resistance. That effect is not significant for some authors that restrict the environmental consequences to a change in bacterial tolerance to antimicrobials but not to resistance (Gerba, 2015). In any case, QACs degraders proliferate and may eventually cause public health problems (Tezel and Pavlostathis, 2015).

Optimal disinfection protocols are critical to minimizing QACs excess after a treatment while assuring safety levels of pathogens. Disinfectant dose concentrations smaller than the optimum are insufficient to achieve the necessary inactivation level and may induce resistance to the disinfectant applied. Larger doses guarantee that most pathogens are inactivated but may induce resistance in surrounding areas where disinfection concentration is lower because of partial coverage (Holah et al., 2002). Moreover, active chemicals may end up in the environment after the treatment and induce cross-resistance to other antimicrobials, including relevant antibiotics. In addition to reducing environmental impact, optimal doses contribute to minimizing the cost of the disinfection treatment.

Nowadays, QACs disinfection is mostly based on minimum inhibitory numbers without any advanced optimization due to the lack of appropriate models. Let us consider for example alkyldimethylbenzylammonium chloride, commonly known as benzalkonium chloride (BAC). This compound is a widely used "over-the-counter" surface disinfectant that may increase tolerance to antibiotics in *E. coli* (Bore et al., 2007). Kinetic models are scarce and mostly limited to time-kill curves without considering the concentration of BAC during the treatment (Ioannou et al., 2007). The few exceptions considering sophisticated models are not focused on the disinfection itself, but on subsequent BAC biodegradation (Zhang et al., 2011; Hajaya and Pavlostathis, 2013). From the authors' knowledge, only Lambert and Johnston (2001) modeled the BAC inhibition of a specific pathogen, in this case *Staphylococcus aureus*. This work is crucial to understand disinfection with soil contamination, but the model cannot be exploited for optimization since BAC after the treatment is not quantified. However, the work presents interesting observations about the dependence of the disinfection effectiveness with the inoculum concentration that requires further research.

In food microbiology, predictive kinetic models are well established with *ad-hoc* software tools that can be exploited to determine optimal operational conditions, but they are primarily focused on non-chemical (abiotic) disinfection (Geeraerd et al., 2005; Garre et al., 2017). Most works modeling the antimicrobial effect describe the inhibition of microbial growth without considering the antimicrobial kinetics. The exception is the research by Reichart (1994). This work develops kinetic models of microbial inactivation together with the dynamics of molecules responsible for the lethal effect. The theory, however, departs from the standard models on water treatment, instead of using standard modeling approaches in food microbiology.

Models considering chemical disinfection are common in water treatment, but disinfectant kinetics are still neglected or too simplistic to study QACs. Most models assume demand-free conditions, that is, the disinfectant is far in excess and remains constant during the treatment. **Figure 1** shows a nested model including common autonomous (without an explicit dependence with time in their derivative form) disinfection models under this demand-free condition. They are extremely useful and flexible to model different inactivation curves, but inadequate when the disinfectant is not constant during the treatment, i.e., in demanding conditions. Most disinfectants in water treatment are volatile and therefore the model modification consists of assuming first-order decay kinetics (Lambert and Johnston, 2000). The few exceptions are the model by Hunt and Mariñas (1999) using second-order kinetics and the model by Fernando (2009) assuming that the specific chemical demand (α) depends on the microorganism density during the treatment.

Another drawback that prevents the direct application of water treatment models (**Figure 1**) is that they assume proportionality between disinfectant and inoculum concentration. They are based on survival or time-kill curves defined in relative terms of log reductions (Ioannou et al., 2007), where the absolute number of active cells is divided by the inoculum concentration. It is therefore implicitly assumed proportionality, i.e., that a 1 log reduction requires the same amount of disinfectant independently of the cell concentration. In other words, if the inoculum doubles, the amount of disinfectant also doubles and therefore the Minimum Inhibitory Concentration (MIC) is proportional to the initial inoculum. However, that contradicts MIC estimations for quaternary ammonium compounds in the literature (Lambert and Johnston, 2001; Ioannou et al., 2007).

This inoculum effect is well-known in antibiotic resistance (Sabath et al., 1975; Thomson and Moland, 2001; Egervärn et al., 2007; Tan et al., 2012; Karslake et al., 2016) with specific descriptions using mathematical models (Udekwu et al., 2009; Bhagunde et al., 2010; Bulitta et al., 2010). Nevertheless, from the author's knowledge, models of the inoculum effect in chemical disinfection are still scarce (Haas and Kaymak, 2003; Kaymak and Haas, 2008) and limited to Lambert and Johnston (2001) for QACs.

In this work, the inoculum effect is studied based on the specific disinfectant uptake, i.e., the mean amount of QACs that is uptaken per cell, that links the pathogen and QAC kinetics. Under the common assumption of proportionality between dose and inoculum concentration, this value is constant with respect to the initial experimental conditions.

The case of study is the optimization *E. coli* inactivation by BAC. The work is organized into three parts: (1) the quantification of the dependence of the specific BAC uptake with respect to the BAC dose and inoculum concentrations; (2) the fit, validation and optimization of the the kinetic model to find the best BAC dose concentration that minimizes the excess of BAC after a treatment and (3) the postulation of possible mechanisms behind the inoculum effect.

2. MATERIALS AND METHODS

2.1. Experimental Materials and Methods

2.1.1. Bacterial Strain and Culturing Conditions

Escherichia coli CECT 4622 was purchased from the Spanish Type Culture Collection. Stock cultures were kept at −80 °C in Brain Heart Infusion Broth (BHI; Biolife, Milan, Italy) containing 50% glycerol 1:1 (v/v). Work cultures were kept at −20 °C in Trypticase Soy Broth (TSB; Cultimed, Barcelona, Spain) containing 50% glycerol 1:1 (v/v). Before the experiments, 100 µL

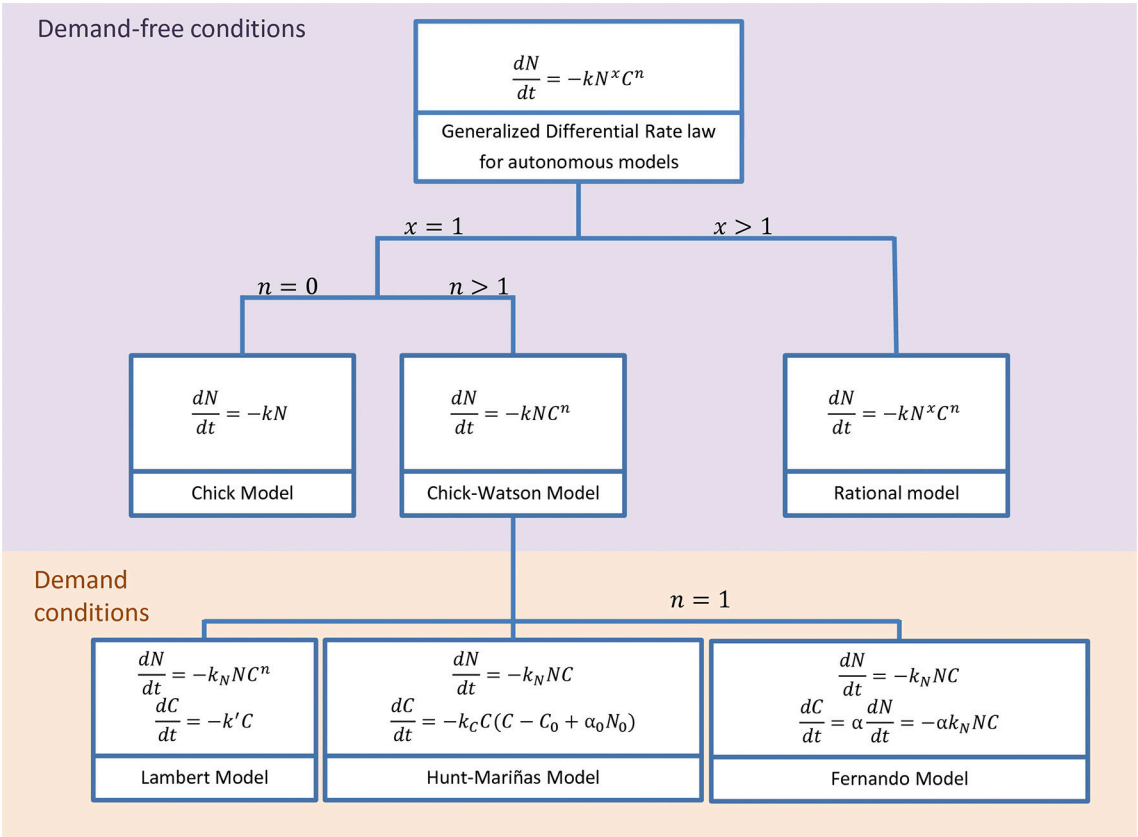


FIGURE 1 | Classical models under disinfectant demand-free conditions assume constant disinfectant concentration and are a special case of the Generalized Differential Rate Law (Gyürék and Finch, 1998; Hunt and Mariñas, 1999). The Integrated form of most of these models can be seen in Gyürék and Finch (1998). Common models using disinfectant demand conditions assume decay independent on the microorganism density: first order decay (Lambert and Johnston, 2000) and second-order rate (Hunt and Mariñas, 1999). Only Fernando (2009) considers that disinfectant decay depends on the microorganism density.

of work cultures was grown overnight at 37 °C in 5 mL of TSB and subcultured overnight in the same conditions so as to ensure proper growth.

2.1.2. Inoculum Preparation

Once activated, the culture was centrifuged (4 min, 8,600g. centrifuge: Sigma, 2-16PK) and the precipitated cells were resuspended in 0.85% (w/v) NaCl. Resuspended cells were adjusted to Abs700 = 0.7 ± 0.001 in 0.85%(w/v) NaCl using a Cecil3000 scanning spectrophotometer (Cecil Instruments, Cambridge, England), corresponding to a concentration of 10^9CFU/mL. This cell suspension was directly used as inoculum in the experiments or serially diluted in sterile 0.85% NaCl to achieve 10^8–10^7CFU/mL the final viable according to the experimental design.

2.1.3. Dynamics of *E. coli* Inactivation With Benzalkonium Chloride

Benzalkonium chloride solutions (Sigma-Aldrich) were prepared in sterile deionized water at three different concentrations for modeling purposes (100, 200, and 300 mg L^{-1}) and two more BAC concentrations for the validation experiments (75 and 250 mg L^{-1}).

Experimental series were prepared by adding 1 mL of BAC to sterile glass vials containing 1 mL of *E. coli* inoculum and allowed to act for 1, 5, 10, 15, and 20 min at 25 °C without shaking. Negative controls were running in parallel by adding NaCl 0.85% (w/v) instead of BAC.

After each time interval of exposition, 500 µL of the culture were neutralized by adding 500 µL of neutralizing solution (composition L-1:10 mL of a 34 g l-1 KH_2PO_4 buffer (pH7.2); 3 g soybean lecithin; 30 mL Tween 80; 5 g $Na_2S_2O_3$; 1 g L-histidine) during 10 min at room temperature and used to determine the number of viable cultivable cells. Quantification of viable counts was carried out by serially diluting the bacterial culture and spreading in triplicate onto Trytone Soy Agar (TSA; Cultimed, Barcelona, Spain). Plates were incubated at 37 °C during 24 h and results were expressed as log CFU/mL.

The rest of the culture (1,500 µL was filter sterilized through a 0.2 µm syringe filter (Sartorius, Gottingen, Germany) and the filtrate was used to determine the concentration of BAC outside the cells following the method described by Scott (1968).

2.2. Kinetic Model and Computational Methods

2.2.1. Kinetic Modeling

The model simulates *E. coli* inactivation and BAC decay kinetics. The inactivation kinetics are based on the generalized Rate Law model by Gyürék and Finch (1998) with $m = 1$. This expression includes most models with demand-free conditions in water treatment (**Figure 1**). BAC kinetics are modeled assuming that each cell adsorbs a certain quantity α of BAC before dying (see section 3 for details). Therefore the final model tested was:

$$\begin{aligned} \frac{dN}{dt} &= -k\,N^x C^n \\ \frac{dC}{dt} &= -\alpha\,k\,N^x C^n \end{aligned} \tag{1}$$

where t is the contact time in minutes, N is the density of viable *E. coli* cells in CFU/ml, C is the concentration of *BAC* in ppm, k is the inactivation rate constant, n the dilution concentration and x an empirical constant used in the rational model.

This a deterministic model that can only be applied when the density of viable cells is sufficient to neglect stochastic fluctuations. Stochastic models are scarce in the literature and usually focused on growth dynamics (Augustin et al., 2015; García et al., 2018) or in thermal, but not chemical, inactivation (Nicolaï and Van Impe, 1996). In this work, the dynamics are assumed deterministic by defining a detection limit of 100 cells. This determines the zone where experimental data present large variability and model simulations large uncertainty to be useful. Therefore any value (simulated or experimental) below this limit will be considered as $\leq$100, without specifying any numerical value.

Integrative forms of these equations are only available for simple cases that can be seen in (Gyürék and Finch, 1998), but not for the case described in this work (BAC decay depends on the inoculum concentration). Therefore the model should be solved using appropriate numerical methods for Ordinary Differential Equations (ODEs).

2.2.2. Computational Methods

Different computational methods are required in this work to simulate model in Equation (1), estimate the unknown parameters from available experimental data and optimize the BAC dose concentration as a function of the inoculum numbers. In this work, AMIGO (Advanced Model Identification using Global Optimization) software was used for the simulation and parameter estimation. This is a multi-platform toolbox implemented in Matlab (Balsa-Canto et al., 2016b). The dose optimization was implemented outside of this toolbox but with the same simulation and optimization methods. CVODES (Hindmarsh et al., 2005) was selected to simulate the model and to evaluate the parametric sensitivities. This solver allows us to calculate the confidence intervals of the parameters. For optimization, a global optimizer based on scatter search (eSS, Enhanced Scatter Search) was used (Egea et al., 2010).

Parameter estimation is based on the maximization of the log-likelihood function (LLF). The idea is to find the vector of parameters that gives the highest likelihood to the measured data Balsa-Canto et al. (2016a). For independent measurements with Gaussian noise the problem becomes to minimize the minimum square error weighted with the standard deviations associated with each measurement:

$$J = \sum_{i=1}^{n_t} \frac{(\log 10(N_i) - \log 10(\hat{N}_i))^2}{\sigma_N^2} + \sum_{i=1}^{n_t} \frac{(C_i - \hat{C}_i)^2}{\sigma_C^2}$$

where N_i and C_i are each of the time measurements for *E. coli* and BAC and $\hat{N}_i$ and $\hat{C}_i$ their respective estimations using model 1 and n_t is the number of time measurements for all experiments. To avoid computational problems derived from the different orders of magnitude a logarithmic scale was used for the viable cells (García et al., 2017b). To solve this optimization problem the standard deviations for *E. coli* (σ_N) and BAC (σ_C) were previously estimated from replicates (2 and 4 replicates for each measurement of viable *E. coli* and one replicate for most of the BAC measurements).

The performance of the estimation is measured using two standard indexes based on mean square errors between model and experimental data for each type of measurements. The first index is the root-mean-square error (RMSE) defined as:

$$RMSE = \sqrt{\left(\frac{1}{n_t}\sum_{i=1}^{n_t}(y_i - \hat{y}_i)^2\right)}$$

where y_i can be referred to BAC or to *E. coli* viable cells. However, BAC and *E. coli* have different orders of magnitude and for a comparison between their fits the coefficient of variation of the RMSE, CV(RMSE), is preferred:

$$CV(RMSE) = \frac{RMSD}{\bar{y}}$$

where $\bar{y} = (1/n_t)\sum_{i=1}^{n_t} y_i$ is the mean of the data values for each type of measurements.

The confidence intervals for the parameters are estimated by:

$$\pm t_{\alpha/2}^{\gamma}\sqrt{C_{ii}}$$

where C_{ii} are the diagonal elements of the confidence matrix, $t_{\alpha/2}^{\gamma}$ is given by Student's t-distribution with γ the number of degrees of freedom and $(1-\alpha)$100% selected to 95%. For non-linear system the Cramér-Rao inequality to compute a bound fo the confidence matrix using the Fisher information matrix (Vilas et al., 2018):

$$C \geq \mathcal{F} \equiv \mathbf{E}\left\{\left(\frac{\partial J}{\partial \Theta}\right)^T\left(\frac{\partial J}{\partial \Theta}\right)\right\}$$

where Θ is the vector of unknown parameters. Relative confidence intervals (calculated by dividing confidence intervals by the estimated value of the parameter) are also calculated

since they are useful when parameters have different orders of magnitude (García et al., 2017a).

To compare the performance among nested models with a different number of parameters, the Akaike Information Criterion (AIC) is used. Its definition using the LFF reads:

$$AIC = 2n_k - 2LLK$$

being n_k the number of unknown parameters. The preferred model is the one with the minimum AIC value (Akaike, 1970).

Validations are performed simulating the model for a new set of data or using the cross-validation method. The latter consists of fitting the available data setting aside some experiment or subset of data (Elsner et al., 1994). The obtained model with its estimated parameters is used to predict the data set aside. The process is repeated until all set of data are validated.

3. RESULTS AND DISCUSSION

Modeling allows us to systematically reproduce and optimize complex systems and to motivate new experiments to improve our knowledge. Kinetics of bacterial inactivation are well-known, with several alternatives that are special cases of the generalized model in **Figure 1**. That is not usually the case for the disinfectant kinetics, and particularly for stable chemicals as QACs. Therefore, next section starts studying BAC uptake at different inoculum and dose concentrations. Section 3.2 focused on the modeling including the description of the mathematical equations, the estimation of the unknown parameters, the assessment of the model predictive capabilities and the optimization of the BAC dose concentrations. Model files, experimental data and scripts to reproduce results can be found in a public repository https://doi.org/10.5281/zenodo.1207616. Finally in section 3.3 the possible mechanisms behind the inoculum effect are discussed and compared with current works in the literature.

3.1. Understanding BAC Uptake for Different Inoculum and Dose Concentrations

3.1.1. BAC Disinfection Is Under Demanding Conditions

To model the chemical demand of BAC by *E. coli*, *E. coli* viable cells and extracellular BAC concentration are measured in six experiment with two different inoculum ($\log_{10}(N_0) \approx 9\text{logs}, 7\text{logs}$) and three different BAC dose concentrations ($C_0 = 100, 200, 300$ ppm). **Figure 2** shows the results arranged in six panels with two columns and three rows. Left and right columns show *E. coli* inactivation and BAC decay, respectively. Different dose concentrations are depicted in different rows. Each panel shows two responses for the two inoculum concentrations tested (blue and red for high and low inoculum concentrations, respectively).

Experiments reveal that BAC is in excess and in demanding conditions for all experiments. After a fast and sharp decay of extracellular BAC, the disinfectant remains constant with values for some cases larger than half the initial dose concentration. Therefore models considering demand-free conditions in **Figure 1** are not valid. They assume that the disinfectant extracellular concentration is constant during the process and independent of the bacterial concentrations.

3.1.2. BAC Uptake Depends on the Inoculum Concentration and Disinfectant Dose

Experiments suggest that the BAC residual at final times (C^*) depends on the dose and inoculum considered. It should be noted that BAC residual is different for each experiment, designed with different inoculum and dose concentrations. Therefore, BAC dynamics cannot be explained using models of first-order decay, so common in water treatment with volatile disinfectants, and a new model has to be proposed.

The objective is to understand how BAC uptake changes as a function of the inoculum concentration and disinfectant dose. The analysis of the disinfectant uptake into the bacterial population is fundamental to understand disinfection in demanding conditions with stable chemicals. Total BAC uptake can be estimated by subtracting the extracellular residual BAC at the end of the experiment C^* from the dose concentration applied C_0, i.e., total BAC uptake is $C_0 - C^*$. Here it is assumed that (Assumption 1) BAC extracellular decay is only due to its uptake into *E. coli* cells. This assumption is supported by the observation that volatilization of QACs is negligible and those compounds are not chemically transformed after application (Tezel and Pavlostathis, 2011).

Unfortunately, total BAC uptake does not show a clear trend with respect to the applied dose. **Figure 3A** shows the calculated BAC uptake with respect to the inoculum concentration. Each circle corresponds to a total uptake of an experiment in **Figure 2**, and different inoculum concentrations are represented with different colors. The trend is not trivial, cannot be estimated from only three points and it is different for each inoculum concentration.

To detect the dependence of BAC uptake with the inoculum concentration new experiments, depicted in **Figure 3B**, were designed. Note that in **Figure 3A** total BAC uptake is larger for larger inoculum concentrations for all cases except for BAC dose of 200 ppm. Results show that total BAC uptake also increases with the inoculum concentration for treatments with 200 ppm. This increase seems to asymptotically approach a saturation level. This trend resembles the one found by Nagai et al. (2003) who estimated extracellular BAC with the Orange II-chloroform method, but for *Pseudomonas fluorescens*.

The use of standard adsorption isotherms to understand BAC uptake was also explored. For both tested inoculum concentrations, as shown in **Figure 3C**, BAC uptake seems to follow a non-linear trend with a maximum uptake. Those patterns differ from the isotherms found by Ioannou et al. (2007) for BAC and didecyldimethylammonium chloride (a similar quaternary ammonium compound). In the latter work, the uptake increased with the equilibrium concentration, was highly dependent on the chemical used and was analyzed only for low dose concentrations. Probably, the isotherms in this work differ because it was analyzed for high dose concentrations, where there are multiple effects that cannot be cast into an isotherm of adsorption.

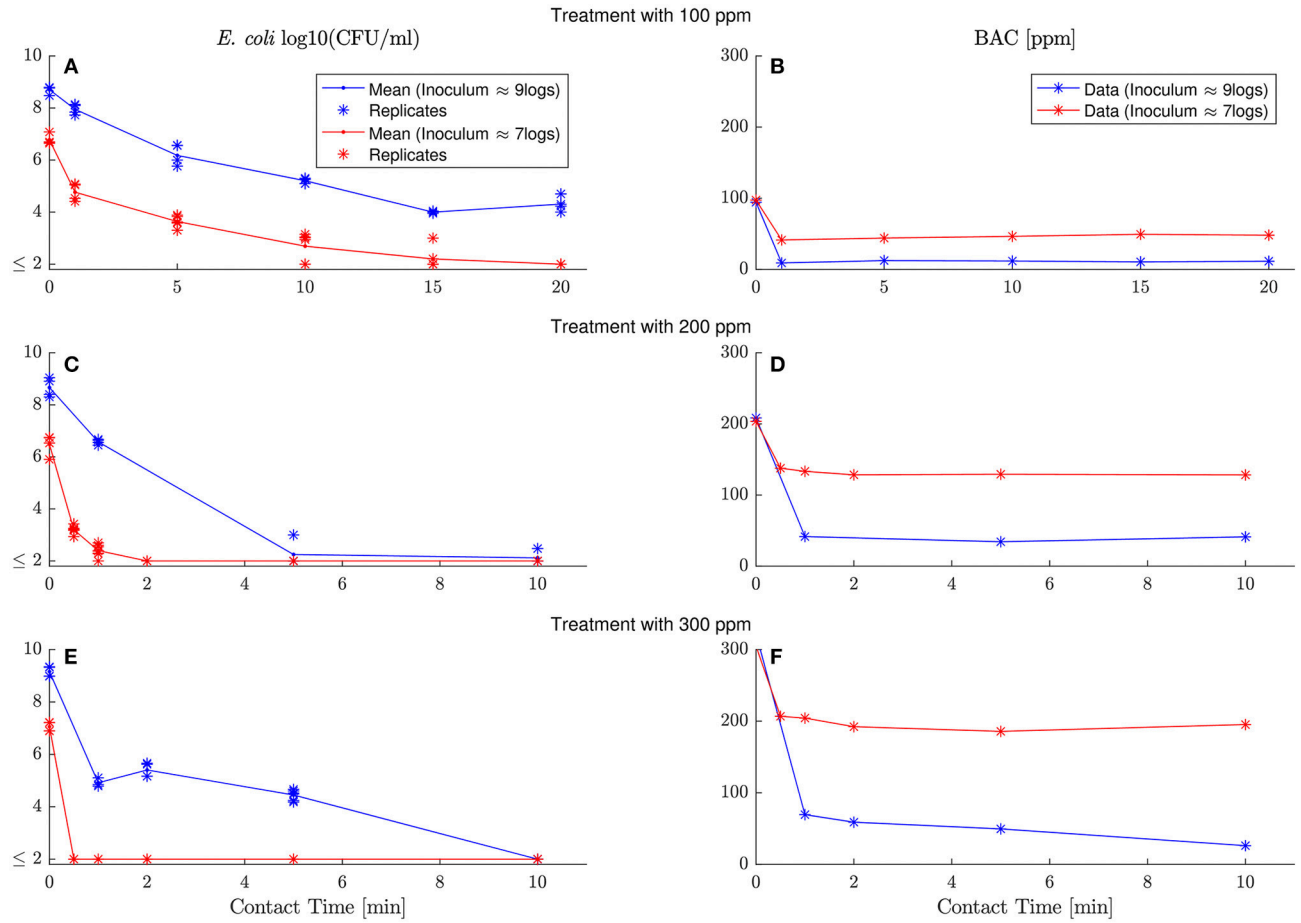


FIGURE 2 | Evolution of *E. coli* viable counts and extracellular BAC concentration with contact time under demanding conditions at different inoculum and dose concentrations. Left and right columns show *E. coli* inactivation and BAC decay, respectively, while each row corresponds to a different dose concentration (100, 200, and 300 ppm). Each panel shows the dynamics with high and low inoculum concentration in, respectively, blue and red. Replicates are represented with asterisks for viable counts, and lines go through their mean values. Detection limit (2 logs) for viable counts is represented with ≤ 2 and gathers all results below this limit. **(A,C,D)** show *E. coli* inactivation by 100, 200 and 300 ppm of initial BAC concentration respectively. **(B–D)** despite BAC decay for the same dose concentration (100, 200 and 300 ppm).

With the data available is not possible to find simple trends of total BAC uptake with inoculum and dose concentrations. The following section describes the use of the specific disinfectant uptake as a better descriptor for chemical-demanding conditions than total BAC uptake or BAC isotherms of adsorption.

3.1.3. Specific BAC Uptake Exhibits a Clear Trend With Respect to the Inoculum and Dose Concentration

Specific BAC uptake (α) is a relative measure of the uptake with respect to the inoculum concentration. Its estimation can be calculated by dividing the total consumption of BAC by the number of cells in the inoculum:

$$\text{specific disinfectant (BAC) uptake} = \alpha = \frac{C_0 - C^*}{N_0} \quad (2)$$

where N_0 is the number of cells in the initial inoculum in CFU per ml. To use this expression it is assumed that (Assumption 2) BAC dose is sufficiently large to kill most of the population. To confirm that the inactivation is complete for the experimental conditions considered in **Figure 2**, it was verified that there were not viable cells after 24 h for the experiment with the highest inocula and lowest dosage (blue lines in panels **Figures 2A,B**). A long time after the exposure was considered because BAC is uptaken within the first minutes and cells can become non-viable with some delay.

Making an analogy with a chemical reaction, the specific BAC uptake (α) represents the stoichiometric coefficient, i.e., a constant relating the dynamics of *E. coli* and BAC interplay:

$$N + \alpha C \rightarrow \hat{N}C_\alpha \quad (3)$$

where N represents CFU per ml and C is the extracellular concentration of BAC. The complex $\hat{N}C_\alpha$ symbolizes concentration of non-viable cells.

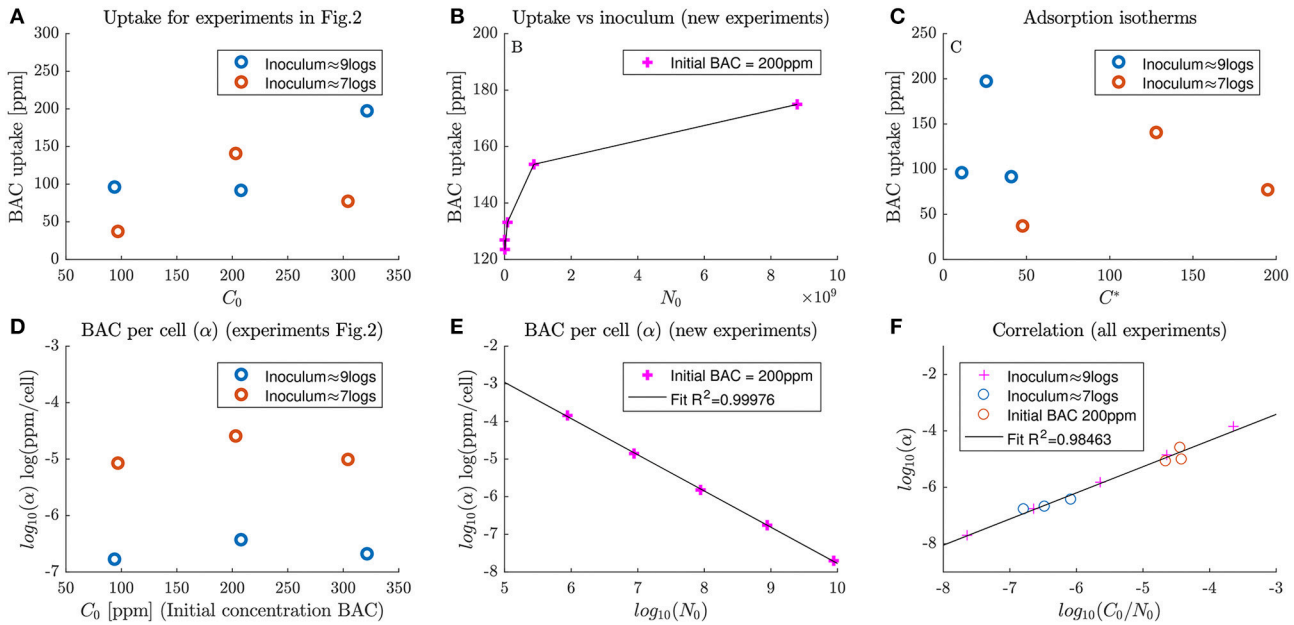


FIGURE 3 | Dependence of BAC uptake and specific BAC uptake (first and second rows of panels respectively) with inoculum size and disinfectant dosage. **(A)** shows the dependence of BAC uptake with dose concentrations at two different inoculum concentrations for experiments in **Figure 2**. **(B)** depicts the dependence of BAC uptake with the inoculum concentration for a set of new experiments. **(C)** shows the isotherms of uptake for experiments in **Figure 2**. **(D)** The dependence of specific BAC uptake with dose concentrations at two different inoculum concentrations for experiments in **Figure 2**. **(E)** depicts the correlation between inoculum and specific BAC uptake for the new experiments at different inocula. Finally **(F)** shows the proposed correlation to explain specific BAC uptake at different inoculum and dose concentrations.

In the experiments, the specific BAC uptake cannot be constant, unlike in pure chemical reactions, and depends on the inoculum and dose concentrations. If the specific BAC update is constant, the total uptake of BAC would increase linearly with respect to the number of cells in the inoculum. That contradicts the results in **Figure 3B**. Another way of noting that the specific BAC uptake is not constant is analyzing experiments in **Figure 2B**. The total uptake of BAC is 80 ppm for an inoculum of 9 logs and 50 ppm for an inoculum of 7 logs. Note that whereas the inoculum size changes two orders of magnitude, total the order of magnitude of BAC uptake remains the same.

The dependence of the specific BAC uptake with respect to the dose concentration for different inocula is depicted in **Figure 3D**. Specific BAC uptake varies several orders of and, contrary to the total BAC uptake in **Figure 3A**, follows the same trend for both inocula. The major differences are because of the inoculum size, although there are also changes with the BAC dosage.

As shown in **Figure 3E**, the specific BAC uptake (α) in logarithmic scale ($log10(\alpha)$) is clearly inversely proportional to the inoculum concentration. Data were the same used in **Figure 3B**. This means that for low inoculum concentrations, each cell uptakes more BAC than in those experiments with high inoculum concentrations. Note that in all cases the uptake is sufficient to make the cell non-viable.

The information from previous figures can be exploited to calculate specific BAC uptake as a function of the BAC dose and inoculum concentrations. Among the different options tested, the best model consisted assumed a linear dependence of the logarithm of α with respect to $\frac{C_0}{N_0}$. As shown in **Figure 3**, this functionality resulted in a R^2 coefficient very close to one.

$$\log_{10}(\alpha) = a + b \log_{10} \frac{C_0}{N_0} \tag{4}$$

meaning that

$$\alpha = 10^{-a} \left(\frac{C_0}{N_0} \right)^b \tag{5}$$

This model predicts that the specific BAC uptake increases with the dose concentration and decreases with the inoculum concentration. The first observation is expected. Higher concentrations of the disinfectant imply larger concentrations of disinfectant uptake. However, the mechanism by which each cell uptakes less disinfectant when population numbers are large is not obvious. Although it seems to be a common pattern observed for different QACs and bacterial strains as it will be discussed in the last section.

3.2. Developing the Predictive Kinetic Model to Optimize BAC Treatment

3.2.1. The Kinetic Model Reproduces Experiments Under Different Dose and Inoculum Concentrations

Models in chemical disinfection are mostly focused on inactivation kinetics of relevant microorganisms. The generalized

differential rate law, in Equation (1), is used to describe the velocity of this reaction. As described in materials and methods, this is a nested model including most of the relevant autonomous models in chemical disinfection.

BAC kinetics are critical to minimize the chemical residuals after a treatment and are calculated from *E. coli* inactivation using the functionality found for the specific BAC uptake in (5). Final dynamic model reads

$$\frac{dN}{dt} = -k N^x C^n$$
$$\frac{dC}{dt} = \alpha\, k N^x C^n = 10^{-a}\left(\frac{C_0}{N_0}\right)^b k N^x C^n \qquad (6)$$

where the set of unknown parameters is:

$$\theta = [a, b, k, x, n].$$

Despite there is a rough estimation of a and b using correlations in previous sections, it is preferable to estimate the whole set of unknown parameters in a single step using all data of *E. coli* and BAC dynamics. This allows us to find the best parameters also to represent the dynamics and mitigates estimation errors due to measurement errors in the residual concentration of BAC (C^*). For considering that specific BAC uptake is constant, the parameter b is fixed to zero being therefore $\alpha = 10^{-a}$.

Comparisons between experimental and simulated data reveal how critical is to assume a dependence of the specific BAC uptake with the inoculum and dose concentrations. **Figure 4** shows the best fit of model (6) assuming that either the specific BAC uptake is constant (dashed line) or depends on the initial experimental conditions (continuous lines). The model reproduces the *E. coli* inactivation kinetics better considering the dependence of specific BAC uptake with the inoculum and dose concentration, but the major differences are in terms of BAC decay. The model assuming that the specific BAC uptake is an invariant stoichiometric coefficient, as in chemical reactions, is not able to reproduce the BAC dynamics for most of the cases, especially for those experiments with low inoculum concentration.

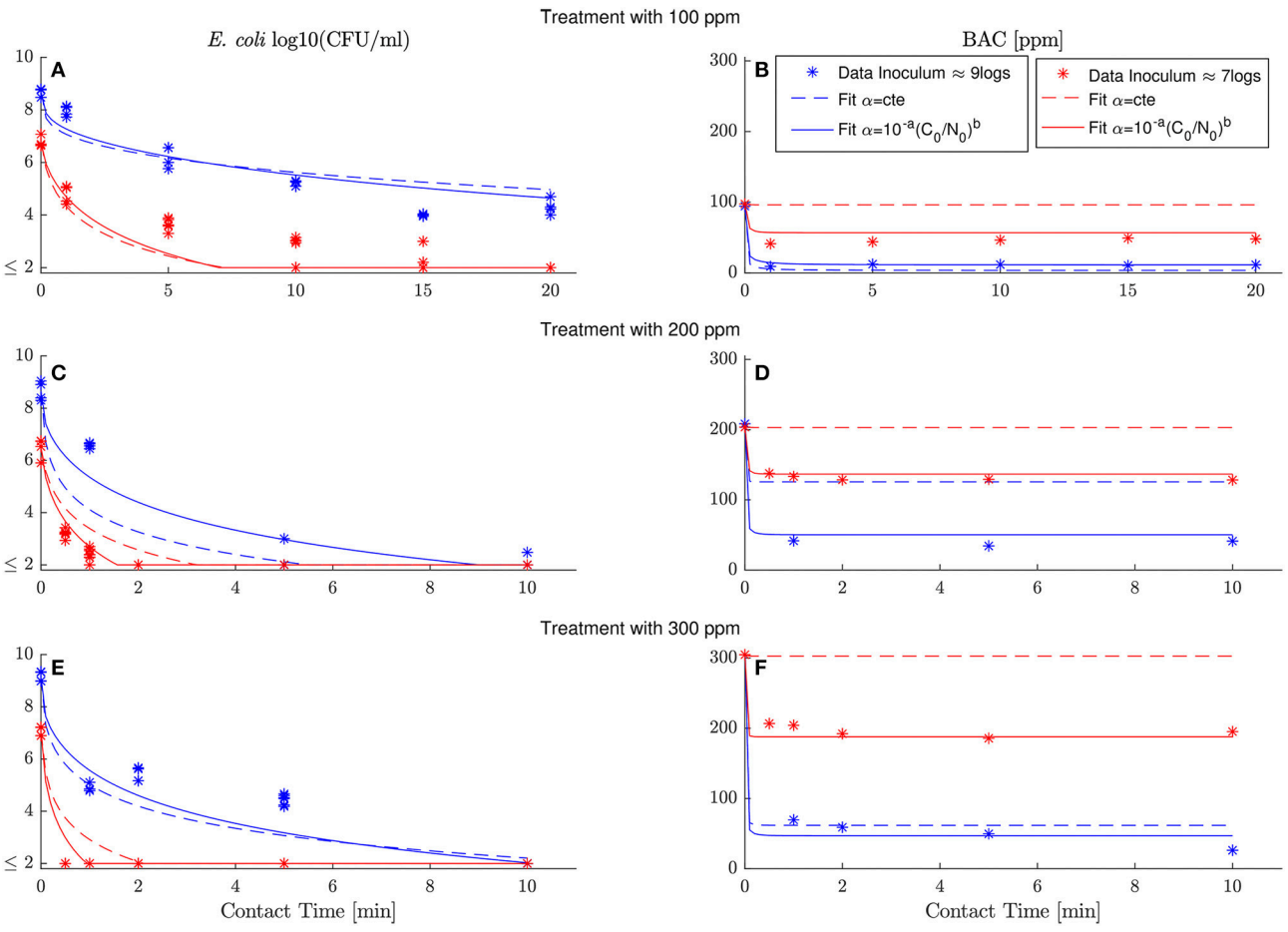


FIGURE 4 | Best fits for model (6) assuming that specific BAC uptake is constant (dashed line) or depends on inoculum and dose concentrations (continuous line). Experimental data marked with asterisks correspond with the results shown in **Figure 2**. High and low inoculum concentration are shown in blue and red, respectively. The model with specific BAC uptake of the form $\alpha = 10^{-a}(C_0/N_0)^b$ fits the data considerably better than the model assuming $\alpha = 10^{-a} = cte$ with $b = 0$. **(A,C,D)** show model simulations and data of *E. coli* inactivation by 100, 200 and 300 ppm of initial BAC concentration, respectively. **(B–D)** model simulations and data of BAC decay for the same dose concentration (100, 200 and 300 ppm).

Standard methods, used to quantify the model ability to reproduce the data, confirm that the proposed model outperforms the model with the classical assumption of constant specific uptake. **Table 1** shows those methods (see Materials and Methods for details) together with the estimated parameters for both models. The root-mean-square error (RMSE), and its coefficient of variation [CV(RMSE)], quantifies how good the model reproduces the data (fit) in absolute and relative terms, respectively. Note that *E. coli* and BAC measurements are of different nature and of different orders of magnitude. Therefore the CV(RMSE) provides a better description of the goodness of fit. As observed in **Figure 4**, and expected due to the larger uncertainty in *E. coli* measurements, the model reproduces the data better for BAC than for *E. coli* and always better than the alternative model. The worse fit for the proposed model is for *E. coli* inactivation at low inoculum and low dose since data in this experiment is close to the detection limit for most times, and therefore with the larger uncertainty. Another way to quantify the model performance is the log-likelihood function (LLF) that is maximized to estimate the parameters. For the type of measured error assumed in this work, it is equivalent to the RMSE weighted with the inverse of the square of the standard deviations. Again, the maximum LLF is observed for the model with specific BAC uptake dependent on dose and inoculum concentrations.

The proposed model reproduces the data without incurring in overparametrization: a usual problem in modeling where fits improve at the expenses of fitting the data noise, outliers and others experimental artifacts (Vilas et al., 2018). To discard overparametrization there are methods considering the model performance as a function of the parameters to be estimated. The Akaike Information Criterion (AIC) is a standard tool to compare nested kinetic models. The proposed model has the minimum Akaike index (**Table 1**) and the best relative confidence intervals for all parameters. The most uncertain parameters, in relative terms, are the dilution term n and the constant of the rational model x. That agrees with the observed variability of the data that is much larger for the numbers of bacterial inactivation than for the extracellular BAC concentration. The parameter with more confidence is b that models the dependence of the specific BAC uptake with the inoculum and dose concentration. Another evidence of the necessity to consider this dependence.

TABLE 1 | Different criteria to assess the capabilities of both models to reproduce the experimental data.

		Proposed model (6) with $\alpha = 10^{-a}\left(\frac{C_0}{N_0}\right)^b$	Model (6) with contant α (i.e., $b = 0$)
Parameters	k	3.75 ± 0.13 (3.41%)	3.22 ± 0.13 (3.86%)
	x	1.25 ± 0.15 (12.00%)	1.34 ± 0.21 (16%)
	n	1.69 ± 0.20 (11.64%)	1.08 ± 0.23 (21.3%)
	a	1.18 ± 0.04 (3.68%)	6.74 ± 0.07 (0.11%)
	b	0.83 ± 0.01 (0.84%)	–
RMSE	*E. coli*	0.54	0.79
	BAC	9.70	60.09
CV(RMSE)	*E. coli*	0.13	0.28
	BAC	0.09	0.57
LLF		−18.09	−42.32
AIC		46.17	94.63

Unknown parameters are shown as: estimation±confidence interval (relative confidence interval). Root-mean-square error, RMSE, and its coefficient of variation, CV(RMSE), are included for E. coli and BAC. Additionally the performance of both models in terms of the log likelihood function (LLF) and the Akaike criterion (AIC) are shown. Last one allows to compare models with different number of estimated parameters.

3.2.2. The Model Predicts New Data

Model-based optimization requires a model with predictive capabilities. Tests in the previous section help us to confirm that the model follows the experimental data used for estimating the unknown parameters (fit). However, for optimization, it is critical to validate if the model with the estimated parameters can predict new data (validation). New data can be inside the rage of the designed experiments for the fit (interpolation), in this work between 7 and 9 logs of inoculum concentration and between 100 and 300 ppm of BAC, or outside (extrapolation). Empirical models commonly predict only interpolation data while mechanistic models or semi-mechanistic models are better reproducing new data outside the range used for the fit.

The proposed model combines mechanistic and empirical arguments. For example, it is based on assumptions 1 and 2 and thanks to this the BAC kinetics are defined. However, the dependence of the specific BAC uptake with the inoculum and dose concentrations is empirical, based on the experimental observations. In this work, the predictive capabilities are tested using the cross-validation method and with two new experiments inside and outside the range of experimental data used for the fit (interpolation and extrapolation).

The experiments in **Figure 2**, six experiments with three BAC dose and two inoculum concentrations, are used to compute the cross-validation. At each step, five experiments of the six are used for the fit and the remaining one for the validation. In the following step, a different experiment is set aside. After the computation, see **Table 2**, there are six sets of estimated parameters and six validation experiments. This technique has the advantage of not requiring new experiments and that it helps to identify problems (if there are) with some subset of data.

Estimated parameters (and confidence intervals) for the cross-validation are similar to the those obtained fitting the six experiments. Larger deviations with respect to the estimated parameters in **Table 1** are, as expected, for extrapolation experiments, like experiment 1 with the lower dose concentration (100 ppm) and high inoculum concentration (9 logs).

The validation experiments of the cross-validation confirm that the proposed model has good prediction capabilities. RMSE and CV(RMSE) are similar and lower for the interpolation experiments (3 and 4). Worse validations are for experiment 1 in terms of *E. coli* CV(RMSE) and experiment 2 for BAC CV(RMSE). But even for those experiments, their kinetics are not so far from the experimental data as shown in the **Figure S1** in Supplemental Data. It should be stressed that validation experiments are obtained with estimated parameters from other

TABLE 2 | Cross validation of final model with experiments in **Figure 2**.

Fit for experiments	**[2,3,4,5,6]**	**[1,3,4,5,6]**	**[1,2,4,5,6]**	**[1,2,3,5,6]**	**[1,2,3,4,6]**	**[1,2,3,4,5]**
k	4.87 ± 0.14	2.94 ± 0.12	4.95 ± 0.12	3.61 ± 0.15	3.64 ± 0.13	3.45 ± 0.18
x	1.23 ± 0.49	1.18 ± 0.15	1.38 ± 0.27	1.25 ± 0.15	1.19 ± 0.16	1.24 ± 0.19
n	2.31 ± 0.53	1.49 ± 0.21	2.01 ± 0.29	1.61 ± 0.22	1.79 ± 0.23	1.56 ± 0.29
a	1.17 ± 0.04	1.24 ± 0.05	1.18 ± 0.04	1.22 ± 0.05	1.12 ± 0.05	1.03 ± 0.06
b	0.83 ± 0.01	0.82 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.84 ± 0.01	0.86 ± 0.01
Validation	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**
RMSE *E. coli*	1.31	0.74	0.95	0.32	1.08	0.60
RMSE BAC	4.26	11.21	11.90	7.59	15.95	22.06
CV(RMSE) *E. coli*	0.22	0.20	0.19	0.11	0.21	0.21
CV(RMSE) BAC	0.17	0.21	0.15	0.05	0.15	0.10

This includes experiments at different BAC dose concentrations: 100 ppm (1 and 2), 200 ppm (3 and 4), and 300 ppm (5 and 6) and with two inoculum concentrations (odd and even numbers correspond to high and low inoculum concentrations, respectively). Table shows the six sets of estimated parameters removing one different experiment each time that is used for the validation. The indexes RMSE and CV(RMSE) are used for analyzing the performance of the validations.

experiments, and therefore their behavior, as well as the RMSE and CV(RMSE), are rarely better than same indexes for the fit and should be carefully compared with results in **Table 1**.

Two additional experiments are carried out to further test the predictive capabilities and weakness of the model. The interpolation consists on a concentration similar to 8 logs for the inoculum and 250 ppm for the BAC dose. The extrapolation experiment was designed with 8 logs for inoculum and 75 ppm for BAC dose concentration.

The model predicts both experiments, especially for BAC decay and interpolation data. First row in **Figure 5** shows the predicted BAC and *E. coli* kinetics. Results are considered satisfactory since BAC decay is predicted particularly good. It should be noted that BAC dynamics are more relevant in the context of this work as for the dose concentrations used a complete inactivation of *E. coli* is assumed, and the prediction always overestimates the *E. coli* numbers being in the safest scenario.

3.2.3. The Model Allows to Optimize Dose Concentration

Model-based optimization also requires, in addition to a predictive model, that the estimated parameters do not change with the experimental conditions. That is a common problem with other models in the literature fitting the data separately for each experiment. Those models also require more data to assume good confidence intervals of the parameter.

For the proposed model, the parameters are the same independently on the inoculum and dose concentration and can be used to optimize doses for a given inoculum. The formal description of the problem is as follows:

$$\min_{C_0} \quad (C^*)^2 \tag{7}$$

$$\text{subject to} \quad N^* <= 100 \text{ (detection limit)} \tag{8}$$

where C^* and N^* is the BAC and *E. coli* numbers at the final time and C_0 is the dose concentration. A final number of *E. coli* less than 100 is required for being the detection limit of the data and knowing that the model tends to overestimate this number, but other criteria can be easily selected. Three scenarios are considered: 5, 30 min, and 24 h to illustrate how optimal treatments change with contact time.

The second-row panel in **Figure 5** shows the minimum BAC dose to reduce the population to 100 viable cells for different inoculum concentrations. The black vertical bars define the range of data used for the fit, and therefore where the model confidence is greater. Data shows how the dose increases exponentially with the inoculum concentration. Probably this is an overestimation of the BAC dose required for inoculum concentrations greater than 9 logs where the model has not been tested.

3.3. Discussing the Mechanisms in BAC Disinfection and the Relevance of Quantifying the Inoculum Effect

3.3.1. Mechanisms Behind the Specific BAC Uptake

Salton's theory, from the sixties, is still the reference when studying the mechanisms behind QACs disinfection (Salton, 1968). This work proposed the following series of events: (i) QAC adsorption to and penetration of the cell wall; (ii) reaction with the cytoplasmic membrane followed by membrane disorganization; (iii) leakage of intracellular lower-weight material; (iv) degradation of proteins and nucleic acids; and (v) cell wall lysis caused by autolytic enzymes.

In the context of Salton's theory, the specific BAC uptake quantified in this work represents the equilibrium between the influx (BAC adsorption or penetration) and the efflux (BAC leaking or cell wall lysis).

It should be stressed that, if efflux is relevant, it occurs at the time scales of the influx and leakage seems to be the main mechanism. Extracellular BAC quickly decays in the experiments within first minutes (**Figure 4**), suggesting that the equilibrium between influx and efflux is fast. On the other hand, attending to some observations while setting-up the methodology to measure

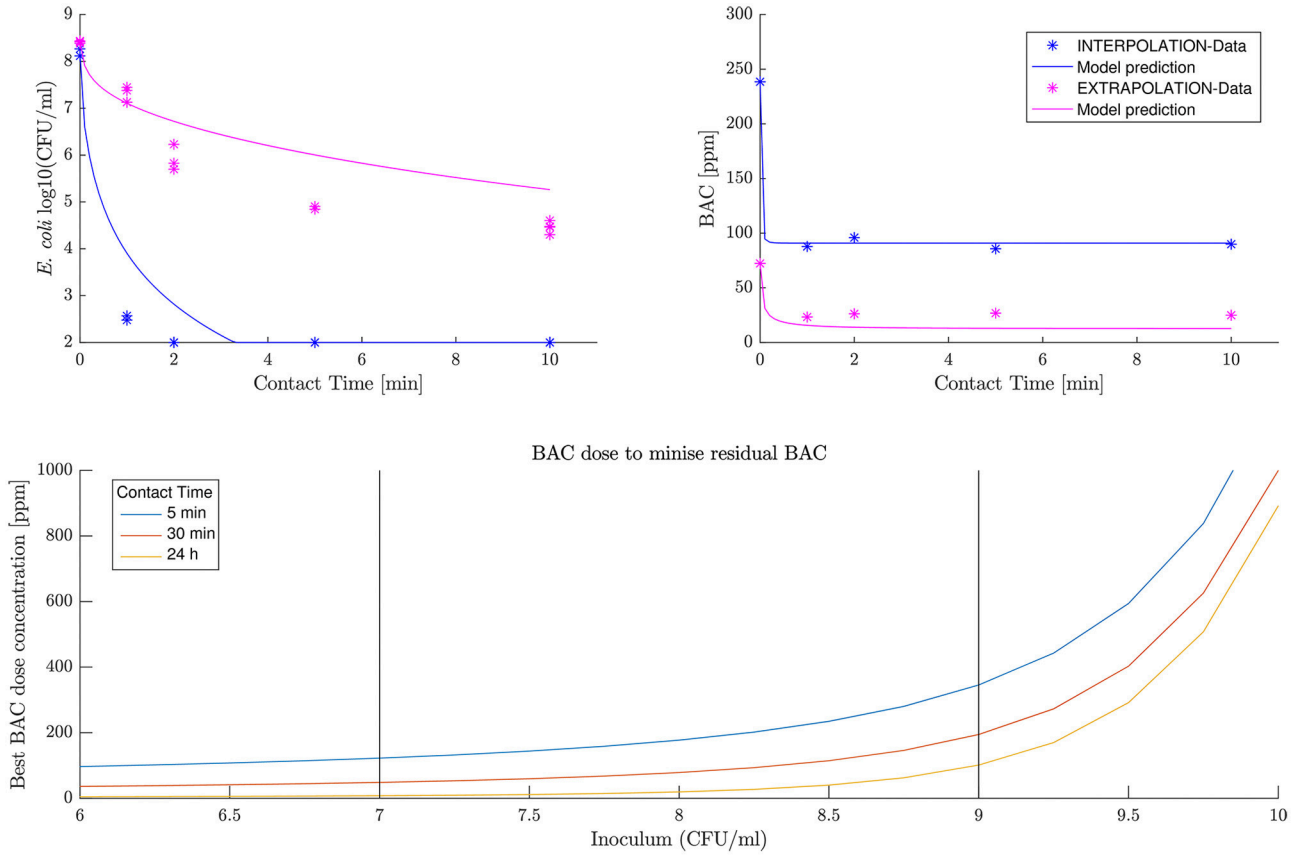


FIGURE 5 | New data prediction (first row) and optimal BAC dose concentration (second row).

extracellular BAC, leakage seems more relevant than cell lysis. Even for high BAC doses, extracellular BAC concentration was substantially larger when cells were separated by centrifugation than by filtering. Since cells are supporting a major pressure with centrifugation, this may indicate that cell membrane retains the BAC (without lysis) and the intracellular material only leaks when sufficient pressure is applied.

However, BAC leakage cannot be fully understood with the available model and data. Overparametrized models have been obtained when trying to expand the model to explicitly consider the influx and efflux of BAC. For a proper definition of the leakage, it is critical to measure another representative variable of this mechanism. A possibility, to be considered for future works, could be to measure an energy-dependent variable linked to efflux pumps, such as in Nagai et al. (2003).

3.3.2. Mechanisms Behind the Inoculum Concentration

The main difficulty to understand the inoculum effect is that it depends on the microbial species and strain and on the antimicrobial type and compound (Udekwu et al., 2009; Karslake et al., 2016).

The effect is critical in drug treatments, where infections exceeding a critical inoculum concentration survive otherwise effective treatments. Works studying antibiotic susceptibility usually postulate that the medium is modulated by the number of bacteria in the population. For example, Karslake et al. (2016) proposed a mechanism based on pH media changes and Datta and Benjamin (1999) in fluctuations of the medium acidity. Other mechanisms such as a decrease in per-cell antibiotic concentration are also being proposed (Udekwu et al., 2009).

Nevertheless, only a few works, from the author's knowledge, have quantified the inoculum effect with BAC (Lambert and Johnston, 2001; Ioannou et al., 2007). This research found that the relationship between the required disinfectant dose and the inoculum level, such as with antibiotics and in this work, was not proportional.

Lambert and Johnston (2001) quantified the inoculum effect when studying inactivation of *Staphylococcus aureus* with BAC using the fractional area. The work found that BAC dose has to be inversely proportional to the inoculum concentration to the power of 0.44. For a similar expression, a value of 0.83 is estimated in this work. It may be speculated that the discrepancy is due to the differences between *E. coli* and *Staphylococcus aureus* or because cell inactivation is measured with different methodologies (viable cells and optical density). Moreover, the proposed model in this work includes other effects, such as the dose concentration, that may cause the differences. In any case,

a similar trend, and therefore quantification, for the inoculum effect was observed in both cases.

On the other hand, Ioannou et al. (2007) proposes the use of the adsorption isotherms to quantify this effect for *Staphylococcus aureus*. Extracellular BAC decay seems too fast for an energy-dependent mechanism and Ioannou et al. (2007) assumed that uptake is mainly because of adsorption. Whereas their results resemble a Langmuir isotherm, data in this work fits better to a C-shaped isotherm (**Figure 3C**). The differences may be again attributed to the microbial species considered. However, it should be also stressed that the BAC doses used in this work were more aggressive than in Ioannou et al. (2007) and therefore other mechanisms could become relevant, such as BAC penetration and leakage, in addition to adsorption.

In fact, experiments in this work using high concentrations of BAC indicates that the specific BAC uptake could be a better descriptor than adsorption isotherms. Note that both are related, but specific BAC uptake may better incorporate other mechanisms that are relevant at significant concentrations of the disinfectant and in antibiotic treatments, such as leakage or pump efflux. Moreover, it directly links the interplay between disinfectant and bacterial inactivation in a simple matter for modeling that can be used as an analogy of a biochemical reaction (3).

It should also be mentioned the theoretical work by Fernando (2009), who also uses the concept of specific disinfectant update (α). However, it assumes that this quantity may change during the treatment because the cell may become less susceptible to the chemical agent. In that case, BAC uptake per cell cannot be calculated using (2). An alternative model was tested (data not shown) assuming a similar dependence of α with viable cells. Good fits were obtained for this case but without improvements. Probably the disinfection process is too fast to observe any change in susceptibility or resistance during the treatment. Also, dependence on initial inoculum, instead of viable cells, has more sense as disinfectant can be adsorbed or enter the cells even if cells are not viable.

Experiments in this work may suggest that the observed differences in specific BAC uptake (or adsorption) are because cells aggregate forming clusters for dense populations, and therefore less membrane surface is exposed to BAC. That would explain results (**Figure 3B**) showing that the specific BAC uptake (BAC uptake per cell) decreased with the number of cells. To test the hypothesis a population of 8 log bacteria was observed by acquiring phase-contrast images, see the **Figure S2** in Supplemental Data. Unfortunately, only cells while and just after division where attached to each other. Therefore the inoculum effect cannot be the attributed to a decrease of membrane exposure for dense populations.

Quorum sensing could be also a plausible explanation of the observed inoculum effect. Quorum sensing circuits regulate gene expression through extracellular signal molecules proportional to the cell density (Miller and Bassler, 2001). Therefore, different inoculum sizes obtained from a stationary phase culture have different concentrations of signal molecules (autoinducers), and different behavior of the bacterial population. In this work, specific BAC uptake changes with the inoculum. This uptake may depend on multidrug resistance efflux pumps, for example, that are regulated by quorum sensing in several bacterial genera, such as *P. aeruginosa* (Köhler et al., 2001) and *E. coli* (Yang et al., 2006).

4. CONCLUDING REMARKS

In this work, the inoculum effect for *E. coli* inactivation by BAC has been quantified. The equation describing the effect is combined with a kinetic model of BAC and *E. coli* to determine the treatment that inactivates most of the population with a minimum dose concentration. Interestingly this optimum dose is not proportional to the inoculum concentration, but it increases exponentially (**Figure 5**). The reason behind is that each cell uptakes less BAC when the inoculum concentration increases. Possible mechanisms are discussed, but more research is needed.

The predictive model of the characteristics here developed allows analyzing different effects, such as the contact time. It may also set the basis to develop a theory for the inoculum effect applicable to other pairs of bacteria-antimicrobials. This will, ultimately, guide the search for the relevant causes of this effect.

AUTHOR CONTRIBUTIONS

MG designed the theoretical study, conducted the computational experiments and drafted the manuscript. MC designed the experimental study and analyzed the data. All authors approved the final version of the manuscript.

FUNDING

The authors acknowledge financial support from the Spanish Government (MINECO) and the European Regional Development Fund (ERDF) through the projects RESISTANCE (DPI2014-54085-JIN).

ACKNOWLEDGMENTS

We especially thank Alberto Gallego López and Sonia Rodríguez Carrera for their technical assistance. We also thank Lucía Sánchez Ruiloba for her phase-contrast images.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01259/full#supplementary-material

Figure S1 | Capabilities of the model to predict dynamics inside the range considered during calibration. Each of the dynamics is calculated from the estimated parameters (**Table 2**) by calibrating the other experiments. The model is able to predict situations not considered during calibration but in the range of the experiments considered for the calibration

Figure S2 | Phase-contrast images of a population of *E. coli* with a density of 8 logs per milliliter.

Supplemental Data

Cross-Validation

The model cross-validation is shown in **Figure S1**. Note that results are validation experiments, i.e., the parameters to simulate the kinetics were determined without considering this set of experimental data.

Code to Reproduce the Computational Results

Model files, experimental data and scripts to reproduce results can be found in a public repository https://doi.org/10.5281/zenodo.1207616.

Phase-Contrast Image of a 8 log Population

To test if aggregation is a plausible explanation for the inoculum effect, a population of 8logs was observed using a DMX1200 camera mounted on an Eclipse TE2000-S inverted microscope, Nikon Japan, with a 40×/0.75 NA objective.

REFERENCES

Akaike, H. (1970). "On a decision procedure for system identification," in *Proceedings of the IFAC Kyoto Symposium on System Engineering Approach to Computer Control* (Kyoto), 485–490.

Augustin, J. C., Ferrier, R., Hezard, B., Lintz, A., and Stahl, V. (2015). Comparison of individual-based modeling and population approaches for prediction of foodborne pathogens growth. *Food Microbiol.* 45, 205–215. doi: 10.1016/j.fm.2014.04.006

Balsa-Canto, E., Alonso, A. A., Arias-Méndez, A., García, M. R., López-Núñez, A., Mosquera-Fernández, M., et al. (2016a). "Modeling and optimization techniques with applications in food processes, bio-processes and bio-systems," in *SEMA SIMAI Springer Series*, Vol. 9, eds I. Higueras, T. Roldán, and J. J. Torrens (Springer International Publishing), 187–216.

Balsa-Canto, E., Henriques, D., Gabor, A., and Banga, J. R. (2016b). AMIGO2, a toolbox for dynamic modeling, optimization and control in systems biology. *Bioinformatics (Oxford, England)* 32, 3357–3359. doi: 10.1093/bioinformatics/btw411

Bhagunde, P., Chang, K.-T., Singh, R., Singh, V., Garey, K. W., Nikolaou, M., et al. (2010). Mathematical modeling to characterize the inoculum effect. *Antimicrob. Agents Chemother.* 54, 4739–4743. doi: 10.1128/AAC.01831-09

Bore, E., Hebraud, M., Chafsey, I., Chambon, C., Skjaeret, C., Moen, B., et al. (2007). Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. *Microbiology* 153, 935–946. doi: 10.1099/mic.0.29288-0

Bulitta, J. B., Yang, J. C., Yohonn, L., Ly, N. S., Brown, S. V., D'Hondt, R. E., et al. (2010). Attenuation of colistin bactericidal activity by high inoculum of Pseudomonas aeruginosa characterized by a new mechanism-based population pharmacodynamic model. *Antimicrob. Agents Chemother.* 54, 2051–2062. doi: 10.1128/AAC.00881-09

Datta, A. R., and Benjamin, M. M. (1999). Cell density dependent acid sensitivity in stationary phase cultures of enterohemorrhagic Escherichia coli O157:H7. *FEMS Microbiol. Lett.* 181, 289–295. doi: 10.1111/j.1574-6968.1999.tb08857.x

Egea, J. A., Martí, R., and Banga, J. R. (2010). An evolutionary method for complex-process optimization. *Comput. Operat. Res.* 37, 315–324. doi: 10.1016/j.cor.2009.05.003

Egervärn, M., Lindmark, H., Roos, S., Huys, G., and Lindgren, S. (2007). Effects of inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. *Antimicrob. Agents Chemother.* 51, 394–396. doi: 10.1128/AAC.00637-06

Elsner, J. B., Schmertmann, C. P., Elsner, J. B., and Schmertmann, C. P. (1994). Assessing forecast skill through cross validation. *Weather Forecast.* 9, 619–624. doi: 10.1175/1520-0434(1994)009<0619:AFSTCV>2.0.CO;2

Fernando, W. (2009). Theoretical considerations and modeling of chemical inactivation of microorganisms: inactivation of Giardia Cysts by free chlorine. *J. Theor. Biol.* 259, 297–303. doi: 10.1016/j.jtbi.2009.03.026

García, M. R., Alonso, A. A., and Balsa-Canto, E. (2017a). "A normalisation strategy to optimally design experiments in computational biology," in *11th International Conference on Practical Applications of Computational Biology and Bioinformatics*, Vol. 616, eds F. Fdez-Riverola, M. S. Mohamad, M. Rocha, J. F. De Paz, and T. Pinto (Porto: Springer International Publishing), 126–136.

García, M. R., Cabo, M. L., Herrera, J. R., Ramilo-Fernández, G., Alonso, A. A., and Balsa-Canto, E. (2017b). Smart sensor to predict retail fresh fish quality under ice storage. *J. Food Eng.* 197, 87–97. doi: 10.1016/j.jfoodeng.2016.11.006

García, M. R., Vázquez, J. A., Teixeira, I. G., and Alonso, A. A. (2018). Stochastic individual-based modeling of bacterial growth and division using flow cytometry. *Front. Microbiol.* 8:2626. doi: 10.3389/fmicb.2017.02626

Garre, A., Fernández, P. S., Lindqvist, R., and Egea, J. A. (2017). Bioinactivation: software for modelling dynamic microbial inactivation. *Food Res. Int.* 93, 66–74. doi: 10.1016/j.foodres.2017.01.012

Geeraerd, A., Valdramidis, V., and Van Impe, J. (2005). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *Int. J. Food Microbiol.* 102, 95–105. doi: 10.1016/j.ijfoodmicro.2004.11.038

Gerba, C. P. (2015). Quaternary ammonium biocides: efficacy in application. *Appl. Environ. Microbiol.* 81, 464–469. doi: 10.1128/AEM.02633-14

Gyürék, L. L., and Finch, G. R. (1998). Modeling water treatment chemical disinfection kinetics. *J. Environ. Eng.* 124, 783–793. doi: 10.1061/(ASCE)0733-9372(1998)124:9(783)

Haas, C. N., and Kaymak, B. (2003). Effect of initial microbial density on inactivation of Giardia muris by ozone. *Water Res.* 37, 2980–2988. doi: 10.1016/S0043-1354(03)00112-X

Hajaya, M. G., and Pavlostathis, S. G. (2013). Modeling the fate and effect of benzalkonium chlorides in a continuous-flow biological nitrogen removal system treating poultry processing wastewater. *Bioresour. Technol.* 130, 278–287. doi: 10.1016/j.biortech.2012.11.103

Hindmarsh, A. C., Brown, P. N., Grant, K. E., Lee, S. L., Serban, R., Shumaker, D. E., et al. (2005). SUNDIALS: suite of nonlinear and differential/algebraic equation solvers. *ACM Trans. Math. Softw.* 31, 363–396. doi: 10.1145/1089014.1089020

Holah, J. T., Taylor, J. H., Dawson, D. J., and Hall, K. D. (2002). Biocide use in the food industry and the disinfectant resistance of persistent strains of Listeria monocytogenes and *Escherichia coli*. *J. Appl. Microbiol.* 92, 111S–120S. doi: 10.1046/j.1365-2672.92.5s1.18.x

Hunt, N. K., and Mariñas, B. J. (1999). Inactivation of *Escherichia coli* with ozone: chemical and inactivation kinetics. *Water Res.* 33, 2633–2641. doi: 10.1016/S0043-1354(99)00115-3

Ioannou, C. J., Hanlon, G. W., and Denyer, S. P. (2007). Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus. *Antimicrob. Agents Chemother.* 51, 296–306. doi: 10.1128/AAC.00375-06

Karslake, J., Maltas, J., Brumm, P., and Wood, K. B. (2016). Population density modulates drug inhibition and gives rise to potential bistability of treatment outcomes for bacterial infections. *PLoS Comput. Biol.* 12:e1005098. doi: 10.1371/journal.pcbi.1005098

Kaymak, B., and Haas, C. N. (2008). Effect of initial microbial density on inactivation of *Escherichia coli* by monochloramine. *J. Environ. Eng. Sci.* 7, 237–245. doi: 10.1139/S07-054

Köhler, T., van Delden, C., Curty, L. K., Hamzehpour, M. M., and Pechere, J. C. (2001). Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 5213–5222. doi: 10.1128/JB.183.18.5213-5222.2001

Lambert, R. J., and Johnston, M. D. (2000). Disinfection kinetics: a new hypothesis and model for the tailing of log-survivor/time curves. *J. Appl. Microbiol.* 88, 907–913. doi: 10.1046/j.1365-2672.2000.01060.x

Lambert, R. J., and Johnston, M. D. (2001). The effect of interfering substances on the disinfection process: a mathematical model. *J. Appl. Microbiol.* 91, 548–555. doi: 10.1046/j.1365-2672.2001.01422.x

Langsrud, S., Sidhu, M. S., Heir, E., and Holck, A. L. (2003). Bacterial disinfectant resistance challenge for the food industry. *Int. Biodeteriorat. Biodegradat.* 51, 283–290. doi: 10.1016/S0964-8305(03)00039-8

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Nagai, K., Murata, T., Ohta, S., Zenda, H., Ohnishi, M., and Hayashi, T. (2003). Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a *Pseudomonas fluorescens* strain. *Microbiol. Immunol.* 47, 709–715. doi: 10.1111/j.1348-0421.2003.tb03440.x

Nicolaï, B., and Van Impe, J. (1996). Predictive food microbiology: a probabilistic approach. *Math. Comput. Simulat.* 42, 287–292. doi: 10.1016/0378-4754(95)00129-8

Reichart, O. (1994). Modelling the destruction of Escherichia coli on the base of reaction kinetics. *Int. J. Food Microbiol.* 23, 449–465. doi: 10.1016/0168-1605(94)90169-4

Sabath, L. D., Garner, C., Wilcox, C., and Finland, M. (1975). Effect of inoculum and of beta-lactamase on the anti-staphylococcal activity of thirteen penicillins and cephalosporins. *Antimicrob. Agents Chemother.* 8, 344–349. doi: 10.1128/AAC.8.3.344

Salton, M. R. J. (1968). Lytic agents, cell permeability, and monolayer penetrability. *J. Gen. Physiol.* 52, 227–252. doi: 10.1085/jgp.52.1.227

Scott, G. V. (1968). Spectrophotometric determination of cationic surfactants with Orange II. *Anal. Chem.* 40, 768–773. doi: 10.1021/ac60260a038

Tan, C., Phillip Smith, R., Srimani, J. K., Riccione, K. A., Prasada, S., Kuehn, M., et al. (2012). The inoculum effect and band-pass bacterial response to periodic antibiotic treatment. *Mol. Syst. Biol.* 8:617. doi: 10.1038/msb.2012.49

Tezel, U., and Pavlostathis, S. G. (2011). "Role of quaternary ammonium compounds on antimicrobial resistance in the environment," in *Antimicrobial Resistance in the Environment*, Chap. 20, eds P. L. KeenMark and H. M. M. Montforts (New York, NY: John Wiley & Sons), 349–387.

Tezel, U., and Pavlostathis, S. G. (2015). Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. *Curr. Opin. Biotechnol.* 33, 296–304. doi: 10.1016/j.copbio.2015.03.018

Thomson, K. S., and Moland, E. S. (2001). Cefepime, piperacillin-tazobactam, and the inoculum effect in tests with extended-spectrum beta-lactamase-producing Enterobacteriaceae. *Antimicrob. Agents Chemother.* 45, 3548–3554. doi: 10.1128/AAC.45.12.3548-3554.2001

Udekwu, K. I., Parrish, N., Ankomah, P., Baquero, F., and Levin, B. R. (2009). Functional relationship between bacterial cell density and the efficacy of antibiotics. *J. Antimicrob. Chemother.* 63, 745–757. doi: 10.1093/jac/dkn554

Vilas, C., Arias-Méndez, A., García, M. R., Alonso, A. A., and Balsa-Canto, E. (2018). Toward predictive food process models: a protocol for parameter estimation. *Crit. Rev. Food Sci. Nutr.* 58, 436–449. doi: 10.1080/10408398.2016.1186591

Yang, S., Lopez, C. R., and Zechiedrich, E. L. (2006). Quorum sensing and multidrug transporters in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2386–2391. doi: 10.1073/pnas.0502890102

Zhang, C., Tezel, U., Li, K., Liu, D., Ren, R., Du, J., et al. (2011). Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. *Water Res.* 45, 1238–1246. doi: 10.1016/j.watres.2010.09.037

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 García and Cabo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 June 2018
doi: 10.3389/fmicb.2018.01370

Detection and Characterization of *Salmonella* Serotypes in the Production Chain of Two Pig Farms in Buenos Aires Province, Argentina

Rocío Colello[1], María J. Ruiz[1], Valeria M. Padín[2], Ariel D. Rogé[2], Gerardo Leotta[3], Nora Lía Padola[1] and Analía I. Etcheverría[1]*

[1] Laboratorio de Inmunoquímica y Biotecnología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET-CICPBA, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina, [2] Servicio Antígenos y Antisueros, Instituto Nacional de Producción de Biológicos, Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos G. Malbrán", Buenos Aires, Argentina, [3] Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout" (UNLP-CONICET LA PLATA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina

The aim of the present study was to determine the prevalence of *Salmonella* in the pork production chain and to characterize *Salmonella* isolates. From 764 samples, 35 (4.6%) were positive for *Salmonella* spp., as determined by biochemical tests and the presence of the *invA* gene. From these, 2.6, 2.0, 8.8, and 8.0% corresponded to samples collected from farms, slaughterhouses, boning rooms and retail markets, respectively. *Salmonella* strains were classified into five serotypes and distributed as follows: *S.* Typhimurium in the pork production chain, *S*. Kentucky in farms and slaughterhouses, *S*. Brandenburg in slaughterhouses, *S*. Livingstone in farms and *S*. Agona in boning rooms and retail markets. Interestingly, the antimicrobial susceptibility testing indicated that all 35 *Salmonella* spp.-positive isolates were resistant to at least one antimicrobial agent, and 30 were multidrug-resistant (MDR) and resistant to different classes of antibiotics. The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) analysis showed clonal relatedness among strains isolated from farms, boning rooms and retail markets. The presence of antibiotic-resistant *Salmonella* in food poses a potential health hazard to consumers.

Keywords: *Salmonella* serotypes, prevalence, pork production chain, MDR, ERIC-PCR

OPEN ACCESS

Edited by:
Maria Schirone,
Università degli Studi di Teramo, Italy

Reviewed by:
Bernadette Dora Gombossy de Melo Franco,
Universidade de São Paulo, Brazil
Francesca Patrignani,
Università degli Studi di Bologna, Italy

****Correspondence:***
Rocío Colello
rocioc@vet.unicen.edu.ar

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *20 March 2018*
Accepted: *06 June 2018*
Published: *28 June 2018*

Citation:
Colello R, Ruiz MJ, Padín VM, Rogé AD, Leotta G, Padola NL and Etcheverría AI (2018) Detection and Characterization of Salmonella Serotypes in the Production Chain of Two Pig Farms in Buenos Aires Province, Argentina. Front. Microbiol. 9:1370. doi: 10.3389/fmicb.2018.01370

INTRODUCTION

Salmonella spp. are important zoonotic pathogens involved in human foodborne illness (Castagna et al., 2005; Sanchez-Maldonado et al., 2017). Most cases of salmonellosis are associated with ingestion of contaminated food such as poultry, milk, beef, pork, eggs, fruits and vegetables (Favier et al., 2013). Contaminated pork meat may be responsible for up to 25% of this illness, being *Salmonella* Typhimurium the most common serotype isolated (Boyen et al., 2008; Kich et al., 2011).

The reservoir of *Salmonella* is the intestinal tract of domestic animals, including pigs. *Salmonella* infection in pigs is sub-clinical; shedding is intermittent for long periods and leading the infection in some farms (Baggesen, 2006). The prevalence of shedding may increase from farm to slaughter because pigs are exposed to a variety of potential stressors during transport, increasing the number

of animals carrying and shedding *Salmonella* as well as its levels in the gastrointestinal tract (Bonardi et al., 2013; Yang et al., 2017). Some slaughter operations, such as handling of the gastrointestinal tract, can influence the bacterial contamination of carcasses, equipment, floors and personnel (Bole-Hribovšek et al., 2008). In addition, environmental *Salmonella* serotypes could produce cross contamination on the slaughter line or during quartering. The molecular tracing of *Salmonella* isolates along the pork production chain represents a suitable tool to evaluate cross contamination (Hernández et al., 2013).

Molecular typing is a useful method for distinguishing among different bacterial isolates that can be used to trace the origins of pathogenic bacteria (Clark et al., 2012). For instance, the enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) analysis is useful to highlight relationships among strains of *Salmonella* isolated from different sources (Swanenburg et al., 1998; de Souza et al., 2015).

Salmonella gastroenteritis is a self-limiting illness although severe cases in immune-compromised, and elderly people or neonates may require effective antimicrobial therapy (White et al., 2001). The use of antimicrobial agents in human and veterinary medicine can lead to the emergence and spread of antimicrobial-resistant *Salmonella*, particularly multidrug-resistant (MDR) strains. Thus, infections with MDR *Salmonella* through contaminated food of animal origin have become a worldwide public health concern (Yang et al., 2017; Zhu et al., 2017).

In Argentina, the National Zoonotic Disease Control Program of the Ministry of Health has incorporated salmonellosis into more important zoonotic diseases of the country (Casas and Geffner, 2014). Nevertheless, few studies report the prevalence of *Salmonella* in the pork production chain in our country, so that the importance of this pathogen in the region is not well-established. Therefore, taking into account the hazard of consuming pork meat contaminated with *Salmonella* and the dissemination of MDR strains (Yang et al., 2017), the aim of this study was to determine the prevalence, serotypes and antibiotic resistance of *Salmonella* strains isolated in the pork production chain, and to assess the possible genetic relationships among *Salmonella* isolates by ERIC-PCR.

MATERIALS AND METHODS

Management of Farms and Animals

The study was conducted in two pig farms which were intensively organized in total confinement. Production stages (gestation, farrowing, weaning and growing/finishing [fattening]) were geographically separated from each other within the same farm. When litters reached 70 days of age and a weight of 35 kg, they were transferred to the fattening or termination area. Each enclosure was divided into rooms and each room consisted of a variable number of pens depending on the size of the group. Partitions between pens were made of concrete. The usual group size varied between 10 and 30 pigs. Pigs and employees moved from one building to another by means of corridors isolated from external traffic. All herds received pelleted feed from the same manufacturer.

Management of Carcasses Before Transport to Retail Markets

Pigs at the finishing production stage were transported to the slaughterhouse. After slaughtering, pork carcasses were chilled for 24–48 h and sent to boning rooms in refrigerated trucks, where they were boned to products such as meat and minced meat. Finally, the products were transferred to retail markets.

Sample Collection

Seven hundred and sixty four samples were collected from two pig production systems, including farms, slaughterhouses, boning rooms and retail markets located in Buenos Aires province, Argentina, from 2012 to 2015.

This study was carried out in accordance with the recommendations of the Animal Welfare Committee from the School of Veterinary Sciences, UNCPBA, 087/02.

Pig Farm Sampling

From a total of 348 samples collected, 277 corresponded to rectal swabs randomly taken from different animals at different production stages, and 71 were obtained from the farm environment by swabbing randomly drinking water, pelleted feed and feces on the floor.

Slaughterhouse Sampling

A total of 147 samples were taken at slaughter. From these, 22 were from rectal swabs after slaughter, 85 from carcasses and 40 from the slaughter environment.

Carcass swabs were taken according to memo No 3496/02 of the National Service of Agrifood Health and Quality (SENASA, for its Spanish acronym) (SENASA, 2002). Five quarter areas of 100 cm^2 each were taken and processed separately (head, external rectum, internal rectum, external thoracic and internal thoracic) (**Figure 1**). Environmental samples were obtained at different points in the slaughter line (pre-washing, scalding, deharing, dressing and cooling) and from knives.

Boning Room Sampling

From a total of 182 samples, 95 were collected from carcasses in the same way as slaughterhouse samples (**Figure 1**), 24 were from meat, 23 from minced meat and 40 from environmental samples obtained by swabbing randomly, refrigerated trucks and meat contact surfaces, such as meat tables, knives, meat mincing machine and vertical band saw machine.

Retail Market Sampling

A total of 87 samples were collected by swabbing randomly meat (43), minced meat (13) and from the environment (31) namely, meat tables, knives, vertical band saw machines and refrigerators).

Microbiological Analysis

Samples were processed according to the FDA Bacteriological Analytical Manual, with modifications. Briefly, each swab was

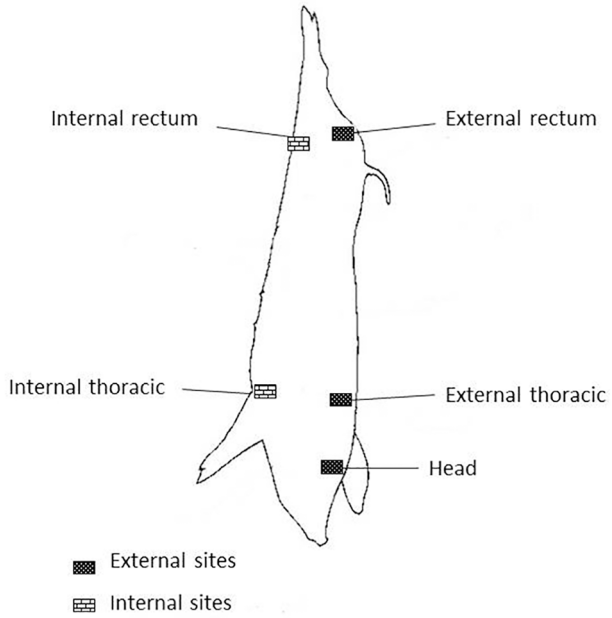


FIGURE 1 | Sites on pig carcass.

homogenized in 225 ml of buffered peptone water and incubated at 37°C for 20 h. Then, 0.1 ml culture medium was inoculated into 10 ml Rappaport-Vassiliadis broth and incubated at 42°C for 24 h. Another 1 ml from the same pre-enrichment culture was inoculated into 10 ml of Tetrathionate Broth Base with iodine solution and incubated at 37°C for 24 h. Each selective enrichment broth was streaked onto Hektoen Enteric agar. Following incubation at 37°C for 24 h, presumptive *Salmonella* colonies were checked by Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA).

invA Gene Detection by PCR

All biochemically typical *Salmonella* isolates were analyzed by PCR to detect the *invA* gene (Rahn et al., 1992). DNA was extracted following methodologies previously described by Parma et al. (2000). Amplification of DNA was performed in a total volume of 50 μl. The reaction mixture contained 500 mM KCl, 100 mM Tris–HCl pH 9, Triton X-100, 25 mM $MgCl_2$, 200 μM of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 1U *Taq*DNA Polymerase Highway® (Inbio) and 5 μl DNA. The initial denaturation at 94°C for 10 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis on 2% agarose gels containing 0.8 μg/ml ethidium bromide in running buffer and visualized by UV transillumination.

Serotyping

Salmonella serotyping was performed according to the White–Kauffmann-Le Minor scheme by slide (O antigen) and tube (H antigen) agglutination, using specific antisera (Instituto Nacional de Producción de Biológicos (INPB) - ANLIS "Dr. Carlos G. Malbrán", Argentina).

Antibiotic Susceptibility

Isolate antibiotic susceptibility profiles were determined by the disk diffusion method according to the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2014). The following antibiotics were assessed: ampicillin (AMP 10 ug), cephalothin (CEF 30 μg), cefotaxime (CTX 30 μg), cefoxitin (FOX 30 μg), amoxicillin/clavulanic acid (AMC 20/10 μg), gentamicin (GEN 10 μg), amikacin (AKN 30 μg), streptomycin (S 300 μg), tetracycline (TET 30 μg), nalidixic acid (NAL 30 μg), trimethoprim/sulfamethoxazole (TMS 1.25/23.75 μg), ciprofloxacin (CIP 5 μg), chloramphenicol (CMP 30 μg), nitrofurantoin (NIT 300 μg), fosfomycin (FOS 50 μg) and colistin (COL 10 μg). *Salmonella* isolates were reported as susceptible, intermediate or resistant (Famiglietti et al., 2005). Multidrug-resistance (MDR) was defined as strain resistance to three or more antibiotic families (Magiorakos et al., 2012).

ERIC-PCR Analysis

All isolates were cultured in TSA (Britania), at 37°C for 24 h; four colonies were taken and then boiled for DNA extraction. The primers used for ERIC-PCR were: ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic et al., 1991). Amplification of DNA was performed in a total volume of 50 μl. The reaction mixture contained 500 mM KCL, 100 mM Tris–HCL pH 9, Triton X-100, 25 mM $MgCl_2$, 200 μM of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 1U *Taq* DNA Polymerase from Highway® and 5 μl DNA. The amplification cycles consisted in an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 1 min, an extension at 72°C for 4 min and a final extension at 74°C for 4 min. The amplification products were separated by 1.5% agarose gel electrophoresis using a 1 kb molecular weight marker plus ladder. Electrophoresis conditions were 100 V for 1 h in Tris-Borate-EDTA with ethyl bromide (0.8 μg/ml).

Data Analysis

DNA fingerprints were analyzed by using a computer program for comparative analysis of DNA electrophoresis patterns (TotalLab Limited 2013). After normalization and alignment of the different DNA profiles, the relative genetic similarity among *Salmonella* spp. isolates was calculated and visualized by cluster analysis. ERIC-PCR products were defined as presence (a score of 1) and absence (a score of 0) of the DNA band. A dendrogram was generated with the BioNumerics vs. 6.6 software (Applied-Maths) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The discrimination index (*D*-value) was calculated by Simpson's diversity index (Hunter and Gaston, 1988).

RESULTS

Prevalence of *Salmonella* spp.

A total of 34 out of 764 samples (4.5%) were *Salmonella* spp.-positive, as determined by biochemical tests and presence of the *invA* gene.

In farms, 3% (11/348) of positive samples were found. From these, 0.7% (2/277) corresponded to the gestation stage and 9% (7/71) to the environment (pelleted feed and floor samples). In slaughterhouses, 2.0% (3/147) of samples were positive. All of them were isolated from the environment (holding pens, holding pen wastewater and rectum of a pig after slaughter). In boning rooms, 8.2% (15/182) of samples were *Salmonella* spp.-positive and namely from the environment (17.5%, 7/40), carcasses (6.3%, 6/95) and meat (4.2%, 2/47). The distribution of positive samples according to the different carcass quarters was 50% (3/6) from the external thoracic region, 16.6% (1/6) from the external rectum, 16.6% (1/6) from the internal rectum and 16.6% (1/6) from heads. In retail markets, 8.0% (7/87) of samples isolated from pork meat and minced meat ready for sale were positive for *Salmonella* spp.

Serotyping

Strains were classified into five serotypes and distributed as follows: *Salmonella* Typhimurium along the pork production chain, *S.* Kentucky in farms and slaughterhouses, *S.* Brandenburg in slaughterhouses, *S.* Livingstone in farms and *S.* Agona in boning rooms and retail markets (**Table 1**). The prevalence of *S.* Typhimurium was significantly higher than that of other serotypes (77.2%), followed by *S.* Agona (11.4%), *S.* Kentucky (5.7%) *S.* Livingstone (2.9%) and *S.* Brandenburg (2.9%).

TABLE 1 | Prevalence of *Salmonella* in farms, slaughterhouses, boning rooms and retail markets, and serotypes identified.

Source/sample isolate	Samples analyzed (n)	Positive samples (%) [(a)]	Serotype [(b)]
Farm			
Different categories	277	2 (0.7)	*S.* Typhimurium (2)
Environment	71	7 (9)	*S.* Typhimurium (5)
			S. Livingstone (1)
			S. Kentucky (1)
Slaughterhouse			
Rectal swabs	22	0 (0)	
Carcasses	85	0 (0)	
Environment	40	3 (7.5)	*S.* Typhimurium (1)
			S. Brandenburg (1)
			S. Kentucky (1)
Boning room			
Carcasses	95	6 (6.3)	*S.* Typhimurium (6)
Meat/minced meat	47	2 (4.2)	*S.* Typhimurium (2)
Environment	40	7 (17.5)	*S.* Typhimurium (5)
			S. Agona (2)
Retail market			
Meat	43	1 (2.3)	*S.* Typhimurium (1)
Minced meat	13	6 (46)	*S.* Typhimurium (5)
Environment	31	0 (0)	*S.* Agona (1)
Total samples	764	34 (4.5)	

[(a)]Percentage of positive sample per type of sample. [(b)]Number of serotypes isolated per sample.

Antibiotic Susceptibility of *Salmonella* Isolates

All of the 34 *Salmonella* isolates tested were resistant to at least one antimicrobial agent, whereas 30 were MDR and resistant to different classes of antibiotics, including β-lactamase, fluoroquinolones, chloramphenicol, aminoglycosides and tetracyclines. Resistance to β-lactams ampicillin (86.1%) and amoxicillin/clavulanic acid (19.4%) was most frequently observed, followed by cephalothin and cefoxitin (16.6%). Percentages of resistance to aminoglycosides such as gentamicin, streptomycin and amikacin were 86, 5.5, and 5.6%, respectively. In the case of tetracycline, 80.5% of isolates showed resistance. Concerning fluoroquinolones, percent resistance was 72.2 and 8.3% to nalidixic acid and ciprofloxacin, respectively. *Salmonella* isolates also exhibited resistance to chloramphenicol (22%), colistin (8.8%) and fosfomycin (2.8%). Some isolates exhibited intermediate sensitivity to cephalothin (38.8%), amoxicillin/clavulanic acid (36.6%) and colistin (27.7%). When antimicrobial resistance was analyzed by source of isolates, farms and retail markets showed the highest rate of resistance to antibiotics of all classes, followed by boning rooms and slaughterhouses. When analyzed by serotype, *S.* Typhimurium and *S.* Agona were the most resistant, followed by *S.* Brandenburg, *S.* Kentucky and *S.* Livingstone (**Table 2**).

Subtyping by ERIC-PCR

All *Salmonella* spp. strains were analyzed by ERIC-PCR. The relationships among isolates on the basis of ERIC fingerprints are presented in **Figure 2**. Multiple DNA fragments of all strains generated with ERIC primers were composed of 6–10 bands ranging between 100 bp and 4 Kb.

The ERIC-PCR analysis and strain clustering produced 18 strains grouped in five clusters (I and V) and 16 strains with unique patterns at a *D*-value of 0.90. Strains with identical profile were isolated from different sources. Strains of clusters II, III, IV, and V presented the same serotype, strains were isolated from boning room (I and IV), farm (V) and boning room and retail markets (II and III). Cluster I had two different serotypes (*S.* Typhimurium (Identical *S.* Typhimurium and *S.* Agona) and two strains isolated from the boning room environment. Cluster II included five strains, two isolated from boning rooms (carcasses and environment) and three isolated from retail markets (meat). Cluster III included three strains from the boning room environment and other three from retail markets (meat and minced meat). Cluster IV comprised three strains from the boning room environment and cluster V contained two strains isolated from farm floor.

TABLE 2 | Antimicrobial resistance of *Salmonella* according to source and serotype.

Source/sample isolate	Serotype	Resistance (%)															
		AMP	CEF	CTX	FOX	A/CL	GEN	AKN	S	TET	NAL	TMS	CIP	CMP	NIT	FOS	COL
Farm																	
Different categories	*S.* Typhimurium (2)	100	0	0	50	50	100	50	0	100	100	0	50	50	0	0	50
Environment	*S.* Typhimurium (5)	100	0	20	20	0	100	0	0	100	100	0	20	20	0	0	0
	S. Livingstone (1)	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0
	S. Kentucky (1)	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Slaughterhouse																	
Rectal swabs	*S.* Typhimurium (1)	100	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0
Carcasses	*S.* Brandenburg (1)	100	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0
Environment	*S.* Kentucky (1)	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0
Boning room																	
Carcasses	*S.* Typhimurium (6)	100	33	0	33	16	100	0	0	100	100	16	0	16	0	0	0
Meat/minced meat	*S.* Typhimurium (2)	100	0	0	50	0	100	0	0	100	0	0	0	0	0	0	0
Environment	*S.* Typhimurium (5)	80	20	20	0		80	0	0	80	80	0	0	0	0	0	0
	S. Agona (2)	0	50	0	0	50	100	50	0	0	0	0	0	50	50	0	0
Retail market																	
Meat	*S.* Typhimurium (1)	100	33	0	0	66	100	0	16	100	100	0	0	16	0	16	0
Minced meat	*S.* Typhimurium (5)	100	0	0	0	0	0	100	0	100	100	0	0	0	0	0	0
	S. Agona (1)	0	0	0	100	100	100	100	100	0	0	0	0	0	0	0	100

DISCUSSION

In this study, the characterization of *Salmonella* strains in the pork production chain is reported. The prevalence of *Salmonella* spp. at different stage of production from other countries are variable and it is important when comparing prevalence since the variation may be due to factors, such as sampling method and samples processing.

Although the routes of access of *Salmonella* onto pork meat differ according to the stage of the process, the main factor is the supply of *Salmonella* colonized pigs onto the slaughter line, with the consequent contamination of carcasses and meat, both sources of foodborne pathogens (Kirchner et al., 2011). Evisceration and subsequent cross-contamination of neighboring carcasses by splash, handling and contact with surfaces are all important aspects (Bole-Hribovšek et al., 2008). In our study, *Salmonella* was detected in 3% of pig farm samples, as opposed to the high prevalence reported by Kich et al. (2011) in Brazil and Bonardi et al. (2013) in Italy. The main factor of pig farm *Salmonella* epidemiology is concerned with the introduction of bacteria, the subsequent transmission to pigs and the introduction of contaminated feed (Vigo et al., 2009). We found positive samples in feed, as reported by Wong et al. (2002), who reported that feed can be considered a risk factor for *Salmonella*.

Our results showed that the prevalence of *Salmonella* in slaughterhouses, boning rooms and retail markets was 2.0, 8.2 and 8.0%, respectively. The information gathered from carcasses in boning rooms and meat from retail markets agreed with that reported in previous studies from different countries showing the high prevalence of *Salmonella* in pig carcasses and meat. For instance, the prevalence of *Salmonella* was 16.7% in China (Li et al., 2013), 10.86% in Spain (Hernández et al., 2013), 13.8% in Germany (Mihaiu et al., 2014) and 24.1% in Argentina (Ibar et al., 2009). Since some carcass areas are more likely exposed to potential contamination or cross contamination, sampling at three or four carcass sites is recommended. The external area involves a particular risk of contamination during the early stages of dressing (Roberts et al., 1984). Our findings showed that the external surface was the most contaminated area, whereas the prevalence of *Salmonella* in equipment was 17.5%, including splitting and mixing machines, processing table and hook. The rol of equipment in carcasses contamination is important, partly due to the possible buildup of bacteria in or on the equipment during working hours (Wong et al., 2002). In retail markets, *Salmonella* recovery was 8.04%, higher than the 0.3 and 4.3% reported by Delhalle et al. (2009) in different pork retailers in Belgium. Contamination levels from pork meat in retail markets depend mainly on the quality of raw materials and products, handling, time and temperature.

All isolates analyzed were genetically confirmed as *Salmonella*-positive by the presence of the *invA* gene. This result is in agreement with that previously reported by other authors (Oliveira et al., 2002; Kumar et al., 2009).

One of the most common serotypes causing human salmonellosis in many countries is *S.* Typhimurium (Campos et al., 2012; Sanchez-Maldonado et al., 2017), which was the main serotype identified in this study. In other reports, this serotype was also found to be predominant in pig and pork products (Botteldoorn et al., 2003; Kich et al., 2011; Bonardi et al., 2013),

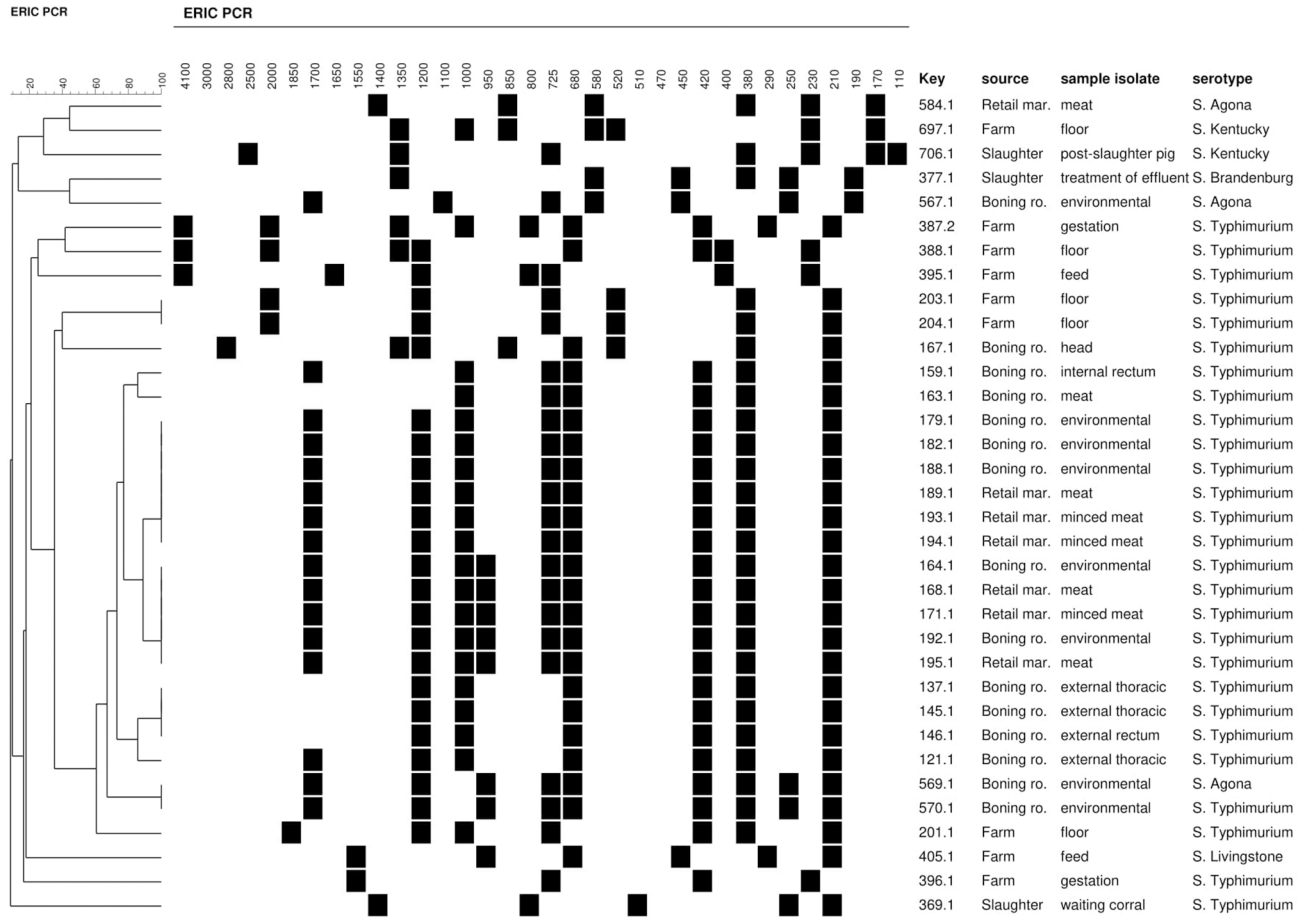


FIGURE 2 | Dendrogram showing genetic relatedness, source, sample type and serotype of *Salmonella* strains isolated along the pork production chain. (*) Multiple DNA fragments of all strains generated with ERIC-PCR are shown on top of the figure. Black, presence of band; White, absence of band.

while other serotypes such as *S.* Agona, *S.* Brandenburg, *S.* Kentucky and *S.* Livingstone were also reported in pigs in previous studies (Botteldoorn et al., 2003; Hernández et al., 2013).

The surveillance of *Salmonella* resistant to antimicrobial vary from 20 to 30% in the 1990s to 70% in some countries in 2000s (Su et al., 2004). The use of antimicrobials in food animals as growth promoters and metaphylactic, prophylactic and therapeutic agents, allows the emergence of antimicrobial-resistant *Salmonella* (Yang et al., 2010). Our findings are similar to those previously described, showing that *Salmonella* isolates from pigs and pork meat are commonly MDR. However, resistance rate was much higher than that reported in the United States and China (Chen et al., 2004), Romania (Mihaiu et al., 2014) and Argentina (Ibar et al., 2009), and the highest frequency was for ampicillin resistance, followed by gentamicin, tetracycline and nalidixic acid. Similar results were found in *Salmonella* isolates from other countries (Thakur et al., 2007; Kich et al., 2011). Fluoroquinolones and cephalosporins are potentially lifesaving treatments for extraintestinal infections. Interestingly, the co-resistance to fluoroquinolones and cephalosporins found in our isolates could limit the effective treatment of *Salmonella* infections in humans, as reported by Li et al. (2013). Colistin is an antimicrobial peptide commercialized in both human and veterinary medicine which has been extensively used orally in pigs for the control of *Enterobacteriaceae* infections (Olaitan et al., 2014; Rebelo et al., 2018). In the present study, 8.8% of strains were colistin-resistant, suggesting the possible loss of colistin effectiveness in human treatment. In addition, is necessary the establishment of a guidelines for the use of colistin in pigs in countries where this drug is approved (Rhouma et al., 2016).

Of the serotypes identified in the present study, *S.* Typhimurium and *S.* Agona showed the highest rates of antimicrobial resistance and MDR. On the other hand, serotypes Kentucky, Livingstone and Brandenburg were relatively more susceptible to antimicrobial agents, indicating that the spread of MDR *S.* Typhimurium isolates is potentially serious, as already reported (Yang et al., 2010; Li et al., 2013).

Swanenburg et al. (1998) standardized ERIC-PCR, a very useful method for quick typing of many *Salmonella* isolates. ERIC analysis showed clonal relatedness among strains

isolated from boning rooms and retail markets, probably due to cross contamination in the deboning process. Five clusters grouped clonal *Salmonella* strains obtained from at least two types of samples. This method is simple, rapid and cheap for typing bacterial strains associated with foodborne outbreaks (Adzitey et al., 2013). However, we could not differentiate even intra-serotype isolates, as reported by Fendri et al. (2013). Future analyses using reliable techniques for discriminating different *Salmonella* serotypes, such as pulsed-field gel electrophoresis, could be appropriate.

Based on our results, isolates from different sources may have originated from a single clone and transmitted along the production chain. That cross-contamination has considerable potential of further spread and dissemination of *Salmonella* spp.

CONCLUSION

Our findings demonstrate the occurrence of *Salmonella* contamination along the pork production chain in Buenos Aires province, Argentina. Surveillance of *Salmonella* in pork meat and characterization of isolates can contribute to the understanding of the epidemiology of this pathogen. Additionally, many *Salmonella* isolates were resistant to multiple antimicrobials, and the presence of this pathogen in the food chain represents a risk for human health. The high rates of MDR *Salmonella* detected suggest that some measures should be taken for the reasonable use of antimicrobials in animal husbandry. These results reinforce the need of an integrated *Salmonella* control program based on pre-harvest good management practices in the farm. A prudent use of antimicrobials and control of critical point systems at post-harvest should be implemented to decrease the hazard of *Salmonella* transmission to consumers. Therefore, implementation of proper hygiene practices during the pork meat production process should be enforced. Isolate characterization should contribute to the understanding of *Salmonella* epidemiology and to conducting food surveillance directed toward this pathogen. Implementation of a comprehensive program covering the entire food value chain continuum from 'farm to fork' is important for *Salmonella* control.

AUTHOR CONTRIBUTIONS

RC conceived, designed, analyzed the experiments, and wrote the manuscript. MR, VP, and AR did some of the experiments. GL, NP, and AE designed some of the experiments, analyzed the data, and revised the manuscript.

FUNDING

This work was supported by PICT 2010-1655, CIC, and SECAT from Argentina.

ACKNOWLEDGMENTS

The authors thank María Rosa Ortiz for technical assistance and A. Di Maggio (IGEVET, UNLP-CONICET LA PLATA), for correcting and editing the manuscript.

REFERENCES

Adzitey, F., Ali, G. R. R., Huda, N., Cogan, T., and Corry, J. (2013). Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from ducks, their rearing and processing environments in Penang, Malaysia. *Food Control* 32, 607–614. doi: 10.1016/j.foodcont.2012.12.016

Baggesen, D. L. (2006). *Opinion of the Scientific Panel on Biological Hazards on the Request from the Commission Related to "Risk Assessment and Mitigation Options of Salmonella in Pig Production*. Question No EFSA-Q-2005–2019. Parma: European Food Safety Authority.

Bole-Hribovšek, V., Chriél, M., Davies, R., Fanning, J., van de Giessen, A. W., Palancar, L. P., et al. (2008). *Salmonella in Holdings with Breeding Pigs in the EU, 2008: Part A: Salmonella Prevalence Estimates*. Parma: European Food Safety Authority.

Bonardi, S., Bassi, L., Brindani, F., D'Incau, M., Barco, L., Carra, E., et al. (2013). Prevalence, characterization and antimicrobial susceptibility of *Salmonella enterica* and *Yersinia enterocolitica* in pigs at slaughter in Italy. *Int. J. Food Microbiol.* 163, 248–257. doi: 10.1016/j.ijfoodmicro.2013.02.012

Botteldoorn, N., Heyndrickx, M., Rijpens, N., and Herman, L. (2003). Detection and characterization of verotoxigenic *Escherichia coli* by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs. *Res. Microbiol.* 154, 97–104. doi: 10.1016/S0923-2508(03)00028-7

Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., and Pasmans, F. (2008). Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. *Vet. Microbiol.* 130, 1–19. doi: 10.1016/j.vetmic.2007.12.017

Campos, J., Pichel, M., Vaz, T., Tavechio, A., Fernandes, S., Muñoz, N., et al. (2012). Building PulseNet Latin America and Caribbean *Salmonella* regional database: first conclusions of genetic subtypes of *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis circulating in six countries of the region. *Food Res. Int.* 45, 1030–1036. doi: 10.1016/j.foodres.2011.10.020

Casas, N., and Geffner, L. (2014). *National Control Programme of Zoonotic Diseases: Surveillance, Prevention and Control of Foodborne Zoonotic Pathogens [Online]*. Available at: www.aazoonosis.org.ar/congreso/web/uploads/resumenes/Casas.doc (accessed May 2018).

Castagna, S. M. F., Muller, M., Macagnan, M., Rodenbusch, C. R., Canal, C. W., and Cardoso, M. (2005). Detection of *Salmonella* sp. from porcine origin: a comparison between a PCR method and standard microbiological techniques. *Braz. J. Microbiol.* 36, 373–377. doi: 10.1590/S1517-83822005000400013

Chen, S., Zhao, S., White, D. G., Schroeder, C. M., Lu, R., Yang, H., et al. (2004). Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. *Appl. Environ. Microbiol.* 70, 1–7. doi: 10.1128/AEM.70.1.1-7.2004

Clark, C. G., Taboada, E., Grant, C. C., Blakeston, C., Pollari, F., Marshall, B., et al. (2012). Comparison of molecular typing methods useful for detecting clusters of *Campylobacter jejuni* and *C. coli* isolates through routine surveillance. *J. Clin. Microbiol.* 50, 798–809. doi: 10.1128/JCM.05733-11

CLSI (2014). *M100-S24 Performance Standards for Antimicrobial Susceptibility Testing, 24th Informational Supplement*, 24 Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

de Souza, A. I., de Freitas Neto, O. C., Batista, D. F., Estupinan, A. L., de Almeida, A. M., Barrow, P. A., et al. (2015). ERIC-PCR genotyping of field isolates of *Salmonella enterica* subsp. *enterica* serovar *Gallinarum* biovars *gallinarum* and *pullorum*. *Avian Pathol.* 44, 475–479. doi: 10.1080/03079457.2015.1086975

Delhalle, L., Saegerman, C., Farnir, F., Korsak, N., Maes, D., Messens, W., et al. (2009). *Salmonella* surveillance and control at post-harvest in the Belgian pork meat chain. *Food Microbiol.* 26, 265–271. doi: 10.1016/j.fm.2008.12.009

Famiglietti, A., Quinteros, M., Vázquez, M., Marín, M., Nicola, F., Radice, M., et al. (2005). Consenso sobre las pruebas de sensibilidad a los antimicrobianos en *Enterobacteriaceae*. *Rev. Argent. Microbiol.* 37, 57–66.

Favier, G. I., Estrada, C. S. L., Otero, V. L., and Escudero, M. E. (2013). Prevalence, antimicrobial susceptibility, and molecular characterization by PCR and pulsed field gel electrophoresis (PFGE) of *Salmonella* spp. isolated from foods of animal origin in San Luis, Argentina. *Food Control* 29, 49–54. doi: 10.1016/j.foodcont.2012.05.056

Fendri, I., Hassena, A. B., Grosset, N., Barkallah, M., Khannous, L., Chuat, V., et al. (2013). Genetic diversity of food-isolated *Salmonella* strains through pulsed field gel electrophoresis (PFGE) and Enterobacterial repetitive intergenic consensus (ERIC-PCR). *PloS One* 8:e81315. doi: 10.1371/journal.pone.0081315

Hernández, M., Gómez-Laguna, J., Luque, I., Herrera-León, S., Maldonado, A., Reguillo, L., et al. (2013). *Salmonella* prevalence and characterization in a free-range pig processing plant: tracking in trucks, lairage, slaughter line and quartering. *Int. J. Food Microbiol.* 162, 48–54. doi: 10.1016/j.ijfoodmicro.2012.12.026

Hunter, P. R., and Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466.

Ibar, M., Vigo, G., Piñeyro, P., Caffer, M., Quiroga, P., Perfumo, C., et al. (2009). Serovariedades de *Salmonella enterica* subespecie *enterica* en porcinos de faena y su resistencia a los antimicrobianos. *Rev. Argent. Microbiol.* 41, 156–162.

Kich, J. D., Coldebella, A., Morés, N., Nogueira, M. G., Cardoso, M., Fratamico, P. M., et al. (2011). Prevalence, distribution, and molecular characterization of *Salmonella* recovered from swine finishing herds and a slaughter facility in Santa Catarina, Brazil. *Int. J. Food Microbiol.* 151, 307–313. doi: 10.1016/j.ijfoodmicro.2011.09.024

Kirchner, M., Marier, E., Miller, A., Snow, L., McLaren, I., Davies, R., et al. (2011). Application of variable number of tandem repeat analysis to track *Salmonella enterica* ssp. *enterica* serovar Typhimurium infection of pigs reared on three British farms through the production cycle to the abattoir. *J. Appl. Microbiol.* 111, 960–970. doi: 10.1016/j.ijfoodmicro.2011.09.024

Kumar, R., Surendran, P., and Thampuran, N. (2009). Distribution and genotypic characterization of *Salmonella* serovars isolated from tropical seafood of Cochin, India. *J. Appl. Microbiol.* 106, 515–524. doi: 10.1111/j.1365-2672.2008.04020.x

Li, R., Lai, J., Wang, Y., Liu, S., Li, Y., Liu, K., et al. (2013). Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. *Int. J. Food Microbiol.* 163, 14–18. doi: 10.1016/j.ijfoodmicro.2013.01.020

Magiorakos, A. P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mihaiu, L., Lapusan, A., Tanasuica, R., Sobolu, R., Mihaiu, R., Oniga, O., et al. (2014). First study of *Salmonella* in meat in Romania. *J. Infect. Dev. Ctries* 8, 50–58. doi: 10.3855/jidc.3715

Olaitan, A. O., Morand, S., and Rolain, J.-M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front. Microbiol.* 5:643. doi: 10.3389/fmicb.2014.00643

Oliveira, S., Santos, L., Schuch, D., Silva, A., Salle, C., and Canal, C. (2002). Detection and identification of salmonellas from poultry-related samples by PCR. *Vet. Microbiol.* 87, 25–35. doi: 10.1016/S0378-1135(02)00028-7

Parma, A., Sanz, M., Blanco, J., Blanco, J., Viñas, M., Blanco, M., et al. (2000). Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. *Eur. J. Epidemiol.* 16, 757–762. doi: 10.1023/A:1026746016896

Rahn, K., De Grandis, S., Clarke, R., McEwen, S., Galan, J., Ginocchio, C., et al. (1992). Amplification of an *invA* gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes* 6, 271–279. doi: 10.1016/0890-8508(92)90002-F

Rebelo, A. R., Bortolaia, V., Kjeldgaard, J. S., Pedersen, S. K., Leekitcharoenphon, P., Hansen, I. M., et al. (2018). Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. *Euro Surveill.* 23, 17-00672. doi: 10.2807/1560-7917.ES.2018.23.6.17-00672

Rhouma, M., Beaudry, F., Thériault, W., and Letellier, A. (2016). Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. *Front. Microbiol.* 7:1789. doi: 10.3389/fmicb.2016.01789

Roberts, T., Hudson, W., Whelehan, O., Simonsen, B., Olgaard, K., Labots, H., et al. (1984). Number and distribution of bacteria on some beef carcasses at selected abattoirs in some member states of the European Communities. *Meat Sci.* 11, 191–205. doi: 10.1016/0309-1740(84)90037-8

Sanchez-Maldonado, A. F., Aslam, M., Service, C., Narváez-Bravo, C., Avery, B. P., Johnson, R., et al. (2017). Prevalence and antimicrobial resistance of *Salmonella* isolated from two pork processing plants in Alberta, Canada. *Int. J. Food Microbiol.* 241, 49–59. doi: 10.1016/j.ijfoodmicro.2016.10.004

SENASA (2002). *Circular 3496/02*. Martinez, CA: Servicio Nacional de Sanidad y Calidad Agroalimentaria.

Su, L.-H., Chiu, C.-H., Chu, C., and Ou, J. T. (2004). Antimicrobial resistance in nontyphoid *Salmonella* serotypes: a global challenge. *Clin. Infect. Dis.* 39, 546–551. doi: 10.1086/422726

Swanenburg, M., Urlings, H., Keuzenkamp, D., and Snijders, J. (1998). Validation of ERIC PCR as a tool in epidemiologic research of *Salmonella* in slaughter pigs. *J. Ind. Microbiol. Biotechnol.* 21, 141–144. doi: 10.1086/422726

Thakur, S., Tadesse, D. A., Morrow, M., and Gebreyes, W. A. (2007). Occurrence of multidrug resistant *Salmonella* in antimicrobial-free (ABF) swine production systems. *Vet. Microbiol.* 125, 362–367. doi: 10.1016/j.vetmic.2007.05.025

Versalovic, J., Koeuth, T., and Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Res.* 19, 6823–6831. doi: 10.1093/nar/19.24.6823

Vigo, G. B., Cappuccio, J. A., Pineyro, P. E., Salve, A., Machuca, M. A., Quiroga, M. A., et al. (2009). *Salmonella enterica* subclinical infection: bacteriological, serological, pulsed-field gel electrophoresis, and antimicrobial resistance profiles—longitudinal study in a three-site farrow-to-finish farm. *Foodborne Pathog. Dis.* 6, 965–972. doi: 10.1089/fpd.2008.0239

White, D. G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., et al. (2001). The isolation of antibiotic-resistant *Salmonella* from retail ground meats. *N. Engl. J. Med.* 345, 1147–1154. doi: 10.1056/NEJMoa010315

Wong, D. L. F., Hald, T., Van Der Wolf, P., and Swanenburg, M. (2002). Epidemiology and control measures for *Salmonella* in pigs and pork. *Livest. Prod. Sci.* 76, 215–222. doi: 10.1016/S0301-6226(02)00121-5

Yang, B., Qu, D., Zhang, X., Shen, J., Cui, S., Shi, Y., et al. (2010). Prevalence and characterization of *Salmonella* serovars in retail meats of marketplace in Shaanxi, China. *Int. J. Food Microbiol.* 141, 63–72. doi: 10.1016/j.ijfoodmicro.2010.04.015

Yang, L., Li, W., Jiang, G.-Z., Zhang, W.-H., Ding, H.-Z., Liu, Y.-H., et al. (2017). Characterization of a P1-like bacteriophage carrying CTX-M-27 in *Salmonella* spp. resistant to third generation cephalosporins isolated from pork in China. *Sci. Rep.* 7:40710. doi: 10.1038/srep40710

Zhu, Y., Lai, H., Zou, L., Yin, S., Wang, C., Han, X., et al. (2017). Antimicrobial resistance and resistance genes in *Salmonella* strains isolated from broiler chickens along the slaughtering process in China. *Int. J. Food Microbiol.* 259, 43–51. doi: 10.1016/j.ijfoodmicro.2017.07.023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Colello, Ruiz, Padín, Rogé, Leotta, Padola and Etcheverría. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 July 2018
doi: 10.3389/fmicb.2018.01475

Characterizing the Antimicrobial Function of a Dairy-Originated Probiotic, *Propionibacterium freudenreichii*, Against Multidrug-Resistant *Salmonella enterica* Serovar Heidelberg in Turkey Poults

Divek V. T. Nair and Anup Kollanoor Johny*

Department of Animal Science, University of Minnesota, Saint Paul, MN, United States

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Fatih Ozogul,
Çukurova University, Turkey
Carmen Wacher,
Universidad Nacional Autónoma de México, Mexico

***Correspondence:**
Anup Kollanoor Johny
anupjohn@umn.edu;
anupkollanoor@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *15 January 2018*
Accepted: *13 June 2018*
Published: *12 July 2018*

Citation:
Nair DVT and Kollanoor Johny A (2018) Characterizing the Antimicrobial Function of a Dairy-Originated Probiotic, Propionibacterium freudenreichii, Against Multidrug-Resistant Salmonella enterica Serovar Heidelberg in Turkey Poults. Front. Microbiol. 9:1475. doi: 10.3389/fmicb.2018.01475

Antimicrobial potential of a dairy-origin probiotic bacteria, *Propionibacterium freudenreichii*, against multidrug-resistant *Salmonella* Heidelberg (SH) in turkey poults was determined in the current study. Employing *in vitro* experiments, two strains (subsp.) of *P. freudenreichii*: *P. freudenreichii freudenreichii* B3523 (PF) and *P. freudenreichii shermanii* B4327 (PS) were tested for their ability to resist low pH (2.5) and bile salts (0.3%). In addition, the ability of the strains to adhere to and invade avian epithelial cells was determined after exposure to *Propionibacterium* strains followed by SH challenge. Moreover, the antibacterial activity of the strains' cell-free culture supernatants (CFCSs) were tested against three major foodborne pathogens, including SH. Furthermore, the susceptibility of the strains to common antibiotics used for human therapy was determined. The hemolytic properties of the strains were determined in comparison to *Streptococcus pyogenes*, a known hemolysis-causing pathogen. Appropriate controls were kept in all studies. Using two *in vivo* experiments, PF was tested against SH colonization of poult ceca and dissemination to liver and spleen. The four treatment groups were: negative control, PF control (PFC), SH control (SC), and a test group (PFS; PF + SH). The poults in the PFC and PFS groups were inoculated with 10^{10} CFU ml^{-1} PF on day 1 through crop gavage and subsequently supplemented through drinking water. On day 7, SC and PFS groups were challenged with SH at 10^{6} CFU ml^{-1}, and after 7 days, cecum, liver, and spleen were collected for determining surviving SH populations. Results indicated that both PF and PS resisted pH = 2.5 and 0.3% bile salts with surviving populations comparable to the control and adhered well onto the avian epithelial cell lines. The strains were susceptible to antibiotics and did not invade the epithelial cells or exhibit hemolytic properties. The CFCSs were highly bactericidal against all tested pathogens. In turkey poults, PF significantly reduced cecal colonization

of SH and the dissemination of the pathogen to the liver, compared to the SH challenge controls ($P < 0.05$). Results revealed that PF, a non-host gastrointestinal tract-derived probiotic, could be an antibiotic alternative to prevent the early colonization of SH in poults, improving the preharvest safety of turkeys.

Keywords: probiotic, turkey safety, antibacterial, antibiotic alternative, *Propionibacterium*, *Salmonella* Heidelberg, multidrug-resistant

INTRODUCTION

Non-typhoidal *Salmonella* is a major bacterial pathogen that causes ∼11% illnesses, 35% hospitalizations, and 28% deaths associated with foodborne outbreaks in the United States, annually (Scallan et al., 2011). Poultry serve as natural reservoir hosts for *Salmonella* and poultry products are commonly implicated in related outbreaks (CDC, 2013; Andino and Hanning, 2015; Antunes et al., 2016). *Salmonella* can survive and colonize in the gastrointestinal tract (GIT) of poultry. Once colonized, *Salmonella* could be shed through their feces leading to environmental (farm) contamination, transmission of the pathogen to fresh incoming flocks, or cross-contamination of the carcasses during faulty evisceration (Foley et al., 2011; Antunes et al., 2016).

Salmonella Heidelberg (SH) is an emerging *Salmonella* serovar in poultry, including turkeys, that has high colonization potential and invasion ability (Nair et al., 2018) compared to the most prevalent serovars such as *S.* Enteritidis (Borsoi et al., 2011). Multistate outbreaks of foodborne salmonellosis occurred in 2011, 2013, and 2014 due to the consumption of poultry products, including ground turkey contaminated with multidrug-resistant (MDR) SH (CDC, 2011, 2014a,b). Moreover, the occurrence of MDR SH in turkeys is a serious concern since it is a major poultry species produced and consumed in the United States and exported globally in massive volumes (USPEA, 2017). This situation is aggravated due to the increased invasiveness of SH isolated from poultry and poultry products, and their multiple drug resistance profiles (Foley et al., 2011; Medeiros et al., 2011; Hoffmann et al., 2014; Antunes et al., 2016). In response to the antibiotic resistance development in foodborne bacteria isolated from animal production, the United States Food and Drug Administration (USFDA) has introduced the Veterinary Feed Directive (VFD) that necessitates veterinary supervision for therapeutic and metaphylactic use of antibiotics in food animals, including poultry (FDA, 2015). This step has resulted in the rigorous search for antibiotic alternatives that can produce meaningful reductions of pathogenic bacteria in food animals, thereby reducing the risk of contaminated animal products entering the food chain.

Dairy-origin *Propionibacterium* are primarily isolated from milk and milk products and ruminants, including dairy cattle (Gutierrez, 1953; Rossi and Dellaglio, 2007; Quigley et al., 2013). These Gram-positive, non-motile bacteria have been used as probiotics in humans with long-term sustainable activity and ability to produce short-chain fatty acids and other metabolites in the GIT (Huang and Adams, 2004). More importantly, *Propionibacterium freudenreichii* spp. is well-characterized among the dairy *Propionibacterium* for its widespread application in the food industry, including production of vitamins, ripening of cheese, and as probiotics (Falentin et al., 2010; Thierry et al., 2011; Cousin et al., 2012; Zárate, 2012; Argañaraz-Martínez et al., 2013; Ganan et al., 2013; Yuksekdag et al., 2014; Rabah et al., 2017). They are classified as a Generally Recognized as Safe (GRAS) and Qualified Presumption of Safety (QPS) status bacteria for use in foods (EFSA, 2013; FDA, 2014). Recently, we found that that *P. freudenreichii* subsp. *freudenreichii* (PF) and *P. freudenreichii* subsp. *shermanii* (PS) have anti-virulence property against *Salmonella* spp., including MDR SH *in vitro* (Nair and Kollanoor-Johny, 2017a). So far, no studies have been conducted to determine the efficacy of *P. freudenreichii* against MDR SH in poultry, including turkeys.

A successful probiotic bacterium should traverse through the adverse digestive and absorptive environment of the poultry GIT to render its beneficial effect on the host. In this process, it should withstand several stresses, including low pH and bile resistance in the intestinal tract (Riley and Austic, 1984; Mahagna et al., 1995; Ao, 2005; Ao et al., 2008; Svihus, 2014). In addition, the probiotic bacterium should possess high affinity and adherence to intestinal epithelial cells and produce secondary metabolites that are responsible also for the antibacterial activity (Patterson and Burkholder, 2003; Alloui et al., 2013; Surendran Nair et al., 2017). Furthermore, the probiotic organism should not develop resistance to commonly used antibiotics and develop pathogenicity in the host species (Salminen et al., 1998; Lin et al., 2007; Musikasang et al., 2009; Smith, 2014; Harimurti and Hadisaputro, 2015). Given these factors, the selection of a potential probiotic is dependant on its tolerance to host physiological stresses since the probiotic qualities are highly strain dependent (Klaenhammer and Kullen, 1999; Saarela et al., 2000; Tuomola et al., 2001; Dunne et al., 2001; Rinkinen et al., 2003; Zárate, 2012). Therefore, the functional properties of the candidate strains of *P. freudenreichii* spp., especially those characteristics that aid in exhibiting antimicrobial activity in the poultry GIT, need to be evaluated to ensure safe application in poultry, in our case, turkeys.

The objectives of the current study, therefore, were (1) to determine the ability of dairy-origin PF and PS to resist various GIT stressors for effective colonization and exhibit antimicrobial activity, *in vitro* and (2) to validate the antimicrobial efficacy of PF on MDR SH colonization of the cecum, and the dissemination of the pathogen to the liver and spleen of turkey poults.

MATERIALS AND METHODS

Ethics Statement

The poult experiments were approved by the Institutional Animal Care and Use Committee, and the use of infectious agents in the experiments was approved by the Institutional Biosafety Committee at the University of Minnesota.

Bacterial Strains and Culture Conditions

Propionibacterium freudenreichii

Two strains of *P. freudenreichii* were used in the study: *P. freudenreichii* subsp. *freudenreichii* B3523 (hereafter PF; USDA ARS NRRL Culture Collection, Peoria, IL, United States) and *P. freudenreichii* subsp. *shermanii* B4327 (hereafter PS; USDA ARS NRRL Culture Collection, Peoria, IL, United States). One hundred microliter of PF or PS stock culture was grown in 10 ml of de Man–Rogosa–Sharpe broth (MRS; catalog no. C5932, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) for 18 h at 41°C. The culture was washed twice with 10 ml of phosphate buffered saline (PBS, pH 7.2) and sedimented by centrifugation (3,600 × *g*, 4°C, 15 min; Allegra X-14R, Beckman Coulter, South Kraemer Boulevard, CA, United States). The pellet was resuspended in 10 ml of PBS, and the bacterial populations in the culture were confirmed by plating 0.1 ml of appropriate dilutions on MRS plates. Viable PF and PS populations were determined after incubating the plates at 41°C (turkey body temperature) for 48 h (Nair and Kollanoor-Johny, 2017a).

Since PF and PS responded similarly in the *in vitro* experiments, PF was selected for the *in vivo* study. PF was made resistant to 50 μg ml^{-1} rifampicin (Rf; catalog no. 50-213-645, Research Products International Corp, 410 E Business Center Dr., Mt Prospect, IL 60056, United States) for selective enumeration and to avoid any confounding inherent *Propionibacterium* in the turkey GIT. The strain was confirmed for resistance to Rf by streaking on MRS containing 50 μg ml^{-1} of Rf (MRS-Rf). For determining the bacterial count, Rf-resistant strain was grown overnight aerobically in 10 ml of MRS supplemented with 50μg ml^{-1} Rf at 37°C. For inoculating birds, a 24 h, Rf-resistant PF (approximately 10^9 CFU ml^{-1}) culture was grown in 1 L MRS broth containing 50 μgml^{-1} Rf and were resuspended in 100 ml PBS after centrifugation at 15,000 rpm for 15 min at 4°C. From this, 1 ml Rf-resistant PF (approximately 10^{10} CFU ml^{-1}) was used to inoculate the day-old poults using crop gavage method. On subsequent days, 10^{10} CFU ml^{-1} Rf-resistant PF was supplemented per gallon of drinking water continuously for 14 days.

Salmonella Heidelberg

A US poultry outbreak isolate of MDR SH was used in the study (GT2011; Nair and Kollanoor-Johny, 2017a,b; Nair et al., 2018). Glycerol stocks of SH stored at −80°C were used for the preparation of working cultures. From the stock cultures, 100 μl was inoculated to 10 ml tryptic soy broth (TSB; catalog no. C7141, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) and incubated for 24 h at 37°C. After three sub-cultures, the third-generation cultures were washed with PBS, centrifuged (3,600 × *g*, 15 min, 4°C) and resuspended in 10 ml PBS. Then the bacterial culture in PBS was serially diluted (1:10) to get a final concentration of 10^7 CFU ml^{-1}. From this, 100 μl was used in the experiments to inoculate the wells containing 2 ml TSB (Kollanoor Johny et al., 2010; Nair and Kollanoor-Johny, 2017a,b; Nair et al., 2018). For the *in vivo* study, GT2011 was made resistant to 50 μg ml^{-1} nalidixic acid sodium salt (NA; CAS. no. 3374-05-8, Alfa Aesar, Haverhill, MA, United States) for selective enumeration to avoid any confounding inherent SH in the turkey GIT. In addition, since the resistance is plasmid encoded for the 2011 ground turkey outbreak strains, any confounding due to the potential loss of plasmids in the GIT was taken care by making the strain NA-resistant. The NA-resistant strain (GT2011NAL; Nair and Kollanoor-Johny, 2017b; Nair et al., 2018) were confirmed for resistance to NA by streaking on xylose lysine desoxycholate (XLD; catalog no. C7322, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) containing 50 μg ml^{-1} of NA (XLD-NA). For inoculating poults, GT2011NAL was grown in 100 ml TSB, and a 16 h broth culture (approximately 10^9 CFU ml^{-1}) was centrifuged (3,600 × *g*, 15 min, 4°C), and the pellet was resuspended in sterile 100 ml PBS (pH 7.2). The culture was serially diluted in PBS to reach final concentration of 10^6 CFU ml^{-1}. Then 2 ml of 10^6 CFU ml^{-1} GT2011NAL was used to inoculate the poults using crop gavage method (Kollanoor-Johny et al., 2012a; Nair et al., 2018).

Escherichia coli O157: H7

Escherichia coli O157: H7 strain CDC EDL 933 (ATCC 43895, Manassas, VA, United States) was used in the study. From the glycerol stock, 100 μl was inoculated to 10 ml TSB and incubated for 24 h at 37°C. After sub-culturing, the third-generation cultures were washed with PBS, centrifuged (3,600 × *g*, 15 min, 4°C) and resuspended in 10 ml PBS. Then the bacterial culture in PBS was serially diluted (1:10) to get a final concentration of 10^7 CFU ml^{-1}. From this, 100 μl was used in the experiments to inoculate the wells containing 2 ml TSB (Amalaradjou et al., 2010; Surendran Nair et al., 2016a,b).

Listeria monocytogenes

Listeria monocytogenes serotype 4b (ATCC) was used in the study. A volume of 100 μl *monocytogenes* inoculum from the glycerol stock cultures were transferred to 10 ml TSB and incubated for 24 h at 37°C to prepare working cultures. After sub-culturing, the third-generation cultures were washed with PBS, centrifuged (3,600 × *g*, 15 min, 4°C), and resuspended in 10 ml PBS. Then the bacterial culture in PBS was serially diluted (1:10) to get a final concentration of 10^7 CFU ml^{-1}. From this, 100 μl was used in the experiments to inoculate the wells containing 2 ml TSB (Upadhyay et al., 2015; Nair and Kollanoor-Johny, 2017a).

In Vitro Study

Determination of Probiotic Resistance to Low pH

Strain PF or PS was grown separately in MRS broth for 18 h at 41°C. The bacterial culture was washed twice with PBS after centrifugation at 3,600 × *g* and 4°C for 15 min. Then the bacterial pellet was resuspended in 10 ml PBS with a pH adjusted to 2.5 using 0.1 N HCl. Bacterial pellet resuspended in PBS with a pH of 7.2 served as negative control. The control and

treatments were incubated at 41°C for 3.5 h. Then at 0 and 3.5 h of incubation, the samples were serially diluted, and 100 μl of appropriate dilutions were plated on MRS agar plates. The survival of PF and PS was determined by enumerating the viable bacteria on plates after 48 h of incubation at 41°C, separately (Owusu-Kwarteng et al., 2015). Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

Determination of Probiotic Resistance to Bile Salts

Strain PF or PS was grown separately in MRS broth for 18 h at 41°C. The bacterial culture was washed twice with PBS after centrifugation at 3,600 × *g* and 4°C for 15 min. Then the bacterial pellets were resuspended in 10 ml PBS containing 0.3% bile salt and an adjusted pH of 8.0 using 0.1 N NaOH. Bacterial pellet resuspended in PBS (pH = 7.2) without bile salt served as negative control. The control and treatments were incubated at 41°C for 3.5 h. Then at 0 and 3.5 h of incubation, the samples were serially diluted, and 100 μl of appropriate dilutions were plated on MRS plates. The survival of PF and PS was determined by enumerating viable bacteria on the plates after 48 h of incubation at 41°C, separately (Owusu-Kwarteng et al., 2015). Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

Determination of Probiotic Hemolytic Activity

Strain PF or PS was grown separately in MRS broth for 18 h at 41°C. The bacterial culture was washed twice with PBS after centrifugation at 3,600 × *g* and 4°C for 15 min. Then the cultures were streaked on Columbia blood agar [Columbia agar (Criterion, Hardy Diagnostics, CA, United States) + 5% (w/v) defibrinated turkey blood (Rockland Immunologicals, PA, United States)]. Columbia blood agar streaked with *Streptococcus pyogenes* (Hardy Diagnostics) with known hemolytic activity was kept as positive control whereas Columbia blood agar streaked with PBS served as negative control (Owusu-Kwarteng et al., 2015). Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

Determination of Probiotic Antimicrobial Activity

Strain PF or PS was grown in MRS broth separately for 72 h at 41°C. The cultures were filter sterilized through a 0.22 μm filtration apparatus to prepare cell-free culture supernatant (CFCS) of PF or PS. The antimicrobial activity of CFCS was determined against three major foodborne pathogens MDR SH, *L. monocytogenes* and *E. coli* O157: H7. The experiment was conducted using 24 well tissue culture plates. A 100 μl bacterial culture at 10^7 CFU ml^{-1} (SH, *L. monocytogenes,* or *E. coli* O157: H7) was added to the wells containing 2 ml TSB having either 5, 10, 15, or 20% (100, 200, 300, and 400 μl; v/v) of CFCS in a 24-well culture plate. Since a lowering in pH was observed in TSB while adding CFCS, the controls were adjusted with 0.1 N HCl to match the pH of treatments containing CFCS and inoculated with the pathogens. Then the treatments and pH-adjusted controls were incubated at 41°C for 24 h, and the optical density (OD = 600 nm) reading was taken at 0 and 24 h (Owusu-Kwarteng et al., 2015; Nair and Kollanoor-Johny, 2017a). Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

Determination of Probiotic Susceptibility to Antibiotics

The antibiotic susceptibility of PF and PS was tested against the common antibiotics that have interpretative criteria either with European Food Safety Authority (EFSA) or Clinical and Laboratory Standards Institute (CLSI) for evaluating minimum inhibitory concentration (MIC; CLSI, 2005, 2015) breakpoints or microbiological cut-off values (MCV; EFSA, 2012). Interpretative MIC or MCV criteria were first sought for *Propionibacterium*, followed by *Lactobacillus* for meaningful comparisons. If criteria were not available for both, interpretation was not expanded comparing *Streptococcus* Groups A, B, C, and G, or other Gram-positive anaerobes. The interpreted antibiotics in the current study include Penicillin (Class – Penicillins; tested range – 0.06–8 μg mL^{-1}; MIC ≤ 8 μg mL^{-1} susceptible; for *Lactobacillus*, no criteria for *Propionibacterium*), Amoxicillin (Class – Penicillins; tested range – 0.25–16 μg mL^{-1}; MIC ≤ 2 μg mL^{-1} susceptible to Ampicillin; same interpretative criteria applied for MIC of Amoxicillin for *Lactobacillus* as per CLSI, 2015), Clindamycin (Class – Lincosamides; tested range – 0.5–4 μg mL^{-1}; MCV = 0.25 μg mL^{-1} susceptible), Erythromycin (Class – Macrolides; tested range – 0.12–4 μg mL^{-1}; MCV = 0.5 μg mL^{-1} susceptible), Gentamicin (Class – Aminoglycosides; tested range – 0.5–8 μg mL^{-1}; MCV = 64 susceptible), Streptomycin (Class – Aminoglycosides; tested range – 8–1024 μg mL^{-1}; MCV = 64 μg mL^{-1} susceptible; **Table 1**, EFSA, 2008), and Tetracycline (Class – Tetracyclines; tested range – 0.25–8 μg mL^{-1}; MCV = 2 μg mL^{-1} susceptible). The tests were conducted using Sensititre™ plates (Trek Diagnostic Systems, Thermo Fisher Scientific, Waltham, MA, United States).

TABLE 1 | Susceptibility of *P. freudenreichii* (PF) and *P. shermanii* (PS) to antibiotics that have MCV or MIC interpretative criteria as per EFSA (2013) or CLSI (2005, 2015), respectively.

Antibiotics	PF	PS
	(MIC-μg ml^{-1})	(MIC-μg ml^{-1})
Penicillins		
Amoxicillin	0.50	1.00
Penicillin	0.25	0.50
Lincosamides		
Clindamycin	<0.50	<0.50
Macrolides		
Erythromycin	<0.12	<0.12
Aminoglycosides		
Gentamicin	<0.50	<0.50
Streptomycin	<8.00	<8.00
Tetracyclines		
Tetracycline	<0.25	<0.25

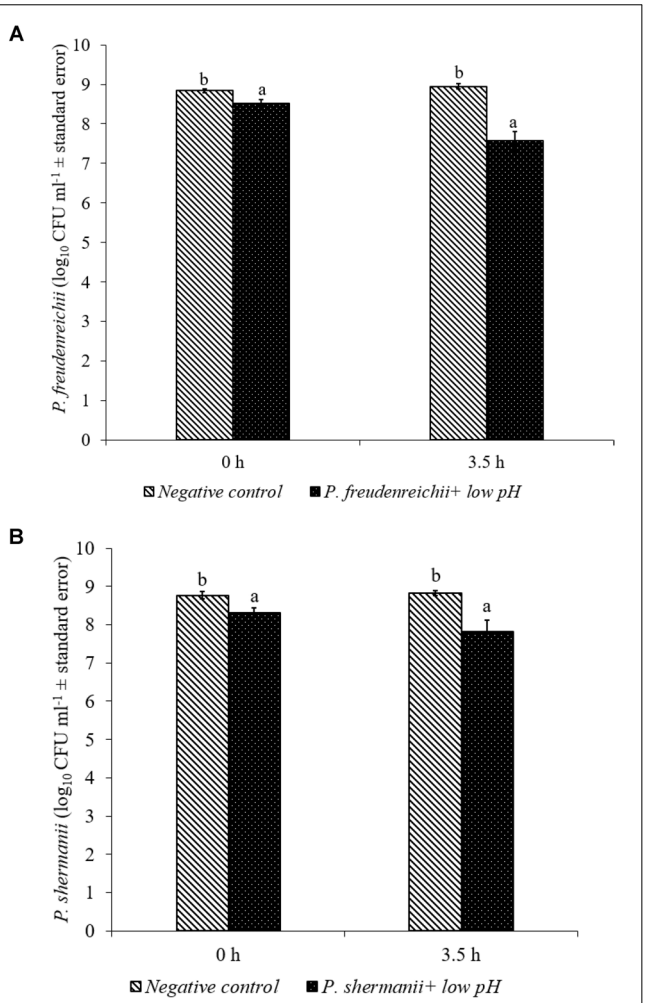


FIGURE 1 | Effect of low pH on the survival of **(A)** *P. freudenreichii* (PF) and **(B)** *P. shermanii* (PS). *Propionibacterium* (10^9 CFU ml^{-1}) was exposed to a low pH = 2.5. Survival of *Propionibacterium* was determined after 3.5 h incubation at 41°C. *Propionibacterium* exposed to a pH = 7.2 for 3.5 h at 41°C served as negative control (n = 6; $^{a-b}P < 0.05$). *Propionibacterium* counts were represented as log_{10} CFU ml^{-1} ± standard error.

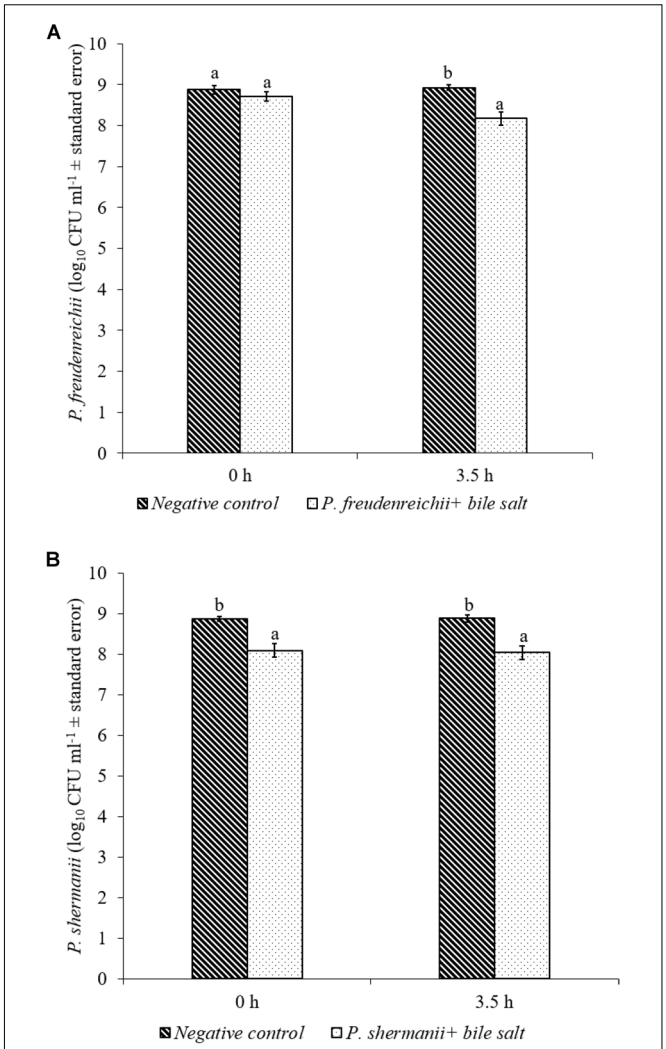


FIGURE 2 | Effect of bile salt on the survival of **(A)** *P. freudenreichii* (PF) and **(B)** *P. shermanii* (PS). *Propionibacterium* (10^9 CFU ml^{-1}) was exposed to 0.3% bile salt at a pH = 8. Survival of *Propionibacterium* was determined after 3.5 h incubation at 41°C. *Propionibacterium* exposed to a pH = 7.2 for 3.5 h at 41°C without bile salt served as negative control (n = 6; $^{a-b}P < 0.05$). *Propionibacterium* counts were represented as log_{10} CFU ml^{-1} ± standard error.

Determination of Potential of Probiotics to Adhere (Associate) to Epithelial Cells

Cell-Association Assay

The adhesion of PF and PS to avian epithelial cells was determined using Budgerigar Abdominal Tumor Cells (BATCs). The bacterial strains were grown separately in sterile cecal filtrate and continuously sub-cultured for three generations at 41°C in 5% CO_2 with agitation (100 rpm) to reach a concentration of 10^9 CFU ml^{-1}. After overnight incubation, cecal filtrate containing 10^9 CFU ml^{-1} PF or PS was used as the inoculum. Sterile turkey cecal filtrates were used for cell association experiments to mimic the cecal environment. The cecal filtrate with PF or PS was added to the wells containing BATCs (10^5 cells/well, >95% confluence; Kollanoor-Johny et al., 2012a; Nair and Kollanoor-Johny, 2017a) in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, United States) and incubated at 37°C under 5% CO_2 for 2 h. Then the wells were washed with DMEM three times, added with 0.1% Triton-X (Invitrogen, Carlsbad, CA, United States) and incubated at 37°C for 15 min. The Triton-X treated cells were homogenized, and cell homogenates were plated on MRS agar plates. The cell-adhered PF or PS was determined after incubating the plates at 37°C for 24 h (Nair and Kollanoor-Johny, 2017a). Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

Gentamicin Protection (Epithelial Invasion) Assay

The BATCs were also used to study the invasion potential of PF and PS to BATCs (Nair and Kollanoor-Johny, 2017a). Briefly,

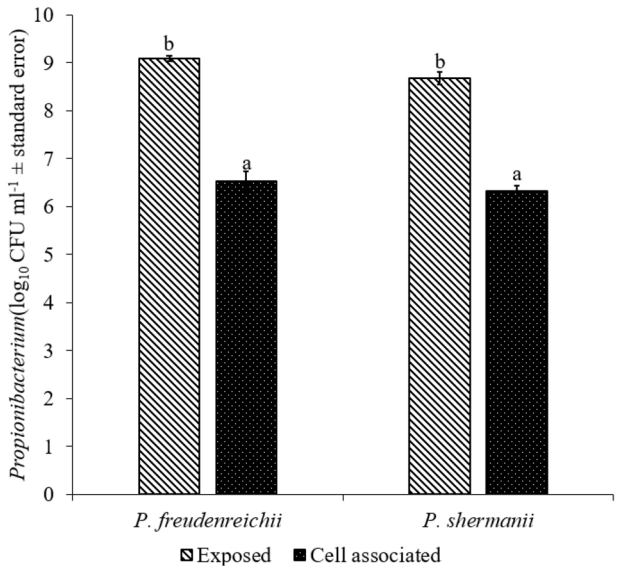


FIGURE 3 | Cell association of *P. freudenreichii* (PF) and *P. shermanii* (PS) on BATCs. BATCs were pre-exposed to 10^9 CFU ml^{-1} of PF or PS for 2 h at 37°C under 5% CO_2. The adherence of the strains to BATCs (cell association) was enumerated after plating cell homogenates on MRS agar plates and incubating at 37°C for 24 h ($n = 6$; $^{a-b}P < 0.05$). *Propionibacterium* counts were represented as log_{10} CFU ml^{-1} ± standard error.

the BATCs were pre-exposed to 10^9 CFU ml^{-1} of PF or PS, separately, for 2 h at 37°C under 5% CO_2 in DMEM: sterile cecal filtrate (1:1). Then the wells were washed three times with DMEM, and fresh whole medium containing 100 μg mL^{-1} of gentamicin (Catalog no. 15750078; Gibco, Invitrogen, Carlsbad, CA, United States) was added to the wells. The wells were incubated at 37°C for 1 h to kill cell surface-attached bacteria. Then the wells were washed with PBS and treated with 0.1% Triton-X to lyse the BATC. After incubation at 37°C with 5% CO_2 for 15 min, the cell homogenates were plated on MRS. The invasion of PF and PS was determined by enumerating viable bacteria after incubating the plates at 37°C for 24 h. Duplicate samples were included for each treatment, and the experiment was repeated at least three times.

In Vivo Study

Experimental Birds, Housing, and Experimental Design

Day-of-hatch, commercial turkey poults (Hybrid Converter), male and female in equal, purchased from a commercial hatchery in Minnesota, were weighed and allocated to isolators in the Research Animal Resources biocontainment (isolation) units at the University of Minnesota. The isolators were maintained with adequate light, heat and floor space specific to the age group. The birds were supplied with *Salmonella*-free *ad libitum* feed (Famo Feeds Inc., 446 Industrial Dr., Freeport, MN, United States) and water according to NRC recommendations.

Two experiments were conducted. In each experiment, day-old poults ($N = 48$) were randomly distributed in four

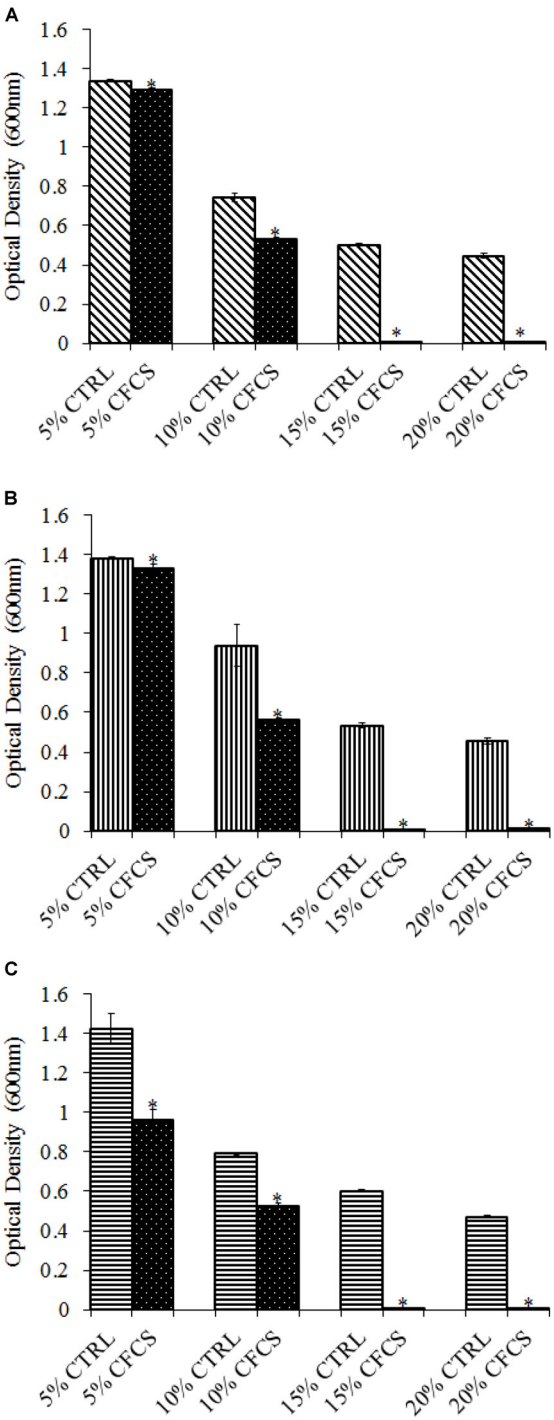


FIGURE 4 | Effect of *P. freudenreichii* (PF) CFCS on **(A)** *S.* Heidelberg **(B)** *L. monocytogenes*, and **(C)** *E. coli* O157: H7. TSB containing *S.* Heidelberg, *L. monocytogenes*, or *E. coli* O157: H7 (10^6 CFU ml^{-1}) were treated with 5, 10, 15, or 20% (v/v) of cell-free culture supernatant (CFCS) of PF. The pH of TSB was adjusted to match the pH of treatments having CFCS were inoculated with the respective pathogen and used as positive controls (CTRL) for each CFCS concentration. The pathogen survival was determined by determining optical density (OD = 600 nm) after incubating samples at 41°C for 24 h ($n = 6$; $^*P < 0.05$). Bacterial counts were represented as OD_{600} ± standard error.

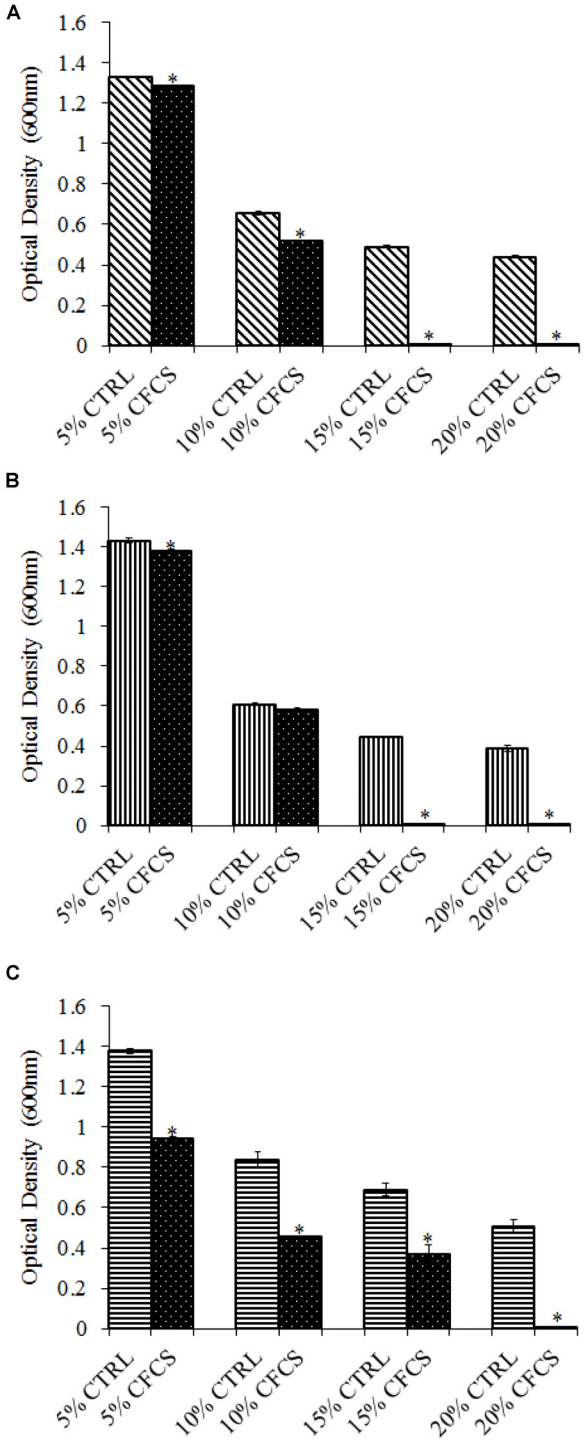


FIGURE 5 | Effect of *P. shermanii* (PS) CFCS on **(A)** *S.* Heidelberg **(B)** *L. monocytogenes*, and **(C)** *E. coli* O157: H7. TSB containing *S.* Heidelberg, *L. monocytogenes*, or *E. coli* O157: H7 (10^6 CFU ml^{-1}) were treated with 5, 10, 15, or 20% (v/v) of CFCS of PS. The pH of TSB was adjusted to match the pH of treatments having CFCS were inoculated with the respective pathogen and used as positive CTRL for each CFCS concentration. The pathogen survival was determined by determining optical density (OD = 600 nm) after incubating samples at 41°C for 24 h ($n = 6$; $^*P < 0.05$). Bacterial counts were represented as OD_{600} ± standard error.

isolator pens with 12 birds each. The treatment groups were: negative control (NC; poults without PF supplementation or SH challenge), PF control (PFC; poults with PF supplementation and without SH challenge), SH control (SC; poults challenged with SH and without PF supplementation), and test group (PFS; poults supplemented with PF and challenged with SH). On day 1, the fecal droppings of the poults from each group were examined for inherent SH, if any. On day 1, the poults in the PFC and PFS groups were inoculated with 10^{10} CFU ml^{-1} PF using crop gavage method. On subsequent days, these groups were supplemented with PF through drinking water. On day 7, SC and PFS groups were challenged with SH at 10^6 CFU ml^{-1} by crop gavage (Nair et al., 2018). Two days after challenge, two poults from each group were euthanized to ensure cecal colonization of SH. Cecum, liver, and spleen were collected to determine PF and SH colonization in the cecum, and SH dissemination to the liver and spleen. The remaining poults from all four groups were euthanized on day 14, and cecum, liver, and spleen were collected for microbiological analysis.

Determination of PF and SH in Turkey Poult Cecum

The colonization of PF and SH in poults were determined after collecting the cecum in 10 ml PBS. The samples were homogenized, serially (1:10) diluted in PBS and 200 µl from appropriate dilutions were plated on MRS-Rf and XLD-NA agar for PF and SH, respectively. Additionally, all samples were enriched in 10 ml selenite cysteine broth (SCB, Hardy Diagnostics, Santa Maria, CA, United States), aerobically, and incubated at 37°C for 6 h, and streaked on XLD-NA plates. The XLD-NA plates were then aerobically incubated at 37°C for 24 h (Kollanoor Johny et al., 2009, 2010; Kollanoor-Johny et al., 2012a,b; Nair and Kollanoor-Johny, 2017a,b).

Determination of SH in Liver and Spleen of Turkey Poults

The liver and spleen samples collected on day 14 were enriched in 10 ml SCB. Enriched samples were incubated 37°C. After 8–12 h incubation, the enriched samples were streaked on XLD and XLD-NA plates, and incubated for 24 h at 37°C to determine the presence of SH in liver and spleen (Nair and Kollanoor-Johny, 2017b).

Statistical Analysis

All *in vitro* experiments were repeated at least three times with duplicate samples per experimental group ($n = 6$/experiment). Each tube was considered an experimental unit for the bile salt resistance, and resistance to low pH experiments. Each well (on a 24-well culture plate) was considered as an experimental unit in the antimicrobial activity determination, and epithelial cell association and invasion assays. Differences between two independent treatments were analyzed using two-tailed *t* tests, and a $P < 0.05$ was considered statistically significant. The results are provided as mean values and standard errors of the means (SEM). The *in vivo* experiment was done two times. A completely randomized design with a factorial treatment structure of 2 × 4 × 3 was used for the *in vivo* experiments. The factors included were two experiments, four treatment groups

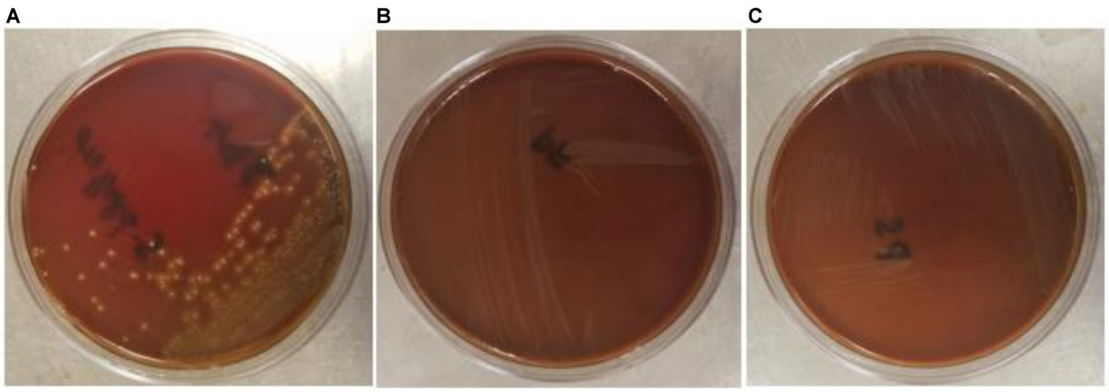


FIGURE 6 | (A–C) Hemolytic activity of *Streptococcus pyogenes*, *P. freudenreichii* (PF) and *P. shermanii* (PS). PF or PS cultures (10^9 CFU ml^{-1}) were streaked on Columbia turkey blood agar. Columbia turkey blood agar streaked with *S. pyogenes* with known hemolytic activity was kept as positive control ($n = 6$; $P < 0.05$).

(NC, PFC, SC, and PFS), and three tissue samples (cecum, liver, and spleen). Among these, PFC and PFS groups were compared to determine the efficacy of PF colonization in the cecum. Similarly, SC and PFS were used to determine the efficacy of PF against SH colonization in the cecum. Bacterial counts were logarithmically transformed ($\log_{10}$ CFU g^{-1}) before analysis. Since there were no significant difference between the experiments, liver and spleen counts data in different groups from two *in vivo* experiments were combined for analysis. On the other hand, there were significant differences between the experiments for the cecal SH counts; data from independent studies were analyzed separately. The data were analyzed using the PROC-MIXED procedure of the statistical analysis software (SAS, version 9.4, SAS Institute Inc., Cary, NC, United States). Differences among the least squares means were detected using Fisher's least significance difference test. The liver and spleen data were analyzed using the Wilcoxon Rank Sum test in SAS to determine the effect of PF on the presence (positive after either direct plating or enrichment) or absence (negative after both direct plating and enrichment) of SH in different organ samples. A $P < 0.05$ was considered statistically significant.

RESULTS

In Vitro Study

In the poultry GIT, the first stress a probiotic bacterium would encounter is the low pH in the proventriculus and gizzard. The results of the current study revealed that both *P. freudenreichii* strains were highly tolerant to the low pH used in the study. The viable counts of PF and PS remained as high as 7.57- and 7.81- $\log_{10}$ CFU ml^{-1}, respectively, after an exposure time of 3.5 h to a pH = 2.5. Viable populations of PF and PS in the controls (pH = 7.2) were at 8.95 and 8.83 $\log_{10}$ CFU ml^{-1}, respectively (**Figures 1A,B**). Similarly, the probiotics would experience the resistance of bile salt while passing through the duodenum. We found that PF and PS exhibited high survivability with viable populations of 8.17- and 8.04- $\log_{10}$ CFU ml^{-1}, respectively, in the treatments when exposed to 0.3% bile salts for 3.5 h, comparable to the controls (**Figures 2A,B**).

The adhesion of a probiotic strain to the host GIT is necessary to elicit its prolonged effect in the host species. In the current study, both *Propionibacterium* strains showed high potential to adhere/associate to the BATCs. PF and PS adhered to BATCs at 6.5 and 6.3 $\log_{10}$ CFU ml^{-1}, respectively, when exposed to ∼9.0 $\log_{10}$ CFU ml^{-1} for 2 h (**Figure 3**). Once adhered to the intestinal cells and perform colonization resistance to the invading microbes, effective probiotics would produce metabolites that may have direct bacteriostatic or bactericidal effects on the pathogens. We found that cell-free culture extracts (CFCSs) of PF and PS were effective against major foodborne pathogens, MDR SH, *E. coli* O157: H7, and *L. monocytogenes*. The CFCS at concentrations of 15 and 20% exhibited the highest antimicrobial activity followed by 10% and 5% (**Figures 4A–C**, **5A–C**).

The safety of the probiotic strains is of utmost importance when used in animals and humans. In this regard, PF and PS did not show hemolytic activity on the Columbia blood agar (**Figures 6A–C**) confirming that the tested *P. freudenreichii* strains are safe to use in turkeys. Additionally, in our study, we observed that both strains were susceptible to the clinically important antibiotics. The minimum inhibitory concentrations (MICs) of tested *P. freudenreichii* strains were in the lower MIC range of the tested antibiotics (**Table 1**). The study also revealed that the tested *P. freudenreichii* strains were not invading the BATCs, providing a good safety margin for their use in turkeys since tissue invasion is one of the mechanisms of bacterial pathogenicity (Kollanoor-Johny et al., 2017).

In Vivo Study

Since PF and PS strains exhibited similar qualities *in vitro*, PF was selected for the *in vivo* experiments. In the current study, 10^{10} CFU ml^{-1} PF was supplemented per gallon of drinking water for 14 days. Of this, approximately 5.4- to 5.7- and 5.4- to 6.0- $\log_{10}$ CFU g^{-1} PF was retained in the cecum of 14-day old turkey poults that received probiotic supplementation in experiments 1 and 2, respectively (**Figures 7A,B**).

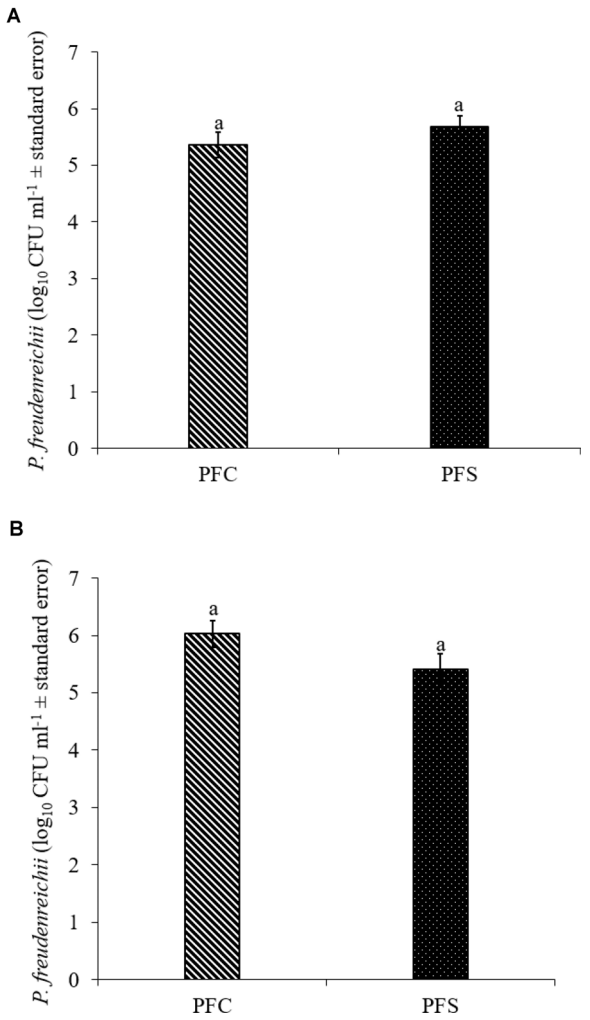


FIGURE 7 | Colonization of *P. freudenreichii* (PF) in 14-day old turkey poults in experiment **(A)** 1 and **(B)** 2. The poults in PFC and PFS groups were supplemented with PF from day 1 to 14. Colonization was determined in PFC and PFS after conducting microbiological analysis of cecal samples (10 poults/group; $^{a}P > 0.05$). PFC: Poults with PF supplementation; PFS: Poults supplemented with PF and challenged with SH. PF counts were represented as $\log_{10}$ CFU ml^{-1} ± standard error.

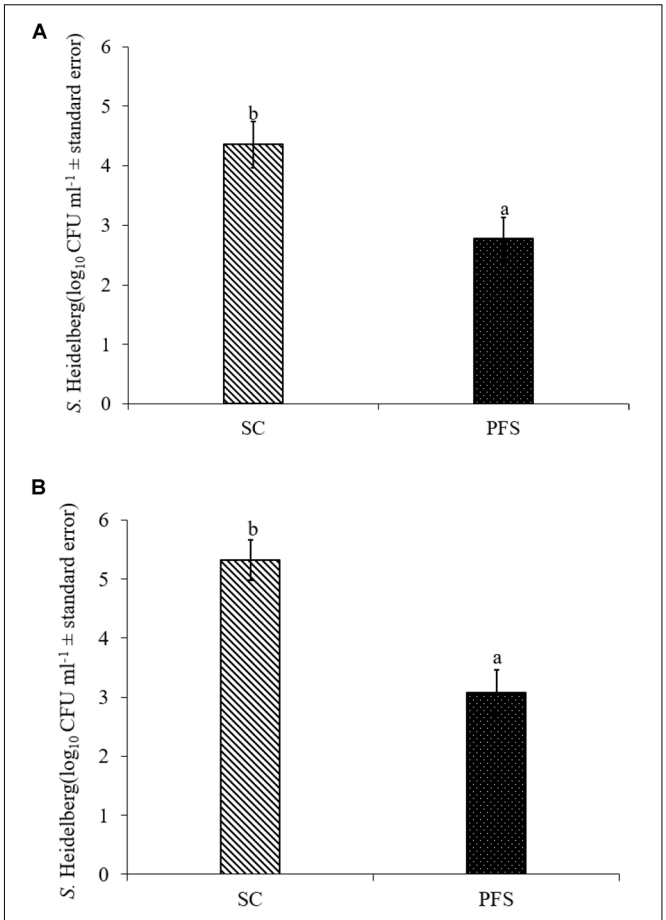


FIGURE 8 | Effect of *P. freudenreichii* (PF) against MDR SH colonization on 14-day old turkey poults in experiments **(A)** 1 and **(B)** 2. The poults in SC group was challenged with SH (10^6 CFU ml^{-1}) on day 7. The PFS group was supplemented with PF from day 1 to 14 and challenged with SH on day 7. The SH recovery was determined in SC and PFS groups after conducting microbiological analysis of cecal samples (10 poults/group; $^{a-b}P < 0.05$). SC, poults challenged with SH; PFS, poults supplemented with PF and challenged with SH. SH counts were represented as $\log_{10}$ CFU ml^{-1} ± standard error.

SH colonized in high numbers in the cecum of turkey poults in the SC groups in both experiments. It was also found that PF significantly reduced SH colonization in turkey poult cecum. The supplementation of PF resulted in 1.6- and 2.2- $\log_{10}$ CFU g^{-1} reduction ($P < 0.05$) of SH on day 14 in the PFS group compared to the SC group in experiments 1 and 2, respectively (**Figures 8A,B**). Additionally, PF supplementation significantly reduced SH dissemination to the liver. On day 14, 70% liver samples were found to be positive for SH in SC groups whereas 35% SH positive liver samples were obtained in PFS groups (**Table 2**). Although, PF supplementation reduced SH dissemination to the spleen on day 14, the reductions were not significantly different ($P = 0.069$).

DISCUSSION

Probiotics provide benefits to a host when physiologically meaningful levels reach the GIT alive and colonize (Patterson and Burkholder, 2003; Chichlowski et al., 2007; Frese et al., 2012). Recently we found that dairy-originated PF and PS possessed the ability to reduce some of the virulence characteristics of *Salmonella* serovars in poultry, including MDR SH *in vitro* (Nair and Kollanoor-Johny, 2017a). Although much information is available on the use of *Propionibacterium* as probiotics in humans, studies that target their benefits in animals are scanty. Before testing the *Propionibacterium* strains in live poultry, we conducted a series of *in vitro* experiments to determine the qualities that might result in efficient colonization in the turkey gut and exhibit antimicrobial activity against SH.

Both *P. freudenreichii* strains were highly tolerant to low pH (**Figures 1A,B**). The underlying mechanisms of pH resistance

TABLE 2 | Effect of *P. freudenreichii* (PF) against MDR SH dissemination to liver and spleen on 14-day-old turkey poults.

	SH positive samples	
Groups	**Liver**	**Spleen**
SC	14/20 (70%)	14/20 (70%)
PFS	7/20 (35%)*	8/20 (40%)

*The poults in SC group was challenged with SH (10^6 CFU ml^{-1}) on day 7. The PFS group was supplemented with PF from day 1–14 and challenged with SH on day 7. The SH dissemination was determined in SC and PFS groups after conducting microbiological analysis of liver and spleen samples (10 poults/group; *indicates $P < 0.05$ between groups within a column).*

are well studied in *P. freudenreichii* spp. The production of SCFAs could gradually reduce the pH of the medium protecting the probiotic bacteria at a pH as low as 2.0. In addition, the upregulation of biotin carboxyl carrier protein and enzymes involved in the DNA synthesis and the universal chaperonins such as GroEL and GroES could play critical roles in pH tolerance (Jan et al., 2001). Also, regulation of F_0F_1-ATPase enzyme, a known molecular response mechanism to low pH conditions in Gram positive organisms, has been implicated by researchers (Cotter and Hill, 2003; Fortier et al., 2003; Corcoran et al., 2005).

The *P. freudenreichii* strains used in this study showed high survival in the presence of 0.3% bile salt (**Figures 2A,B**). Adaption of *P. freudenreichii* to the stress induced by the bile has been attributed to bile salt stress-response proteins. In addition, production of superoxide dismutase and cysteine synthase were also identified in *P. freudenreichii* as stress response proteins mainly responding to the oxidative damage caused by bile acids (Leverrier et al., 2003). In addition, upregulation of active efflux of bile acids and salts by transporters are also implicated (Piddock, 2006; Ruiz et al., 2013).

Once the probiotic bacteria triumphs over the low pH and bile salts, the adhesion of *P. freudenreichii* spp. to intestinal cells is necessary for its colonization in the GIT and to exhibit antimicrobial effects (Patterson and Burkholder, 2003; Hossain et al., 2017). The colonization of probiotic bacteria could competitively inhibit the intestinal adhesion of pathogenic enterobacteria spp. such as *Salmonella* and *Campylobacter* (Hossain et al., 2017). In the current study, PF and PS showed higher potential to adhere to the BATCs (**Figure 3**). Once attached efficiently to the GIT, the probiotics exhibit their beneficial effects, one critical activity of importance to food safety being the antimicrobial activity against pathogenic microbes. It is reported that the cell-free supernatants derived from the probiotics are primarily responsible for the antibacterial activity due to the multitude of bioactive molecules, including bacteriocins. In line with this, the CFCSs of PF and PS were found to be active against pathogens, SH, *L. monocytogenes*, and *E. coli* O157: H7 (**Figures 4A–C**, **5A–C**). The CFCS of *P. freudenreichii* could contain SCFAs and propionicins that directly inhibit pathogens (Gwiazdowska and Trojanowska, 2006; Stackebrandt et al., 2006; Dunkley et al., 2009; Argañaraz-Martínez et al., 2013).

Safety to the host is a critical issue while considering a probiotic for human consumption or animal feeding purposes. In that regard, β-hemolytic activity is one of the characteristic features of pathogenic bacteria such as *S. pyogenes*, the reference pathogenic bacteria used in our study. The β-hemolytic activity of *S. pyogenes* is attributed to hemolysins such as streptolysin S and streptolysin O (Saroj et al., 2016; Spellerberg and Brandt, 2016). The β-hemolysis of *S. pyogenes* was evidenced by the destruction of RBCs around bacterial colonies in the blood agar plates whereas PF and PS did not show any sign of hemolysis (**Figures 6A–C**). In addition, we also evaluated the ability of *P. freudenreichii* spp. to invade the poultry epithelial cells, which is another property of pathogenic bacteria. The cell culture results indicated that *P. freudenreichii* spp. did not invade the BATCs assuring safety.

Susceptibility to common antibiotics is one of the desirable qualities of a probiotic bacterium. In our study, we observed that both strains were susceptible to the clinically important antibiotics (**Table 1**). However, the lack of extensive MIC standards for *Propionibacterium* for making meaningful interpretation of susceptibility profiles was a challenge (Mayrhofer et al., 2010). With the available MIC interpretative criteria, the tested strains were found to be susceptible to Clindamycin, Erythromycin, Gentamicin, Streptomycin, Tetracycline, and Penicillin (CLSI, 2005; EFSA, 2012). Under the molecular taxonomy model published by EFSA (2012), a probiotic with an MIC ≤cut-off could be considered acceptable as a feed additive. Since susceptibility to antibiotics depends upon the strain and/or species of probiotics (Danielsen and Wind, 2003), and lack of availability of such data in *Propionibacterium*, more studies are warranted in this area.

The *in vivo* experiments revealed that PF colonized turkey poults cecum and reduced SH colonization in the cecum (**Figures 8A,B**; $P < 0.05$), and decreased the pathogen dissemination to liver (**Table 2**, $P < 0.05$). The inhibitory action of PF on SH could be due to a competitive exclusion effect (Patterson and Burkholder, 2003; Hossain et al., 2017) or the production of secondary metabolic products, including propionate, acetate, and bacteriocins (Al-Zoreky et al., 1991; Holo et al., 2002; Argañaraz-Martínez et al., 2013) or both. Our previous study (Nair and Kollanoor-Johny, 2017a) also revealed that PF at a concentration ≥7 $\log_{10}$ CFU ml^{-1} was effective in inactivating 5 $\log_{10}$ CFU ml^{-1} SH in a co-culture medium after 24 h incubation at 37°C. Similarly, the CFCS of PF was effective in reducing the motility of SH, which is a virulence factor (Nair and Kollanoor-Johny, 2017a).

The results of the study indicated that PF reduced the dissemination of SH to liver ($P \leq 0.05$). The colonization of PF in large numbers in the cecum of turkey poults might have resulted in reduced attachment of SH to the cecum, eventually resulting in the inhibition to cross the intestinal barrier and dissemination to the internal organs. The colonization ability of PF and the resultant SH exclusion from the avian epithelial cell lines (*in vitro*) were previously proven and the current *in vivo* results corroborate with those findings (Nair and Kollanoor-Johny, 2017a). Moreover,

the persistence of PF through the attachment on to the intestinal cell wall could prolong the production and release of antimicrobial metabolites such as bacteriocins (Zárate, 2012).

Overall, the results indicated that PF and PS exhibited probiotic qualities *in vitro* that could benefit their use in poultry. The tested strains showed high survival in low pH and bile salts, indicating high tolerance to the adverse GIT environment in poultry. In addition, *P. freudenreichii* spp. showed high adhesion to the avian epithelial cells. The CFCS of *P. freudenreichii* spp. exhibited antibacterial activity against major foodborne pathogens, including SH. Regarding the safety of use in turkeys, *P. freudenreichii* strains did not exhibit hemolytic properties, were susceptible to common antibiotics, and did not invade avian epithelial cells *in vitro*. Furthermore, the *in vivo* experiments revealed that PF could colonize well in the cecum of turkey poults for a period of 14 days when supplemented through drinking water that resulted in SH reduction in cecum and dissemination to liver.

AUTHOR CONTRIBUTIONS

AKJ conceived the idea and designed the experiments with DN. DN performed *in vitro* studies and jointly conducted the *in vivo* study with AKJ. DN conducted the statistical analysis. DN and AKJ jointly wrote the manuscript.

FUNDING

The research was funded through the Minnesota State Special Funds provided to the Principal Investigator, AKJ, through the Minnesota Agricultural Experimentation Station.

REFERENCES

Alloui, M. N., Szczurek, W., and Świątkiewicz, S. (2013). The usefulness of prebiotics and probiotics in modern poultry nutrition: a review. *Ann. Anim. Sci.* 13, 17–32. doi: 10.2478/v10220-012-0055-x

Al-Zoreky, N., Ayres, J. W., and Sandine, W. E. (1991). Antimicrobial activity of Microgard against food spoilage and pathogenic microorganisms. *J. Dairy Sci.* 74, 758–763. doi: 10.3168/jds.S0022-0302(91)78222-2

Amalaradjou, M. A. R., Narayanan, A., Baskaran, S. A., and Venkitanarayanan, K. (2010). Antibiofilm effect of *trans*-cinnamaldehyde on uropathogenic *Escherichia coli*. *J. Urol.* 184, 358–363. doi: 10.1016/j.juro.2010.03.006

Andino, A., and Hanning, I. (2015). *Salmonella enterica*: survival, colonization, and virulence differences among serovars. *Sci. World J.* 2015:520179. doi: 10.1155/2015/520179

Antunes, P., Mourão, J., Campos, J., and Peixe, L. (2016). Salmonellosis: the role of poultry meat. *Clin. Microbiol. Infect.* 22, 110–121. doi: 10.1016/j.cmi.2015.12.004

Ao, T. (2005). *Exogenous Enzymes and Organic Acids in the Nutrition of Broiler Chicks: Effects on Growth Performance and in Vitro and in Vivo Digestion*. Doctoral dissertations, University of Kentucky, Lexington, KY.

Ao, T., Cantor, A. H., Pescatore, A. J., and Pierce, J. L. (2008). *In vitro* evaluation of feed-grade enzyme activity at pH levels simulating various parts of the avian digestive tract. *Anim. Feed Sci. Technol.* 140, 462–468. doi: 10.1016/j.anifeedsci.2007.04.004

Argañaraz-Martínez, E., Babot, J. D., Apella, M. C., and Chaia, A. P. (2013). Physiological and functional characteristics of *Propionibacterium* strains of the poultry microbiota and relevance for the development of probiotic products. *Anaerobe* 23, 27–37. doi: 10.1016/j.anaerobe.2013.08.001

Borsoi, A., Ruschel do Santos, L., Beatriz Rodrigues, L., Luiz de Souza Moraes, H., Tadeu Pippi Salle, C., and Pinheiro do Nascimento, V. (2011). Behavior of *Salmonella* Heidelberg and *Salmonella* Enteritidis strains following broiler chick inoculation: evaluation of cecal morphometry, liver and cecum bacterial counts and fecal excretion patterns. *Braz. J. Microbiol.* 42, 266–273. doi: 10.1590/S1517-83822011000100034

CDC (2011). *Salmonella Heidelberg Infections Linked to Ground Turkey*. Available at: https://www.cdc.gov/salmonella/2011/ground-turkey-11-10-2011.html

CDC (2013). Surveillance for foodborne disease outbreaks in the United States, 1998–2008. *Morb. Mortal. Wkly. Rep.* 62, 1–34.

CDC (2014a). *Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Foster Farms Brand Chicken*. Available at: https://www.cdc.gov/salmonella/heidelberg-10-13

CDC (2014b). *Outbreak of Salmonella Heidelberg Infections Linked to Tyson Brand Chicken*. Available at: https://www.cdc.gov/salmonella/heidelberg-01-14

Chichlowski, M., Croom, J., McBride, B. W., Havenstein, G. B., and Koci, M. D. (2007). Metabolic and physiological impact of probiotics or direct-fed-microbials on poultry: a brief review of current knowledge. *Int. J. Poult. Sci.* 6, 694–704. doi: 10.3923/ijps.2007.694.704

CLSI (2005). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Proposed Guideline. CLSI Document M45-P*. Available at: https://clsi.org/media/1450/m45ed3_sample.pdf

CLSI (2015). *M07-A10: Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically; Approved Standard*. Available at: https://clsi.org/media/1632/m07a10_sample.pdf

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2005). Survival of probiotic *Lactobacilli* in acidic environments is enhanced in the presence of metabolizable sugars. *Appl. Environ. Microbiol.* 71, 3060–3067. doi: 10.1128/AEM.71.6.3060-3067.2005

Cotter, P. D., and Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* 67, 429–453. doi: 10.1128/MMBR.67.3.429-453.2003

Cousin, F. J., Foligné, B., Deutsch, S. M., Massart, S., Parayre, S., Le Loir, Y., et al. (2012). Assessment of the probiotic potential of a dairy product fermented by *Propionibacterium freudenreichii* in Piglets. *J. Agric. Food Chem.* 60, 7917–7927. doi: 10.1021/jf302245m

Danielsen, M., and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol.* 82, 1–11. doi: 10.1016/S0168-1605(02)00254-4

Dunkley, K. D., Callaway, T. R., Chalova, V. I., McReynolds, J. L., Hume, M. E., Dunkley, C. S., et al. (2009). Foodborne *Salmonella* ecology in the avian gastrointestinal tract. *Anaerobe* 15, 26–35. doi: 10.1016/j.anaerobe.2008.05.007

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., et al. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am. J. Clin. Nutr.* 73, 386s–392s. doi: 10.1093/ajcn/73.2.386s

EFSA (2008). *Update of the Criteria Used in the Assessment of Bacterial Resistance to Antibiotics of Human or Veterinary Importance*. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2008.732

EFSA (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA panel on additives and products or substances used in animal feed (FEEDAP). *EFSA J.* 10:2740.

EFSA (2013). EFSA panel on biological hazards (BIOHAZ) scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update): QPS 2013 update. *EFSA J.* 11:3449.

Falentin, H., Deutsch, S. M., Jan, G., Loux, V., Thierry, A., Parayre, S., et al. (2010). The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1T, a hardy *Actinobacterium* with food and probiotic applications. *PLOS One* 5:e11748. doi: 10.1371/journal.pone.0011748

FDA (2014). *Agency Response Letter GRAS Notice No. GRN 000415*. Available at: https://wayback.archive-it.org/7993/20171031010359/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm335742.htm

FDA (2015). *Fact Sheet: Veterinary Feed Directive Final Rule and Next Steps*. Available at: https://www.fda.gov/animalveterinary/developmentapprovalprocess/ucm449019.htm

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., and Ricke, S. C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Appl. Environ. Microbiol.* 77, 4273–4279. doi: 10.1128/AEM.00598-11

Fortier, L. C., Tourdot-Maréchal, R., Diviès, C., Lee, B. H., and Guzzo, J. (2003). Induction of *Oenococcus oeni* H^+-ATPase activity and mRNA transcription under acidic conditions. *FEMS Microbiol. Lett.* 222, 165–169. doi: 10.1016/S0378-1097(03)00299-4

Frese, S. A., Hutkins, R. W., and Walter, J. (2012). Comparison of the colonization ability of autochthonous and allochthonous strains of lactobacilli in the human gastrointestinal tract. *Adv. Microbiol.* 2, 399–409. doi: 10.4236/aim.2012.23051

Ganan, M., Martinez-Rodriguez, A. J., Carrascosa, A. V., Vesterlund, S., Salminen, S., and Satokari, R. (2013). Interaction of *Campylobacter* spp. and human probiotics in chicken intestinal mucus. *Zoonoses Public Health* 60, 141–148. doi: 10.1111/j.1863-2378.2012.01510.x

Gutierrez, J. (1953). Numbers and characteristics of lactate utilizing organisms in the rumen of cattle. *J. Bacteriol.* 66, 123–128.

Gwiazdowska, D., and Trojanowska, K. (2006). Antimicrobial activity and stability of partially purified bacteriocins produced by *Propionibacterium freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*. *Le Lait* 86, 141–154. doi: 10.1051/lait:2006001

Harimurti, S., and Hadisaputro, W. (2015). "Probiotics in poultry," in *Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas*, ed. M.-T. Liong (Basel: Springer International Publishing), 1–19.

Hoffmann, M., Zhao, S., Pettengill, J., Luo, Y., Monday, S. R., Abbott, J., et al. (2014). Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. *Genome Biol. Evol.* 6, 1046–1068. doi: 10.1093/gbe/evu079

Holo, H., Faye, T., Brede, D. A., Nilsen, T., Ødegård, I., Langsrud, T., et al. (2002). Bacteriocins of propionic acid bacteria. *Le Lait* 82, 59–68. doi: 10.1051/lait:2001005

Hossain, M. I., Sadekuzzaman, M., and Ha, S. D. (2017). Probiotics as potential alternative biocontrol agents in the agriculture and food industries: a review. *Food Res. Int.* 100, 63–73. doi: 10.1016/j.foodres.2017.07.077

Huang, Y., and Adams, M. C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy *Propionibacteria*. *Int. J. Food Microbiol.* 91, 253–260. doi: 10.1016/j.ijfoodmicro.2003.07.001

Jan, G., Leverrier, P., Pichereau, V., and Boyaval, P. (2001). Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* 67, 2029–2036. doi: 10.1128/AEM.67.5.2029-2036.2001

Klaenhammer, T. R., and Kullen, M. J. (1999). Selection and design of probiotics. *Int. J. Food Microbiol.* 50, 45–57. doi: 10.1016/S0168-1605(99)00076-8

Kollanoor Johny, A., Baskaran, S. A., Charles, A. S., Amalaradjou, M. A. R., Darre, M. J., Khan, M. I., et al. (2009). Prophylactic supplementation of caprylic acid in feed reduces *Salmonella* Enteritidis colonization in commercial broiler chicks. *J. Food Prot.* 72, 722–727. doi: 10.4315/0362-028X-72.4.722

Kollanoor Johny, A., Darre, M. J., Donoghue, A. M., Donoghue, D. J., and Venkitanarayanan, K. (2010). Antibacterial effect of *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken cecal contents *in vitro*. *J. Appl. Poult. Res.* 19, 237–244. doi: 10.3382/japr.2010-00181

Kollanoor-Johny, A., Frye, J. G., Donoghue, A., Donoghue, D. J., Porwollik, S., McClelland, M., et al. (2017). Gene expression response of *Salmonella enterica* serotype Enteritidis phage type 8 to subinhibitory concentrations of the plant-derived compounds trans-cinnamaldehyde and eugenol. *Front. Microbiol.* 8:1828. doi: 10.3389/fmicb.2017.01828

Kollanoor-Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A., Babapoor, S., March, B., et al. (2012a). Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds *trans*-cinnamaldehyde and eugenol. *Appl. Environ. Microbiol.* 78, 2981–2987. doi: 10.1128/AEM.07643-11

Kollanoor-Johny, A., Mattson, T., Baskaran, S. A., Amalaradjou, M. A. R., Hoagland, T. A., Darre, M. J., et al. (2012b). Caprylic acid reduces *Salmonella* Enteritidis populations in various segments of digestive tract and internal organs of 3-and 6-week-old broiler chickens, therapeutically. *Poult. Sci.* 91, 1686–1694. doi: 10.3382/ps.2011-01716

Leverrier, P., Dimova, D., Pichereau, V., Auffray, Y., Boyaval, P., and Jan, G. (2003). Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. *Appl. Environ. Microbiol.* 69, 3809–3818. doi: 10.1128/AEM.69.7.3809-3818.2003

Lin, W. H., Yu, B., Jang, S. H., and Tsen, H. Y. (2007). Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe* 13, 107–113. doi: 10.1016/j.anaerobe.2007.04.006

Mahagna, M., Nir, I., Larbier, M., and Nitsan, Z. (1995). Effect of age and exogenous amylase and protease on development of the digestive tract, pancreatic enzyme activities and digestibility of nutrients in young meat-type chicks. *Reprod. Nutr. Dev.* 35, 201–212. doi: 10.1051/rnd:19950208

Mayrhofer, S., Van Hoek, A. H., Mair, C., Huys, G., Aarts, H. J., Kneifel, W., et al. (2010). Antibiotic susceptibility of members of the *Lactobacillus acidophilus* group using broth microdilution and molecular identification of their resistance determinants. *Int. J. Food Microbiol.* 144, 81–87.

Medeiros, M. A., Oliveira, D. C., Rodrigues Ddos, P., and Freitas, D. R. (2011). Prevalence and antimicrobial resistance of *Salmonella* in chicken carcasses at retail in 15 Brazilian cities. *Rev. Panam. Salud Publica* 30, 555–560. doi: 10.1590/S1020-49892011001200010

Musikasang, H., Tani, A., H-kittikun, A., and Maneerat, S. (2009). Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. *World J. Microbiol. Biotechnol.* 25, 1337–1345. doi: 10.1007/s11274-009-0020-8

Nair, D. V. T., and Kollanoor-Johny, A. (2017a). Effect of *Propionibacterium freudenreichii* on *Salmonella* multiplication, motility, and association with avian epithelial cells. *Poult. Sci.* 96, 1376–1386. doi: 10.3382/ps/pew367

Nair, D. V. T., and Kollanoor-Johny, A. (2017b). Food grade pimenta leaf essential oil reduces the attachment of *Salmonella enterica* Heidelberg (2011 ground turkey outbreak isolate) on to turkey skin. *Front. Microbiol.* 8:2328. doi: 10.3389/fmicb.2017.02328

Nair, D. V. T., Thomas, J. V., and Kollanoor Johny, A. (2018). Effect of various inoculum levels of multidrug-resistant *Salmonella enterica* serovar Heidelberg (2011 ground turkey outbreak isolate) on cecal colonization, dissemination to internal organs, and deposition in skeletal muscles of commercial turkeys after experimental oral challenge. *Front. Microbiol.* 8:2680. doi: 10.3389/fmicb.2017.02680

Owusu-Kwarteng, J., Tano-Debrah, K., Akabanda, F., and Jespersen, L. (2015). Technological properties and probiotic potential of *Lactobacillus fermentum* strains isolated from West African fermented millet dough. *BMC Microbiol.* 15:261. doi: 10.1186/s12866-015-0602-6

Patterson, J. A., and Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poult. Sci.* 82, 627–631. doi: 10.1093/ps/82.4.627

Piddock, L. J. (2006). Multidrug-resistance efflux pumps: not just for resistance. *Nat. Rev. Microbiol.* 4, 629–636. doi: 10.1038/nrmicro1464

Quigley, L., O'sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., et al. (2013). The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 37, 664–698. doi: 10.1111/1574-6976.12030

Rabah, H., Rosa do Carmo, F. L., and Jan, G. (2017). Dairy *Propionibacteria*: versatile probiotics. *Microorganisms* 5:24. doi: 10.3390/microorganisms5020024

Riley, W. W., and Austic, R. E. (1984). Influence of dietary electrolytes on digestive tract pH and acid-base status of chicks. *Poult. Sci.* 63, 2247–2251. doi: 10.3382/ps.0632247

Rinkinen, M., Westermarck, E., Salminen, S., and Ouwehand, A. C. (2003). Absence of host specificity for *in vitro* adhesion of probiotic lactic acid bacteria to intestinal mucus. *Vet. Microbiol.* 97, 55–61. doi: 10.1016/S0378-1135(03)00183-4

Rossi, F., and Dellaglio, F. (2007). Quality of silages from Italian farms as attested by number and identity of microbial indicators. *J. Appl. Microbiol.* 103, 1707–1715. doi: 10.1111/j.1365-2672.2007.03416.x

Ruiz, L., Margolles, A., and Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Front. Microbiol.* 4:396. doi: 10.3389/fmicb.2013.00396

Saarela, M., Mogensen, G., Fonden, R., Mättö, J., and Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* 84, 197–215. doi: 10.1016/S0168-1656(00)00375-8

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., et al. (1998). Demonstration of safety of probiotics—a review. *Int. J. Food Microbiol.* 44, 93–106. doi: 10.1016/S0168-1605(98)00128-7

Saroj, S. D., Maudsdotter, L., Tavares, R., and Jonsson, A. B. (2016). *Lactobacilli* interfere with *Streptococcus pyogenes* hemolytic activity and adherence to host epithelial cells. *Front. Microbiol.* 7:1176. doi: 10.3389/fmicb.2016.01176

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17, 1–21. doi: 10.3201/eid1701.P11101

Smith, J. M. (2014). A review of avian probiotics. *J. Avian Med. Surg.* 28, 87–94. doi: 10.1647/2012-031

Spellerberg, B., and Brandt, C. (2016). "Laboratory diagnosis of *Streptococcus pyogenes* (group A *Streptococci*)," in *Streptococcus pyogenes*: *Basic Biology to Clinical Manifestations*, eds J. J. Ferretti, D. L. Stevens, and V. A. Fischetti (Oklahoma City, OK: University of Oklahoma Health Sciences Center).

Stackebrandt, E., Cummins, C. S., and Johnson, J. L. (2006). "Family Propionibacteriaceae: the genus *Propionibacterium*," in *The Prokaryotes*, eds M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer, and E. Stackebrandt (New York, NY: Springer), 400–418.

Surendran Nair, M., Amalaradjou, M. A., and Venkitanarayanan, K. (2017). Chapter One- anti-virulence properties of probiotics in combating microbial pathogenesis. *Adv. Appl. Microbiol.* 98, 1–29. doi: 10.1016/bs.aambs.2016.12.001

Surendran Nair, M., Kollanoor-Johny, A., Ananda-Baskaran, S., Norris, C., Lee, J. Y., and Venkitanarayanan, K. (2016a). Selenium reduces enterohemorrhagic *Escherichia coli* O157: H7 verotoxin production and globotriaosylceramide receptor expression on host cells. *Future Microbiol.* 11, 745–756. doi: 10.2217/fmb.16.16

Surendran Nair, M., Lau, P., Belskie, K., Fancher, S., Chen, C. H., Karumathil, D. P., et al. (2016b). Potentiating the heat inactivation of *Escherichia coli* O157: H7 in ground beef patties by natural antimicrobials. *Front. Microbiol.* 7:15. doi: 10.3389/fmicb.2016.00015

Svihus, B. (2014). Function of the digestive system. *J. Appl. Poult. Res.* 23, 306–314. doi: 10.3382/japr.2014-00937

Thierry, A., Deutsch, S. M., Falentin, H., Dalmasso, M., Cousin, F. J., and Jan, G. (2011). New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *Int. J. Food Microbiol.* 149, 19–27. doi: 10.1016/j.ijfoodmicro.2011.04.026

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E., and Salminen, S. (2001). Quality assurance criteria for probiotic bacteria. *Am. J. Clin. Nutr.* 73, 393s–398s. doi: 10.1093/ajcn/73.2.393s

Upadhyay, A., Upadhyaya, I., Karumathil, D. P., Yin, H. B., Surendran Nair, M., Bhattaram, V., et al. (2015). Control of *Listeria monocytogenes* on skinless frankfurters by coating with phytochemicals. *LWT Food Sci. Technol.* 63, 37–42. doi: 10.1016/j.lwt.2015.03.100

USPEA (2017). *Economic Data*. Available at: https://www.uspoultry.org/economic_data/

Yuksekdag, Z. N., Darilmaz, D. O., and Beyatli, Y. (2014). Dairy *Propionibacterium* strains with potential as biopreservatives against foodborne pathogens and their tolerance–resistance properties. *Eur. Food Res. Technol.* 238, 17–26. doi: 10.1007/s00217-013-2066-y

Zárate, G. (2012). *Dairy Propionibacteria: Less Conventional Probiotics to Improve the Human and Animal Health*. Rijeka: INTECH Open Access Publisher.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Nair and Kollanoor Johny. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 July 2018
doi: 10.3389/fmicb.2018.01628

The Fimbrial Gene *z3276* in Enterohemorrhagic *Escherichia coli* O157:H7 Contributes to Bacterial Pathogenicity

Bicheng Zhang[1,2,3,4,5], *Xiaohan Sun*[1,2,3,4,5], *Hongjie Fan*[6], *Kongwang He*[1,2,3,4,5] *and Xuehan Zhang*[1,2,3,4,5]*

[1] *Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing, China,* [2] *Key Laboratory of Engineering Research of Veterinary Bio-products of Agricultural Ministry, Nanjing, China,* [3] *National Center for Engineering Research of Veterinary Bio-products, Nanjing, China,* [4] *Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing, China,* [5] *Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, China,* [6] *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China*

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a zoonotic pathogen of worldwide importance that causes foodborne infections in humans. It is not capable of expressing type I fimbrial because of base deletion in the *fim* operon. BLAST analysis shows that the open reading frame *z3276*, a specific genetic marker of EHEC O157:H7, encodes a sequence with high amino acid identity to other *E. coli* type I fimbrial, but its definitive function in EHEC O157:H7 remains unclear. We are here to report that a *z3276* mutant (Δ*z3276*) was constructed using the reference EHEC O157:H7, the mutant Δ*z3276* was biologically characterized, and the pathogenicity of Δ*z3276* was assessed in mice in comparison with the wild-type (WT) strain. Motility and biofilm formation assays revealed a smaller twitching motility zone for Δ*z3276* on the agar surface and significantly decreased biofilm formation by Δ*z3276* compared with the parental strain. The adhesion and invasion ability of Δ*z3276* to HEp-2 cells showed no significant change, but the invasion ability of Δ*z3276* to IPEC-J2 cells was attenuated. Furthermore, in the animal study, we observed shortened and lower fecal shedding among the Δ*z3276* mutant-infected animals compared with those infected WT strain. The data in this study indicate that this unique gene of *z3276* in EHEC O157:H7 seems to play an important role in bacterial pathogenicity.

Keywords: EHEC O157:H7, genetic marker, *z3276*, motility, biofilm formation, invasion, pathogenicity

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Baoguang Li,
United States Department of Health and Human Services, United States
Amy Michele Grunden,
North Carolina State University, United States

***Correspondence:**
Xuehan Zhang
liuxuehan1996@hotmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 March 2018*
Accepted: *28 June 2018*
Published: *19 July 2018*

Citation:
Zhang B, Sun X, Fan H, He K and Zhang X (2018) The Fimbrial Gene z3276 in Enterohemorrhagic Escherichia coli O157:H7 Contributes to Bacterial Pathogenicity. Front. Microbiol. 9:1628. doi: 10.3389/fmicb.2018.01628

INTRODUCTION

Since first being recognized in 1982 following an outbreak of hemorrhagic colitis in the United States (Riley et al., 1983), Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has emerged as a significant cause of serious human gastrointestinal disease worldwide (Tarr et al., 2005). The clinical manifestations of EHEC O157:H7 infections range from self-limiting diarrhea to hemorrhagic colitis, which can lead to severe complications known as hemolytic uremic syndrome, which is associated with a mortality rate of 2–10% (Kaper et al., 2004; Tarr et al., 2005; Makvana and Krilov, 2015).

Multiple fimbrials and fimbrial gene clusters have been implicated in contributing to the adherence of EHEC O157:H7 to host cells and its virulence (Li et al., 1997; Low et al., 2006; Lloyd et al., 2012; Russell et al., 2018). The hemorrhagic coli pilus of EHEC O157:H7, a type IV pilus, has been associated with intestinal adherence and invasion and can induce proinflammatory cytokine secretion in intestinal epithelial cells (Xicohtencatl-Cortes et al., 2007; Ledesma et al., 2010; Mazariego-Espinosa et al., 2010). Type I fimbrial, composite surface fibers present in various types of pathogenic *E. coli* (Uropathogenic *E. coli* and Diffusely adherent *E. coli*), are important bacterial adhesion organelles present in most Gram-negative bacterial strains that facilitate bacterial colonization (Epler Barbercheck et al., 2018). These fimbrial are encoded by the *fim* gene cluster and are exported by the chaperone/usher pathway, in which FimC is the periplasmic chaperone, FimD is the outer membrane usher, and FimA and FimH are the major structure for adhesion (Epler Barbercheck et al., 2018; Russell et al., 2018). However, EHEC O157:H7 is not able to express type I fimbrial despite containing the *fim* operon because of a 16-bp deletion at the 5' end of *fim*A and a C to A mutation at position 467 in the *fim*H gene (Li et al., 1997; Low et al., 2006). A 1035-bp open reading frame (ORF), named *z3276*, has been firstly reported in the EHEC O157:H7 genome (Perna et al., 2001). The bioinformatics studies indicated that the *z3276* was properly unique in the genome and identified as a specific marker of EHEC O157:H7 (Perna et al., 2001; Ravan and Amandadi, 2015). It was used to successfully developed detection method, i.e., real-time PCR (Li and Chen, 2012; Li et al., 2017). The protein encoded by the *z3276* gene shows identity to type I fimbrial, but its definitive function in EHEC O157:H7 remains unclear.

To investigate the function of Z3276, a mutant (Δ*z3276*) of EHEC O157:H7 with the *z3276* gene deleted was constructed. Our results suggested that *z3276* played important roles in the bacterial motility, biofilm formation, invasion of specific cell types and colonization *in vivo* of EHEC O157:H7.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids involved in this study are presented in **Table 1**. *E. coli* C118 and SM10 strains were used to prepare the complement cells for pMEG375 and its recombinant plasmids. Theses *E. coli* strains were routinely grown in Luria-Bertani (LB) broth or on LB agar at 37°C, whereas the *E. coli* EDL933 parent strain, the Δz3276 mutant strain, and the complement strain (cΔ*z3276*) were cultured in tryptone soya broth (TSB) or on tryptic-soy agar (TSA) at 37°C to prepare bacterial cultures for comparative adherence assays, invasion assay and twitching motility assay. When required, the antibiotics kanamycin (100 μg/mL), ampicillin (100 μg/mL), chloramphenicol (34 μg/mL) and/or gentamicin (50 μg/mL) were added to the media.

Cell Line Culture Conditions

To investigate the Z3276 role in the adherence and invasion to non-intestinal cells and intestinal cells, perhaps by recognition of a common surface receptor, we employed human laryngeal carcinoma cell line HEp-2 (BioVector NTCC Inc.) and porcine neonatal jejunal epithelial cell line IPEC-J2 (a gift from Dr. Zhu Guoqiang, Yangzhou University). These cells were cultured in antibiotic-free RPMI1640 medium, supplemented with 10% newborn calf serum (Sigma-Aldrich) at 37°C in a humidified incubator in an atmosphere of 5% CO_2.

Phylogeny Analysis of the Z2376 Amino-Acid Sequence

To explore the homology of Z2376 protein in the protein database of GenBank, its amino-acid sequence was submitted to GenBank to identify its phylogenetic relationships and conserved domain. The phylogenetic tree was generated by BLAST analysis.

Antigen Cloning, Expression, and Purification

The *z3276* ORF is between 2,951,428 and 2,952,462 nucleotide of EHEC O157:H7 complete genome (GenBank, CP015855.1), far from the disabled *fim* operon, located in the 5,444,749 nucleotide. The PCR product of the complete ORF of *z3276* with primers (P6-F/R) were amplified from chromosomal EHEC O157:H7 and introduced into pCold I to generate recombinant bacteria BL21 (DE3)/pCold I-*z3276*. The recombinant bacterium was grown overnight, subcultured into fresh medium, and further grown for 2 h at 37°C; and isopropyl β-d-thiogalactopyranoside (IPTG) was added and incubation was continued for 24 h. The bacterial cultures were harvested by centrifugation and resuspended in PBS, containing 1 mM Pefabloc, 0.5 mg/mL lysozyme, 10 μg/mL DNase I, and 10 μg/mL RNase A. Cell lysates were ultrasonicated for 5 min with 30 s intervals on ice. Centrifuged supernatants were purified using His●Bind Resin Chromatography according to the manufacturer's instructions (GE Healthcare Life Sciences).

Polyclonal Antibody Preparation

For anti-recombinant Z3276 protein polyclonal antibody preparation, Jiangsu Academy of Agricultural Sciences Institutional Animal Care and Use Committee approved the animal procedures (SYXK2015-0066) in the context of the guidelines of the Jiangsu Province Animal Regulations (Government Decree No. 45). New Zealand white rabbits were obtained from Jinling rabbit Farm (Nanjing, China), housed in cages (W 50 cm, L 30 cm, H 40 cm). They were provided with food and water *ad libitum*. At the end of the study, they were euthanized by intravenous injection of barbiturate, exsanguinated, and blood was clotted at 37°C for 0.5 h, chilled at 4°C for overnight. The purified recombinant protein Z3276 was used as antigen for polyclonal antibody preparation. Polyclonal antibody against Z3276 protein was obtained by immunizing New Zealand White rabbits subcutaneously at multiple sites with approximately 0.5 mg of purified protein emulsified 1:1 in Freund's complete adjuvant. The rabbits received one booster

TABLE 1 | Bacterial strains and plasmids used in this study.

	Genotypes and phenotypes	Sources or reference
Strains		
EDL933	Wild-type EHEC O157:H7 EDL933 strain, SmR	Current lab
BL21 (DE3)	Host for expressing recombinant plasmid	TaKaRa
Trans5α	Host for cloning recombinant plasmid	TaKaRa
CC118	Δ(ara-leu) araD ΔlacX74 ga1K phoA20 thi-1 rpsE rpoB argE(Am) recA1, λpir	Current lab
SM10	thi-1 thr leu tonA lacY supE recA::RP4-2-Tc:Mu, KmR, λpir	Current lab
CC118-1	CC118 (pMEG375-*z3276*FRGm), CmR	This work
SM10-1	SM10 (pMEG375-*z3276*FRGm), SmS, GmR, CmR	This work
Δ*z3276*	Stable Δ*z3276* mutant, SmR, GmR, CmS	This work
cΔ*z3276*	Stable CΔ*z3276* mutant, SmR, GmR, ApR	This work
Plasmids		
pMEG375	sacRB mobRP4 oriR6K, CmR, ApR	Current lab
pMEG375-z3276FRGm	pMEG-375:: Δ*z3276*:: Gm, CmR	This work
pFastBac	ApR, GmR	Current lab
pMD19-T	ApR	TaKaRa
pMD19-T-*z3276*	ApR	This work
pCold I	ApR	TaKaRa
pCold I-*z3276*	ApR	This work

injection with the same antigen concentration emulsified 1:1 with Freund's incomplete adjuvant 14 days later and then were bled 10 days after the booster was administered. Sera were stored in −80°C freezer.

For anti-EHEC O157:H7 polyclonal antibody preparation, Jiangsu Academy of Agricultural Sciences Institutional Animal Care and Use Committee approved the animal procedures (SYXK2013-0014) in the context of the guidelines of the Jiangsu Province Animal Regulations (Government Decree No. 45). During feeding and study, health status of mice was monitored twice a day and recorded the clinical signs (ruffled hair coat, hunched posture, and diarrhea). If animals displayed clinical signs of illness, they were euthanized by cervical dislocation. Streptomycin-treated Balb/c mice were used to orally feed 3×10^8 CFU live EHEC O157:H7 strain 86-24 each week for 4 weeks. Mice were bled 2 weeks after the last booster. Sera were stored in a −80°C freezer.

Development Indirect ELISA for Detection Z3276 Expression *in Vivo* and *in Vitro*

For detection Z3276 expression *in vivo*, ELISAs were performed in 96-well plates (Costar, United States) coated with recombinant Z3276 antigen (0.6 μg/mL) and incubated at 4°C overnight. Plates were washed and blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween 20 (PBST). The mouse negative sera and anti-EHEC O157:H7 sera were, respectively, added to antigen-coated wells and incubated at 37°C for 1 h. Sera were removed prior to adding goat anti-mouse IgG-HRP (1/5000 in PBST) (Boster, Wuhan, China) for 45 min at 37°C. The substrate solution TMBS (Sigma) was added into the washed wells. After 5 min, 2 M H_2SO_4 was used to stop the ELISA followed by read absorbance at 450 nm.

For detecting Z3276 expression *in vitro*, the method was the same as above, with some differences: (1) For coating antigen preparation, the EHEC O157:H7 strain 86-24 was cultured in TSB medium for 6 h, and inactivated with 0.3% formaldehyde solution at 37°C for 24 h until no live bacteria remained. The inactivated bacteria were washed two times with 0.01 M PBS (pH = 7.4) and used as antigen for coating plate; (2) the coating concentration was 10^8 CFU/mL; (3) The primary antibody was the recombinant Z3276 protein polyclonal antibody from rabbits.

Construction of Δ*z3276* and the Complemented Strain

The mutant was generated using the suicide plasmid as described previously (Dozois et al., 2003). Briefly, DNA sequences flanking *z3276* were amplified from the chromosomal DNA of EHEC O157:H7 strain EDL933 by PCR with two pairs of specific primers (P1-F/R and P2-F/R) carrying *BamH* I/*EcoR* I and *Sal* I/*EcoR* I restriction enzyme sites, respectively (**Table 2**). A DNA fragment containing the gentamicin resistance-encoding gene cassette was also obtained by PCR amplification from FastBac plasmid with primers (P3-F/R) carrying *EcoR* I restriction enzyme sites (**Table 1**). Gel-purified PCR products were cloned into pMEG375 to construct a suicide plasmid designated pMEG375-*z3276*FRGm, which was then transformed into *E. coli* strain CC118, named CC118-1. The positive plasmid pMEG375-*z3276*FRGm was transformed into a λpir lysogen of strain SM10, named SM10-1. The SM10-1 was hybridized with 86-24 strain on agar plate to produce Δ*z3276* mutant by homologous recombination. The Δ*z3276* was confirmed by PCR screening with *z3276*-specific primers (P4-F/R) (**Table 2**), DNA

TABLE 2 | Primers used in this study.

Primer	Nucleotide sequences (5′–3′)	Restriction sites	PCR product (bp)
P1-F/R	taaggatcctcgggaatttctaag	*BamH* I	343
	ggggaattcgtgaaatacgattaa	*EcoR* I	
P2-F/R	ggggaattcatttctgaacatcat	*EcoR* I	407
	taagtcgacgacggactgacacctta	*Sal* I	
P3-F/R	gaattcaccgtggaaacgcatgaag	*EcoR* I	811
	gaattcacggcttgaacgaattgtt	*EcoR* I	
P4-F/R	tttagtaaaagtgtcgtgtttact	/	898
	aatcgtatttcacgttgattaatg	/	
P5-F/R	atgatgttcagaaatagaatatta	/	1035
	ttaatcgtatttcacgttgattaa	/	
P6-F/R	cgcggatcctttagtaaaagtgtc	*BamH* I	916
	ggcaagcttaatcgtatttcacgtt	*Hind* III	

sequencing and Western blot analysis. The full-length *z3276* gene was amplified by PCR (P5-F/R) from EDL933 genomic DNA to generate a complementation strain cΔ*z3276*. The PCR primers used are listed in **Table 2**. The amplified 1344-bp DNA fragment of the *z3276* gene was inserted into the pMD19-T vector, and the recombinant plasmid was confirmed by DNA sequencing and restriction enzyme digestion. The positive pMD19-T-*z3276* was introduced into the Δ*z3276* strain to overexpress the Z3276 protein for reconfirmation of its function.

Crystal Violet Biofilm Assay

A static biofilm formation assay was performed in 96-well polystyrene plates as previously reported (Lourenço et al., 2013; Eberly et al., 2017). Briefly, overnight cultures were diluted to an OD_{600} of 0.05 in TSB medium and Minca medium, respectively (300 μL) and incubated for 10 h without shaking at 37°C. After the cell density had been measured (turbidity at 630 nm), the plates were washed three times with PBS to remove all planktonic cells, and then 300 μL of 0.1% crystal violet was added to each well. After 20 min at room temperature, the microplate was emptied and washed three times with PBS. Next, 300 μL of 95% ethanol was added to resolve the stained biofilm cells. The total number of biofilm cells (absorbance at 570 nm) was measured and the total biofilm (OD_{570}) was normalized with cell growth (OD_{630}).

Bacterial Adherence to Intestinal and Non-intestinal Cells

Cell adherence assays were performed as previously described with some modifications (Segura and Gottschalk, 2002). Bacteria were centrifuged, washed twice with PBS, and resuspended at 10^7 CFU/mL in RPMI 1640 medium without antibiotics. We employed human laryngeal carcinoma cell line HEp-2 cells and porcine neonatal jejunal epithelial cell line IPEC-J2 cells. Monolayers of these cells lines grown in 24-well plates were infected at a multiplicity of infection of 10 bacteria per cell. The plates were centrifuged at 800 × *g* for 10 min and incubated in RPMI 1640 medium with 2% newborn calf serum for 3 h at 37°C with 5% CO_2. After washing three times with PBS and digesting with a mixture of 0.25% tryptase and 0.02% EDTA, adherent bacteria were plated onto sorbitol MacConkey (SMAC) agar plates to count the CFU. All assays were performed in triplicate and repeated three times. The results were expressed as the adherence rate relative to that of the WT set as 100%.

Adherence Inhibition Assays

Anti-Z3276 polyclonal antiserum, for which the sensitivity and specificity were demonstrated by western blot analysis, was made in our laboratory. Briefly, we constructed a pcold I-*z3276* plasmid that could express abundant Z3276 protein in BL21 (DE3) cells. Purified Z3276 protein was used to immunize rabbits. Prior to adding onto the cell monolayer, 10 μL of the bacterial inoculum were incubated with 1:10, 1:50, and 1:100 dilutions of the anti-Z3276 and pre-immune bacterial suspensions at 37°C with gentle agitation. The number of adherent bacteria per cell in each sample was determined by plating onto SMAC plates at 37°C.

Invasion Assay

The procedure for the invasion assay was the same as that for the adherence assay except the one ceftriaxone (10 μg/mL) and kanamycin (100 μg/mL) were added and incubated with the cells for 2 h to kill any extracellular bacteria before digestion. To confirm that all extracellular bacteria were killed by the antibiotic, 200 μL of the final PBS wash solution was plated on SMAC agar. Invasion frequencies were calculated as the number of bacteria surviving incubation with antibiotics divided by the total number of bacteria present in the absence of the antibiotic. All assays were performed in triplicate and repeated three times. The results were expressed as the invasion rate relative to that of the WT set as 100%.

In Vivo Experiments

We used a streptomycin-treated mouse model to investigate the colonization ability of the strains. BALB/c mice were bought from experimental animal center of Yangzhou University (Yangzhou, China), housed in microisolator cages, provided with food and water *ad libitum*. To the study, the health status of mice was monitored twice a day and recorded the clinical signs (ruffled hair coat, hunched posture, and diarrhea). Thirty 6-week-old female BALB/c mice (n = 30) were divided into three groups and orally challenged, after anesthetized by inhaling diethyl ether, with WT, Δ*z3276*, and cΔ*z3276* strains at a dose of 10^{10} CFU in PBS, respectively. Mice infected with sterile PBS were used as controls. Fecal samples were taken on alternate days to monitor for shedding over 2 weeks.

Twitching Motility Assay

Motility assays were performed as described previously with some modifications (Mattick, 2002). Briefly, 100 μL of an overnight culture was re-inoculated into 5 mL of sterile TSB and incubated at 37°C without shaking until logarithmic phase. One microliter of each bacterial culture was dropped onto semi-solid agar plates, which were incubated for 20 h at 37°C before

analysis. Motility was observed by measuring the diameter of the motility halo. Non-motile strains grew only at the site of inoculation.

Statistical Analysis

Where appropriate, data were expressed as the mean ± SEM. The difference from two groups was analyzed using the Student's *t*-test and GraphPad Prism 5 software.

RESULTS

Phylogeny Analysis of Z2376 Protein

The BLAST analysis indicates that Z3276 amino-acid sequence has high identity between 91 and 100% to fimbrial proteins in GenBank (**Figure 1**), suggesting a conserved fimbrial domain in the Z3276 protein.

Protein Expression and Purification

Recombinant plasmid pCold I-*z3276* was sequenced to indicate that *z3276* has 100% identity to reference sequences (GenBank CP015855.1) using Genscript Biotechnology, Co., Ltd. (Nanjing, China). Recombinant bacteria BL21/pCold I-*z3276* was induced by IPTG. SDS-PAGE showed that recombinant Z3276 (33 kDa) was successfully expressed with 35% proportion to whole bacterial protein in contrast to naïve bacteria.

Development of pAb Against Z3276 and EHEC O157:H7

Anti-Z3276 was successfully prepared from rabbits 229 and 230, and the titers were 2.6×10^6 and 7.9×10^5, respectively, using indirect ELISA. Anti-EHEC O157:H7 was successfully prepared from 80% (4/5) mice, and the highest titer and the lowest titer were 9×10^4 and 3×10^3, respectively.

Indirect ELISA for Detection Z3276 Expression *in Vivo* and *in Vitro*

The ELISA plate coated with Z3276 was used to incubate with anti-EHEC O157:H7 sera and negative sera. The anti-EHEC O157:H7 sera gave an OD450 value greater than 1.0, compared with lower OD450 values from negative sera (**Table 3**). The detection data indicated Z3276 protein could express *in vivo* and perhaps on the bacterial surface.

The ELISA plate coated with the inactivated EHEC O157:H7 was used to incubation with anti-Z3276 sera and negative sera. The anti-EHEC O157:H7 sera gave OD450 value was greater than 1.0, compared with lower OD450 value from negative sera (**Table 4**). The detection data indicated Z3276 protein could express under the experimental conditions.

Construction of a *z3276*-Defective Mutant

The Δ*z3276* mutant was constructed by homologous recombination and was confirmed by PCR (**Figure 2A**)

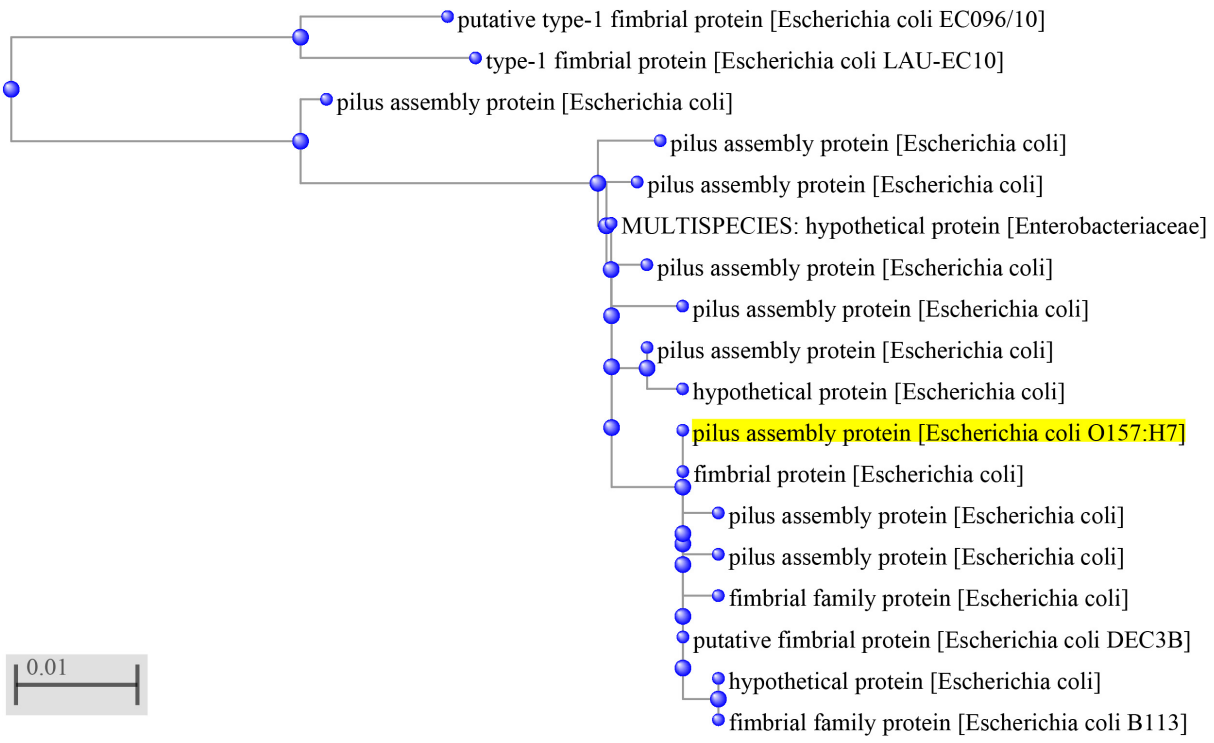


FIGURE 1 | Phylogenetic relationships between Z3276 protein and other fimbrial proteins. The phylogenetic tree was generated by BLAST in GenBank with Z3276 protein (yellow) encoded by the entire *z3276 gene* from EHEC O157:H7 genome. The scale bar represents 0.01 nucleotide substitutions per site.

TABLE 3 | Z3276 expression *in vivo* using indirect ELISA.

	1:200	1:400	1:800	1:1600
Negative mouse sera	0.142	0.117	0.085	0.051
Anti-EHEC O157:H7 sera	1.445	1.201	0.923	0.631

TABLE 4 | Z3276 expression *in vitro* using indirect ELISA.

	1:200	1:400	1:800	1:1600
Negative rabbit sera	0.191	0.138	0.056	0.029
Anti-Z3276 sera	2.282	1.931	1.602	1.242

and Western blot analysis (**Figure 2B**). PCR results confirmed that the Δ*z3276* mutant was negative for *z3276* gene, and no reaction was detected between Δ*z3276* and anti-Z3276 serum by the Western blot assay. In term of growth stability, the Δ*z3276* and cΔ*z3276* strains could be subcultured very well at least for 20 passages.

Z3276 Contributes to Biofilm Formation

To test whether Z3276 was important for biofilm formation by EHEC O157:H7 on abiotic substrata, we compared the abilities of the WT, Δ*z3276*, and cΔ*z3276* strains to form biofilms in 96-well polystyrene plates. The results indicated that Δ*z3276* was reduced in its ability to form biofilms compared with the WT strain and complemented strains (**Figure 3**) regardless of culture media used. Mutant strains lost more abilities to form biofilms when cultured in TSB medium ($P < 0.01$) than in Minca medium ($P < 0.05$). Complemented strains regained stronger biofilm-forming abilities in a greater extent than WT strains had ($P > 0.05$).

Anti-Z3276 Serum Blocked the Adherence of the WT Strain to Both HEp-2 and IPEC-J2 Cells

No significant difference was detected in the adherence ability of the Δ*z3276* mutant compared with the WT strain to both HEp-2 and IPEC-J2 cells ($P > 0.05$, $P > 0.05$, respectively) (**Figure 4A**). However, an obvious reduction was observed in the adherence of the WT strain to HEp-2 and IPEC-J2 cells after incubation with an anti-Z3276 antibody at a 1:2 dilution (antibody titer = 1:12,800) ($P < 0.05$, $P < 0.05$, respectively), and a 1:10 dilution ($P < 0.05$, $P < 0.05$, respectively) (**Figure 4B**), but not at a 1:100 dilution ($P > 0.05$, $P > 0.05$, respectively) (**Figure 4B**).

Expression of Z3276 Could Trigger the Invasion of IPEC-J2 Cells

Invasion of host cells is a crucial step in bacterial pathogenesis. Compared with the WT strain, we found no change in the rate of invasion of HEp-2 cells for the Δ*z3276* mutant ($P > 0.05$), whereas an 81% reduction in invasion of IPEC-J2 cells was observed for the Δ*z3276* mutant ($P < 0.05$) (**Figure 5**). These results indicated that *z3276* may be involved in the pathogenicity of EHEC O157:H7 in specific cell types.

Δ*z3276* Mutant Showed Decreased Colonization in Mice

After treating mice with streptomycin (5 g/L for the first 3 days and then 0.5 g/L) and mitomycin C (2.5 mg/kg) to enhance their sensitivity (Zhang et al., 2011, 2014), we challenged with the WT and Δ*z3276* via oral–gastric inoculation. The results revealed shortened and lower fecal shedding for the Δ*z3276* compared with the WT strain ($P < 0.05$) (**Figure 6**), which indicated that Z3276 protein may be involved in colonization *in vivo*.

Motility of the 86-24 WT and the Δ*z3276* Mutant Strains

Twitching motility is a phenomenon associated with virulence in many Gram-negative bacteria (Mattick, 2002), and is mediated by the retraction and extension of flexible pili by bacteria growing on a semi-solid surface. In this study, both the WT and Δ*z3276* were motile on agar. A 15-mm twitching motility zone on the semi-solid agar surface was observed for the WT, compared with a 50% reduction in the zone for the Δ*z3276* mutant strain ($P < 0.05$) (**Figure 7**).

DISCUSSION

EHEC is a zoonotic pathogen, of which O157:H7 is the most important serotype responsible for a number of outbreaks in

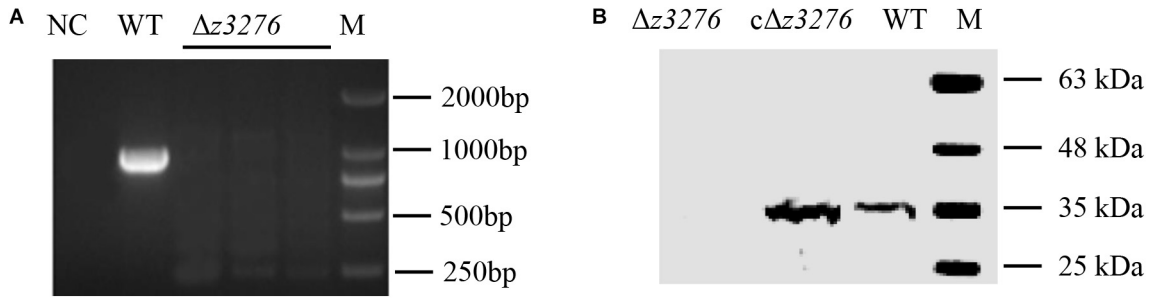


FIGURE 2 | Confirmation of the Δ*z3276* in EHEC O157:H7 strain 86-24. **(A)** Confirmation of the Δ*z3276* by PCR using primers P4-F/R. M, 2000-bp marker. The predicted PCR product size is 898 bp. **(B)** Confirmation of the Δ*z3276* by Western blot analysis. The predicted protein size is 33 kDa.

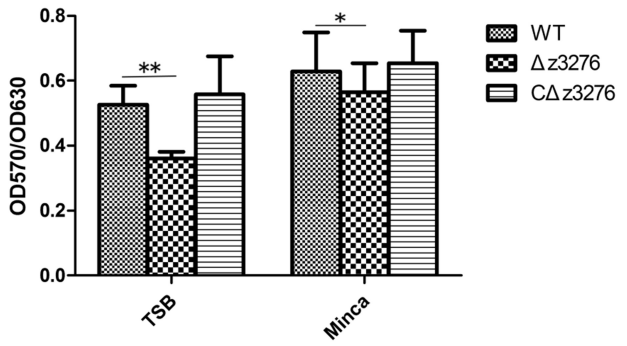


FIGURE 3 | Δ*z3276* decreases biofilm formation in TSB medium and modified Minca medium at 37°C without shaking after 10 h in 96-well plates. Total biofilm formation (OD_{570}) was normalized by bacterial growth (OD_{630}). The experiment was performed in triplicate, and data represent the mean ± SEM of three independent experiments. *Indicates significance at $P < 0.05$; **Indicates extreme significance at $P < 0.01$.

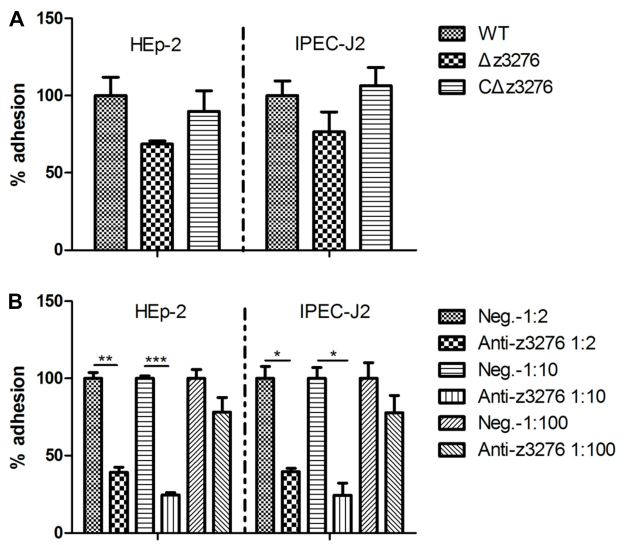


FIGURE 4 | Interaction of the WT and Δ*z3276* with HEp-2 and IPEC-J2 cells. **(A)** The Δ*z3276* mutant showed no change of adherence to either cell line compared with the parental strain. **(B)** Adherence of WT EHEC O157:H7 strain to HEp-2 and IPEC-J2 cells reduced after pre-incubation with blocking antibody (1:2 and 1:10 dilution). The WT strain adhesion index was assumed to be 100%. The results shown are the means + SEM of three independent experiments. *Indicates significance at $P < 0.05$, ** and ***Indicate extreme significance at $P < 0.01$ and $P < 0.001$.

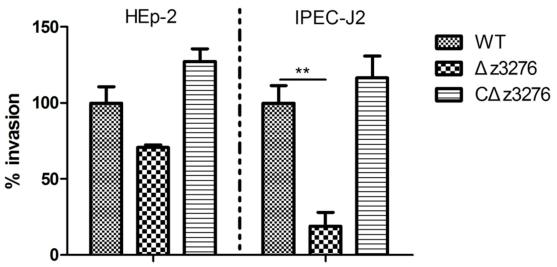


FIGURE 5 | The ability of Δ*z3276* to invade IPEC-J2 cells was decreased compared with the wild-type strain. Ceftriaxone (10 μg/mL) and kanamycin (100 μg/mL) were added to ensure that only intracellular bacteria were obtained. The WT strain adhesion index was assumed to be 100%. The results shown are the means ± SEM of three independent experiments. **Indicates extreme significance at $P < 0.01$.

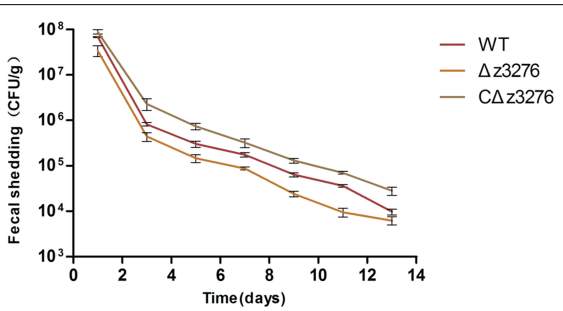


FIGURE 6 | Δ*z3276* showed shortened and lower fecal shedding in mice. Mice were orally challenged with strains WT, Δ*z3276*, cΔ*z3276*, or PBS. Fecal samples were collected on alternate days over a 2-week period. The CFUs in each sample were determined by plating onto SMAC plates at 37°C. The results shown are the averages of five mice per group.

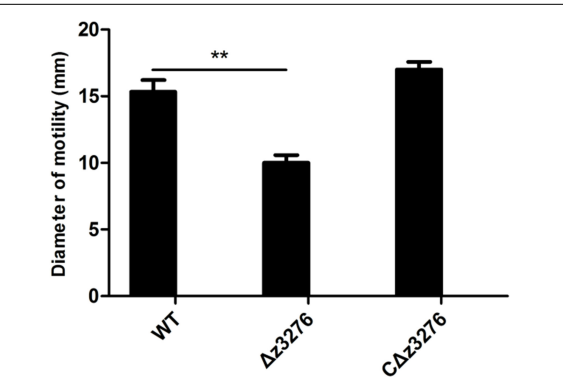


FIGURE 7 | Swimming motility diameter of WT, Δ*z3276,* and cΔ*z3276*. Data represent the means ± SEM of three separate experiments. **Indicates extreme significance at $P < 0.01$.

animals, poultry, and humans worldwide, leading to serious public health concern. The locus of enterocyte Effacement Island, a prophage producing two Shiga toxins and a 60 MDa virulence plasmid pO157 are considered the major determinants of EHEC O157:H7 pathogenesis (Sjogren et al., 1994; Schmedt et al., 1996). EspP was recently recognized as a new member of the serine protease autotransporters of Enterobacteriaceae family and, contributing to increased hemorrhaging into the intestinal tract (Khan et al., 2009) and evasion from immune system-mediated elimination (Orth et al., 2010). In addition to above-mentioned defining virulence factors of EHEC O157:H7, there are other factors that may also contribute to its pathogenicity.

Increasing evidence suggests that fimbrial play an important role in the initial stages of EHEC O157:H7 infections (Xicohtencatl-Cortes et al., 2007). Martinez and coworkers

showed that the tip adhesin Fim H of type 1 fimbrial was sufficient to trigger invasion of uropathogenic *E. coli* into bladder epithelial cells (Martinez et al., 2000). However, the potential role of type 1 fimbrial in the pathogenicity of EHEC O157:H7 has not been reported, due to a nucleotide deletion and mutation in the *fim* operon encoding type 1 fimbrial (Li et al., 1997). Among the complete genome sequences available in current databases, the *z3276* gene was only detected in EHEC O157:H7 (Li and Chen, 2012), and its amino acid sequence showed high homology with other *E. coli* type I fimbrial. Therefore, it is possible that *z3276*-encoding protein is a compensatory mechanism for type I fimbrial.

Biofilms help bacteria colonize inert surfaces, whilst protecting the bacterial cells from the host immune defense system as well as from antibiotic drugs (Gocer et al., 2017; Russell et al., 2018). In this study, an obvious reduction in biofilm formation was observed with a Δ*z3276* mutant compared with the WT strain in modified Minca medium and TSB, but no change was detected in Mueller–Hinton broth (MHB), brain heart infusion broth (BHI) or LB broth, suggesting that Z3276 production *in vivo* may be modulated by the composition of the medium and other conditions. *Candida albicans* biofilms are highly resistant to the actions of clinically important antifungal agents due to major multidrug efflux pumps encoded by *C. albicans* and the important role of biofilms in the drug resistance of planktonic cells (Cao et al., 2005). Moreover, *E. coli* biofilm bacteria showed weaker inflammatory responses and enhanced resistance to some antimicrobial peptides, and the increased *in vivo* survival of biofilm bacteria in a clinically relevant model of catheter infection has been reported (Lloyd et al., 2012; Chalabaev et al., 2014). Therefore, we can speculate that the *z3276* gene in EHEC may confer the bacterium with resistance against antibiotics, thereby increasing its survival time in the host or the environment, which is critical to the transmission and infection.

Adherence is the first step in bacterial invasion. In the present study, we explored whether Z3276 plays a role in the adherence between the bacteria and cultured intestinal (IPEC-J2 cells) and non-intestinal (HEp-2 cells) epithelial cells. Qualitative analysis from IPEC-J2 cells infected with Δ*z3276* and WT showed no significant difference in the levels of adherence, with values of 1.2×10^5 CFU/mL and 1.6×10^5 CFU/mL, respectively. The same result was observed with HEp-2 cells. One possibility is that HEp-2 cells and IPEC-J2 cells do not possess a suitable receptor to interact with *z3276*-encoding protein, however, *z3276*-encoding protein may contribute to the adherence to other cell types or to abiotic surfaces. For instance, the Δ*z3276* and its parental strain showed similar levels of invasion of HEp-2 cells. In contrast, the Δ*z3276* mutant showed 81% decrease in invasion ability to IPEC-J2 cells compared with the WT strain. Thus, we speculate that there might be certain moieties that can recognize Z3276 specifically and "carry" the bacterium into cells.

A mouse model was used to evaluate the colonization ability of the Δ*z3276* and the WT strains *in vivo*. The data of the animal study demonstrated that the Δ*z3276*-infected mice rendered the animals lower bacterial counts and higher clearance efficiency, indicating that the ability of Δ*z3276* mutant to colonize the host and thereby survive in the host was impaired. This may be partially due to the reduced capacity to invade epithelial cells. The z3276 gene in EHEC may encode a fimbrial protein, or termed as tip adhesin (in the distal end of the pili), which has been previously reported to mediate the specific attachment to tissues or surfaces (Lindberg et al., 1987; Kaper et al., 2004; Low et al., 2006; Gu, 2017).

Besides as a colonization factor, Z3276 was also shown here to be involved in the twitching motility of EHEC O157:H7. This property may contribute to the pathogenicity of EHEC O157:H7, as it has been demonstrated that flagellum/fimbrial-mediated motility is essential for enhancing pathogen–host interactions and for promoting the subsequent adherence and colonization of several other Gram-negative pathogens (La Ragione et al., 2000; Wright et al., 2007; Mahajan et al., 2009).

In summary, this study confirmed that the *z3276* gene in EHEC O157:H7 encodes multifunctional structures with properties that may contribute to host colonization and bacterial survival in the environment.

AUTHOR CONTRIBUTIONS

XZ and KH conceived and designed the experiments. BZ and XS performed the experiments. HF analyzed the data. BZ and XZ wrote the paper.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant No. 31572503), the Jiangsu Key Research and Development Plan (Grant No. BE2017341-1), and the National Key Research and Development Program of China (Grant No. 2018YFD0500500).

REFERENCES

Cao, Y. Y., Cao, Y. B., Xu, Z., Ying, K., Li, Y., Xie, Y., et al. (2005). cDNA microarray nalysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrob. Agents Chemother.* 49, 584–589. doi: 10.1128/AAC.49.2.584-589.2005

Chalabaev, S., Chauhan, A., Novikov, A., Iyer, P., Szczesny, M., Beloin, C., et al. (2014). Biofilms formed by gram-negative bacteria undergo increased lipid a palmitoylation, enhancing in vivo survival. *mBio* 5:e1116-14. doi: 10.1128/mBio.01116-14

Dozois, C. M., Daigle, F., and Curtiss, R. (2003). Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. *Proc. Natl. Acid. Sci. U.S.A.* 100, 247–252. doi: 10.1128/mBio.01116-14

Eberly, A. R., Floyd, K. A., Beebout, C. J., Colling, S. J., Fitzgerald, M. J., Stratton, C. W., et al. (2017). Biofilm formation by uropathogenic *Escherichia coli* is favored under oxygen conditions that mimic the bladder environment. *Int. J. Mol. Sci.* 18:E2077. doi: 10.3390/ijms18102077

Epler Barbercheck, C. R., Bullitt, E., and Andersson, M. (2018). Bacterial adhesion pili. *Subcell. Biochem.* 87, 1–18. doi: 10.1007/978-981-10-7757-91

Gocer, H., Emir, D., Onger, M. E., and Dabak, N. (2017). Effects of bone cement loaded with teicoplanin, N-acetylcysteine or their combination on Staphylococcus aureus biofilm formation: an in vitro study. *Eklem Hastalik. Cerrahisi* 28, 13–18. doi: 10.5606/ehc.2017.52507

Gu, H. (2017). Role of flagella in the pathogenesis of *Helicobacter pylori*. *Curr. Microbiol.* 74, 863–869. doi: 10.1007/s00284-017-1256-4

Kaper, J. B., Nataro, J. P., and Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2, 123–140. doi: 10.1038/nrmicro818

Khan, A. B., Naim, A., Orth, D., Grif, K., Mohsin, M., Prager, R., et al. (2009). Serine protease espP subtype alpha, but not beta or gamma, of Shiga toxin-producing *Escherichia coli* is associated with highly pathogenic serogroups. *Int. J. Med. Microbiol.* 299, 247–254. doi: 10.1016/j.ijmm.2008.08.006

La Ragione, R. M., Sayers, A. R., and Woodward, M. J. (2000). The role of fimbrial and flagella in the colonization, invasion and persistence of *Escherichia coli* O78:K80 in the day-old-chick model. *Epidemiol. Infect.* 124, 351–363. doi: 10.1017/S0950268899004045

Ledesma, M. A., Ochoa, S. A., Cruz, A., Rocha-Ramirez, L. M., Mas-Oliva, J., Eslava, C. A., et al. (2010). The hemorrhagic coli pilus (HCP) of *Escherichia coli* O157:H7 is an inducer of proinflammatory cytokine secretion in intestinal epithelial cells. *PLoS One* 5:e12127. doi: 10.1371/journal.pone.0012127

Li, B., and Chen, J. Q. (2012). Real-time PCR methodology for selective detection of viable *Escherichia coli* O157:H7 cells by targeting Z3276 as a genetic marker. *Appl. Environ. Microbiol.* 78, 5297–5304. doi: 10.1128/AEM.00794-12

Li, B., Liu, H., and Wang, W. (2017). Multiplex real-time PCR assay for detection of *Escherichia coli* O157:H7 and screening for non-O157 Shiga toxin-producing E. *coli*. *BMC Microbiol.* 17:215. doi: 10.1186/s12866-017-1123-1122

Li, B. G., Koch, W. H., and Cebula, T. A. (1997). Detection and characterization of the fimA gene of *Escherichia coli* O157:H7. *Mol. Cell. Probes* 11, 397–406. doi: 10.1006/mcpr.1997.0132

Lindberg, F., Lund, B., Johansson, L., and Normark, S. (1987). Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. *Nature* 328, 84–87. doi: 10.1038/328084a0

Lloyd, S. J., Ritchie, J. M., and Torres, A. G. (2012). Fimbriation and curliation in *Escherichia coli* O157:H7: a paradigm of intestinal and environmental colonization. *Gut Microbes* 3, 272–276. doi: 10.4161/gmic.20661

Lourenço, A., de Las Heras, A., Scortti, M., Vazquez-Boland, J., Frank, J. F., and Brito, L. (2013). Comparison of *Listeria monocytogenes* Exoproteomes from biofilm and planktonic state: Lmo2504, a protein associated with biofilms. *Appl. Environ. Microbiol.* 79, 6075–6082. doi: 10.1128/AEM.01592-13

Low, A. S., Holden, N., Rosser, T., Roe, A. J., Constantinidou, C., Hobman, J. L., et al. (2006). Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic *Escherichia coli* O157:H7. *Environ. Microbiol.* 8, 1033–1047. doi: 10.1111/j.1462-2920.2006.00995.x

Mahajan, A., Currie, C. G., Mackie, S., Tree, J., McAteer, S., McKendrick, I., et al. (2009). An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157:H7 with bovine intestinal epithelium. *Cell. Microbiol.* 11, 121–137. doi: 10.1111/j.1462-5822.2008.01244.x

Makvana, S., and Krilov, L. R. (2015). *Escherichia coli* infections. *Pediatr. Rev.* 36, 167–170. doi: 10.1542/pir.36-4-167

Martinez, J. J., Mulvey, M. A., Schilling, J. D., Pinkner, J. S., and Hultgren, S. J. (2000). Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *EMBO J.* 19, 2803–2812. doi: 10.1093/emboj/19.12.2803

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu. Rev. Microbiol.* 56, 289–314. doi: 10.1146/annurev.micro.56.012302.160938

Mazariego-Espinosa, K., Cruz, A., Ledesma, M. A., Ochoa, S. A., and Xicohtencatl-Cortes, J. (2010). Longus, a type IV pilus of enterotoxigenic *Escherichia coli*, is involved in adherence to intestinal epithelial cells. *J. Bacteriol.* 192, 2791–2800. doi: 10.1128/JB.01595-09

Orth, D., Ehrlenbach, S., Brockmeyer, J., Khan, A. B., Huber, G., Karch, H., et al. (2010). EspP, a serine protease of enterohemorrhagic *Escherichia coli*, impairs complement activation by cleaving complement factors C3/C3b and C5. *Infect. Immun.* 78, 4294–4301. doi: 10.1128/IAI.00488-10

Perna, N. T., Plunkett, G. III, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., et al. (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533. doi: 10.1038/35054089

Ravan, H., and Amandadi, M. (2015). Analysis of yeh fimbrial gene cluster in *Escherichia coli* O157:H7 in Order to find a genetic marker for this serotype. *Curr. Microbiol.* 71, 274–282. doi: 10.1007/s00284-015-0842-6

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., et al. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* 308, 681–685. doi: 10.1056/NEJM198303243081203

Russell, C. W., Fleming, B. A., Jost, C. A., Tran, A., Stenquist, A. T., Wambaugh, M. A., et al. (2018). Context-Dependent requirements for FimH and other canonical virulence factors in gut colonization by extraintestinal pathogenic *Escherichia coli*. *Infect. Immun.* 86, e746–e717. doi: 10.1128/IAI.00746-17

Schmedt, H., Kernnach, C., and Karch, H. (1996). Analyysis of the EHEC hly operon and its location in the physical map of the large plasmid of enteroheamorrhagic *Escherichia coli* O157:H7. *Microbiology* 142, 907–914. doi: 10.1099/00221287-142-4-907

Segura, M., and Gottschalk, M. (2002). *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. *Infect. Immun.* 70, 4312–4322. doi: 10.1128/IAI.70.8.4312-4322.2002

Sjogren, R., Neill, R., Rachmilewitz, D., Fritz, D., Newland, J., Sharpnack, D., et al. (1994). Role of shiga-like toxin I in bacterial enteritis: comparison between isogenic *Escherichia coli* strains induced in rabbits. *Gastro* 106, 306–317. doi: 10.1016/0016-5085(94)90587-8

Tarr, P. I., Gordon, C. A., and Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 365, 1073–1086. doi: 10.1016/S0140-6736(05)71144-2

Wright, K. J., Seed, P. C., and Hultgren, S. J. (2007). Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. *Cell. Microbiol.* 9, 2230–2241. doi: 10.1111/j.1462-5822.2007.00952.x

Xicohtencatl-Cortes, J., Monteiro-Neto, V., Ledesma, M. A., Jordan, D. M., Francetic, O., Kaper, J. B., et al. (2007). Intestinal adherence associated with type IV pili of enterohemorrhagic *Escherichia coli* O157:H7. *J. Clin. Invest.* 117, 3519–3529. doi: 10.1172/JCI30727

Zhang, X., Yu, Z., Zhang, S., and He, K. (2014). Immunization with H7-HCP-tir-intimin significantly reduces colonization and shedding of *Escherichia coli* O157:H7 in goats. *PLoS One* 9:e91632. doi: 10.1371/journal.pone.0091632

Zhang, X. H., He, K. W., Zhang, S. X., Lu, W. C., Zhao, P. D., Luan, X. T., et al. (2011). Subcutaneous and intranasal immunization with Stx2B-Tir-Stx1B-Zot reduces colonization and shedding of *Escherichia coli* O157:H7 in mice. *Vaccine* 29, 3923–3929. doi: 10.1016/j.vaccine.2011.02.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Zhang, Sun, Fan, He and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 July 2018
doi: 10.3389/fmicb.2018.01511

Combinational Inhibitory Action of *Hedychium spicatum* L. Essential Oil and γ-Radiation on Growth Rate and Mycotoxins Content of *Fusarium graminearum* in Maize: Response Surface Methodology

Naveen K. Kalagatur[1], Jalarama R. Kamasani[2], Chandranayaka Siddaiah[3], Vijai K. Gupta[4], Kadirvelu Krishna[5] and Venkataramana Mudili[5]**

[1] Food Microbiology Division, Defence Food Research Laboratory, Mysuru, India, [2] Freeze Drying and Processing Technology Division, Defence Food Research Laboratory, Mysuru, India, [3] Department of Biotechnology, University of Mysore, Mysuru, India, [4] Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia, [5] DRDO-BU-Centre for Life Sciences, Coimbatore, India

OPEN ACCESS

Edited by:
Pierina Visciano,
Università di Teramo, Italy

Reviewed by:
Sunil D. Saroj,
Symbiosis International University, India
Vivek K. Bajpai,
Dongguk University Seoul, South Korea
Katarzyna Anna Marchwińska,
Poznań University of Economics, Poland

***Correspondence:**
Naveen K. Kalagatur
knaveenkumar.kalagatur@yahoo.co.in
Venkataramana Mudili
ramana.micro@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 14 September 2017
Accepted: 18 June 2018
Published: 31 July 2018

Citation:
Kalagatur NK, Kamasani JR, Siddaiah C, Gupta VK, Krishna K and Mudili V (2018) Combinational Inhibitory Action of Hedychium spicatum L. Essential Oil and γ-Radiation on Growth Rate and Mycotoxins Content of Fusarium graminearum in Maize: Response Surface Methodology. Front. Microbiol. 9:1511. doi: 10.3389/fmicb.2018.01511

Nowadays, contamination of agricultural commodities with fungi and their mycotoxins is one of the most annoying with regard to food safety and pose serious health risk. Therefore, there is a requisite to propose suitable mitigation strategies to reduce the contamination of fungi and mycotoxins in agricultural commodities. In the present study, combinational inhibitory effect of *Hedychium spicatum* L. essential oil (HSEO) and radiation was established on growth rate, production of deoxynivalenol (DON) and zearalenone (ZEA) by *Fusarium graminearum* in maize grains. The HSEO was obtained from rhizomes by hydrodistillation technique and chemical composition was revealed by GC-MS analysis. A total of 48 compounds were identified and major compounds were 1,8-cineole (23.15%), linalool (12.82%), and β-pinene (10.06%). The discrete treatments of HSEO and radiation were effective in reducing the fungal growth rate and mycotoxins content, and the complete reduction was noticed at 3.15 mg/g of HSEO and 6 kGy of radiation. Response surface methodology (RSM) was applied to evaluate the combinational inhibitory effect of HSEO and radiation treatments on fungal growth rate and mycotoxins content. A total of 13 experiments were designed with distinct doses of HSEO and radiation by central composite design (CCD) of Stat-Ease Design-Expert software. In combinational approach, complete reductions of fungal growth, DON, and ZEA content were noticed at 1.89 mg/g of HSEO and 4.12 kGy of radiation treatments. The optimized design concluded that combinational treatments of HSEO and radiation were much more effective in reducing the fungal growth and mycotoxins content compared to their discrete treatments ($p < 0.05$). Responses of the design were assessed by second-order polynomial regression analysis and found that quadratic model was well fitted. The optimized design has larger F-value and adequate precision, smaller p-value, decent regression coefficients (R^2) and found statistically significant ($p < 0.05$). In addition, correlation matrix, normal plot residuals, Box-Cox, and actual

vs. predicted plots were endorsed that optimized design was accurate and appropriate. The proposed combinational decontamination technique could be highly applicable in agriculture and food industry to safeguard the food and feed products from fungi and mycotoxins.

Keywords: mycotoxins, *Fusarium graminearum*, *Hedychium spicatum*, deoxynivalenol, zearalenone, essential oil, γ-radiation, response surface methodology

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by filamentous fungi on agricultural products, which cause acute or chronic toxic effects in farm animals and humans called mycotoxicosis (Schirone et al., 2016; Du et al., 2017). The contamination of agricultural products with fungi occurs during pre-and post-harvesting stages due to inappropriate and unhygienic practices (Bernhoft et al., 2012). The Food and Agricultural Organization (FAO) of the United Nations estimate that ∼25% of agricultural products are contaminated with fungi and mycotoxins worldwide (Pitt and Hockings, 2009). The fungal infestation brings intolerable alterations in appearance, color, texture, flavor, and nutrition of food (Pitt and Hockings, 2009). Therefore, the incidence of fungi and mycotoxins contamination in agricultural products have become one of the foremost issues of farmers, food industry, and government concerning the food safety (Annunziata et al., 2017; Udomkun et al., 2017).

Among the mycotoxigenic fungi, *Fusarium graminearum* has received a wide attention because of its ability to produce a variety of mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA) under diverse climate conditions (Pasquali et al., 2016). Several studies have addressed the toxic effects of DON and ZEA in *in-vitro* and *in-vivo* experiments and documented their genotoxicity, hepatotoxicity, neurotoxicity, immunotoxicity, nephrotoxicity, reproductive, and developmental toxicity, carcinogenicity, etc. (Zinedine et al., 2007; Venkataramana et al., 2014; Schumann et al., 2016; Kalagatur et al., 2017; Gonçalves et al., 2018; Muthulakshmi et al., 2018; Reddy et al., 2018). Moreover, International Agency for Research on Cancer (IARC) has studied the carcinogenic property of DON and ZEA in laboratory animals and classified as the group 3 carcinogens (IARC, 1999).

The DON and ZEA have been detected in wide range of agricultural commodities, such as barley, corn, corn silage, hay, oats, rice, sesame seed, sorghum, and wheat (CAST, 2003; Zinedine et al., 2007). Particularly, Fusarium head blight (FHB) in wheat and barley, and Gibberella ear rot in maize caused by *F. graminearum* is a devastating plant disease of temperate regions and results in yield loss and mycotoxins contamination (Wilson et al., 2018). Most lately, Xu et al. (2018) from North China Plain have detected as high as 95.7% of maize germ contamination with DON and measured average attendance of DON in processed products of maize germs as 163.7–1175.2 μg/kg. In Hungary, Tima et al. (2018) have surveyed the occurrence of DON in maize, wheat and its by-products during the period 2008–2015 and noticed overall mean of 2,159 ± 2,818 μg/kg, which was annoyingly much higher than maximum allowed limit. Besides, Mallmann et al. (2017) have summarized the occurrence of DON and ZEA in barley and wheat grains of Southern Brazil during the middle of 2008–2015 and noticed 67% of DON and 41% of ZEA contamination. Similarly, Tralamazza et al. (2016) have observed 99% of DON and 84% of ZEA contamination in wheat samples originated from Brazil. Also, Ji et al. (2014) have detected 74.4% of wheat contaminated with DON in the FHB epidemic region of Jiangsu province, China. In our previous study (Mudili et al., 2014) 72–94 μg/kg of DON in freshly harvested maize grains from Andhra Pradesh, Karnataka, and Tamil Nadu states of Southern India was noticed. Furthermore, Mishra et al. (2013) have also measured the exposure and risk assessment of DON in the Indian individuals, and DON was identified in 30% of cereal samples and in that 7% of samples were surpassed the FSSR (Food Safety and Standard Regulation, India) limit of 1 mg/kg. In this context, *F. graminearum* and its toxins, DON, and ZEA have a great threat to the agricultural and food industry, especially in warm, and humid climate country like India (Ramana et al., 2011; Mishra et al., 2013; Divakara et al., 2014; Mudili et al., 2014; Aiyaz et al., 2016). In this scenario, there is a need to develop safe strategies to combat the problems of mycotoxigenic *F. graminearum* as well as DON and ZEA contamination in agricultural products.

Since last decade, agriculture and food industry have applied various decontamination practices to safeguard the agricultural products from fungi and mycotoxins (Zinedine et al., 2007; Kanapitsas et al., 2016; Kalagatur et al., 2018a,b). Unfortunately, some methods were undesirable, and only few of them were acceptable by WHO, FAO, EU, JECFA, and other regulatory agencies with some constraints. The application of synthetic fungicidal agents could drive the drug-resistant fungi, environmental pollution, health risk in animals and humans, and its use in food has been not passable and restricted worldwide (Kretschmer et al., 2009). Henceforth, there is a huge demand for bio-fungicides as an alternative to synthetic fungicidal agents (Akocak et al., 2015; George et al., 2016; Iram et al., 2016; Kumar et al., 2016; Sellamani et al., 2016; Kalagatur et al., 2017; Muniyandi et al., 2017). On the other hand, chemical decontamination methods bring unacceptable changes in the nutritional, sensory, and functional qualities of food and produce adverse toxic residues (Pitt and Hockings, 2009). The physical decontamination methods are most potent due to its spontaneous and strong effects. Particularly, radiation treatment is substantially efficient decontamination technique. The WHO and FDA of the United Nations have recognized that

under well-established Good Manufacturing Practices (GMP) the application of γ-radiation in a low dose for disinfestations and enhancement of shelf life of agricultural commodities is nutritionally adequate and acceptable (Calado et al., 2014). Thus, a combination of biological and physical decontamination methods is likely to be a safe and much effective in controlling the fungal growth and mycotoxins content.

To the best of our knowledge, the combinational inhibitory action of essential oil and γ-radiation on the growth rate, production of DON and ZEA by *F. graminearum* has been not reported earlier. Therefore, the present study was aimed to establish the combinational inhibitory effect of *Hedychium spicatum* L. essential oil (HSEO) and radiation for the aforementioned purposes. The *H. spicatum* is a hardy and small perennial plant that belong to family Zingiberaceae and popularly known as "perfume ginger" or "spiked ginger lily" (Joshi et al., 2008). The HSEO is a rich source of diverse medicinal compounds, and it is widely used in herbal medicine to treat a variety of ailments, including nausea, stomachache, dysentery, local inflammations, asthma, bad breath, bronchitis, and rheumatic problems (Koul et al., 2005). The HSEO was obtained from rhizomes by hydrodistillation technique and its chemical profile was revealed by GC-MS analysis. The discrete inhibitory action of HSEO and radiation on the growth rate, production of DON, and ZEA by *F. graminearum* was evaluated in maize grains. Their combinational inhibitory action on aforementioned purposes was evaluated by response surface model adopting central composite design (CCD) and obtained model was validated experimentally.

MATERIALS AND METHODS

Chemicals and Reagents

Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), and peptone were obtained from HiMedia (Mumbai, India). Certified standards of DON and ZEA were obtained from Sigma-Aldrich (Bengaluru, India). Immunoaffinity columns specific for DON and ZEA were procured from Vicam (Waters business, USA). The other chemicals used in the study were analytical grade and obtained from Merck Millipore (Bengaluru, India).

Fungi Cultural Conditions

Fusarium graminearum (MTCC 1893) capable for the synthesis of DON and ZEA was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), India, and grown on SDA for 7 days at 28°C. The fungal spores were collected by soft scrape in sterile peptone solution supplemented with 0.01% Tween 80. The spore number was counted by hemocytometer and their number was set to 1×10^6 spores per mL.

Collection and Characterization of *H. spicatum* L. Essential Oil

Plant Material Collection and Essential Oil Extraction

The rhizomes of *H. spicatum* were obtained from agricultural field, Ooty, Tamil Nadu, India. Plants were dried under shade for 2 weeks at 27 ± 2°C and ground to a fine powder (voucher no: PEO 37). The essential oil was extracted from 250 g of fine powder by hydrodistillation technique using Clevenger-type apparatus as per the procedure of European Pharmacopoeia (Council of Europe, 1997). The collected essential oil was dried over anhydrous sodium sulfate to get rid of moisture and safeguarded in an amber glass vial at 4°C for further analysis.

Chemical Characterization of HSEO by GC-MS

The chemical composition of HSEO was revealed by GC–MS analysis using PerkinElmer Clarus 600 C (PerkinElmer, Inc., Waltham, USA) equipped with DB-5MS column (30 m × 0.25 mm; 0.25 μm film thickness) as per our previous reported methodology (Kalagatur et al., 2015). The chemical constituents of HSEO were identified by comparing their mass spectra (MS) with NIST/Wiley library and retention indices (RI) literature of Adams (2007). The quantification (%) of chemical constituents was obtained from the GC peak areas devoid of correction factors.

Radiation Treatment

The radiation treatment was carried out in Gamma radiation chamber-5000 (Freeze Drying and Processing Technology Division, Defense Food Research Laboratory, Mysuru, India) and cobalt 60 was used as a source of γ-radiation with an effective dose frequency of 5.57 kGy per h at 35°C. The absorbed radiation dose was determined by a Ceric-cerous dosimeter that was fixed to the surface of the bottom and top of the test sample. The equivalence of radiation dose was stated as D_{max}/D_{min} and it was 1.01 (Reddy et al., 2015).

Assessment of Discrete and Combinational Inhibitory Effects of HSEO and Radiation Treatments on Growth Rate, Production of DON and ZEA by *F. graminearum* in Maize

Experimental Design

The decisive aim of the present study was to evaluate the discrete and combinational inhibitory effects of HSEO and radiation treatments on the growth rate, production of DON and ZEA by *F. graminearum* in maize grains. The maize grains were obtained from the local agricultural market of Mysuru, Karnataka state, India, and 100 g was packed in plastic bags. The bags were autoclaved at 121°C for 20 min and grains were dried out in a hot air oven at 60°C. The autoclaving process used for removal of background microbial flora could affect the texture of the grains and which could have an impact on the results presented in this study.

The fungal spore suspension of 10 μL (1×10^6 spores per mL) was diluted to 1 mL with sterile peptone water solution and aseptically inoculated to the grain samples and mixed for 15 min at 140–160 rpm in a rotary shaker (Aziz et al., 2007). Following, grain samples were separately exposed to different doses of HSEO and radiation treatments to evaluate their discrete inhibitory effect. The HSEO was directly applied to maize grains and thoroughly vortexed and mixed for 15 min at 140–160 rpm in a rotary shaker as per methodology of Velluti et al. (2003). The combinational exposure of HSEO and radiation

treatments with distinctive doses was accomplished by CCD of the response surface methodology (RSM) statistical program adopting State–Ease Design-Expert version 10.0.6.0 software application (Anderson and Whitcomb, 2016). In this study, grain samples initially treated with a distinctive concentration of HSEO were exposed to different doses of radiation treatment as per CCD. The grain samples were incubated for 14 days at water activity of 0.70 and temperature of 28°C. The sample unexposed with HSEO and radiation treatments were referred as a control. Following the incubation period, the growth of fungi, quantification of DON, and ZEA were determined by colony forming units (CFU) and UHPLC, respectively.

The combinational inhibitory effect of HSEO and radiation (independent factors) on the reductions of CFU, DON, and ZEA content (response factors) was assessed by polynomial regression analysis of RSM. The lower and higher range of HSEO (0–3.15 mg/g) and radiation treatments (0–6 kGy) was fixed based on the dose required for complete inhibition of fungal growth and mycotoxins as per the study of their discrete exposure. The coded factors, name, units, range, levels, mean, and standard deviation of independent variables were shown in Supplementary Table 1. The order of experiments within the block was randomized and executed independently for six times. The regression analysis of attained responses i.e. log CFU, DON, and ZEA were assessed by fitting with suitable models represented by the second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i \neq j=1}^{n} \beta_{ii} x_i x_{ij}$$

where, "0" was the value of the fitted response at the center point of the design; i, ii, and ij were the linear, quadratic and cross-product (interaction effect) regression terms, respectively and "n" denoted the number of independent variables.

Determination of Fungal Growth by Dilution Plating Technique

The fungal growth in maize grains was determined as per methodology of Aziz et al. (2007) with minor modifications. Following the incubation period, 10 g of maize grains were collected from the plastic bags and suspended in 90 mL of sterile peptone water and mixed for 15 min at 140 – 160 rpm in a rotary shaker. The decimal dilutions were prepared in sterile peptone water and spread plated on SDA plates and incubated at 28°C for 3 days. The fungal growth was determined in colony forming units (CFU) and results were expressed in log CFU/g.

Quantification of DON and ZEA by UHPLC

Following the incubation period, 25 g maize grains were collected from the test samples and ground to a fine powder under aseptic conditions and suspended in 250 mL solution of acetonitrile and water (v/v, 6:4). The blend was mixed for 15 min at 140–160 rpm and the supernatant was collected by centrifugation at 6000 rpm for 5 min. The supernatant was subjected to clean-up with immunoaffinity columns of DON and ZEA in according to the instructions of the manufacturer (Vicam, Waters business, USA). The quantification DON and ZEA were done as per our previous reported methodology (Mudili et al., 2014; Kalagatur et al., 2015) using UHPLC system (Nexera, Shimadzu, Japan). The quantification of DON and ZEA was deducted from their respective standard calibration curve and concentration was expressed in μg/g.

Statistical Analysis

The experiments were performed in six independent replicates. The statistical analysis was done according to the one-way ANOVA and significant differences were determined by Tukey's post hoc multiple comparison test and value of $p < 0.05$ was considered significant. The statistical analysis and graphical illustrations were attained adopting the software program GraphPad Prism trial version 7 (GraphPad Software, Inc., USA). The statistical analysis for the optimization of RSM was done by CCD following the State–Ease Design-Expert trial version 10 software program (Stat-Ease, Inc., Minneapolis, USA). The responses of the polynomial regression at a significance level of $p < 0.05$ were considered to design statistical model (Anderson and Whitcomb, 2016). The accuracy of the model was evaluated by measuring the coefficient of determination (R^2) and lack of fit.

RESULTS AND DISCUSSION

Chemical Composition of HSEO

In the present study, the chemical composition of HSEO was revealed by GC-MS and a total of 48 compounds were identified constituting to 96.84% of total weight (**Table 1**). The major compounds were 1,8-cineole (23.15%), linalool (12.82%), β-pinene (10.06%), γ-terpinene (8.16%), terpinolene (5.04%), α-terpinene (3.81%), and α-terpineol (3.35%). In our study, β-eudesmol, furanoid, β-himachalene, hedycaryol, eremoligenol, agarospirol, 8-epi-β-bisabolol, and α-cadinol were not detected in accordance with the previous reports of Joshi et al. (2008) and Sabulal et al. (2007). While, diverse compounds, such as terpinolene, α-terpinyl acetate, γ-eudesmol, and γ-muurolene were identified in our study. The chemical compounds and their concentration of essential oil depend on the genetics of plant, part of the plant used, geographical origin, nutrients, harvesting time, and analytical method employed (Gobbo-Neto and Lopes, 2007). Therefore, in our study chemical compounds and their concentration values were varied in comparison to the previous reports.

Inhibitory Effect of HSEO and Radiation Treatments on Growth Rate, Production of DON and ZEA by *F. graminearum* in Maize

Discrete Treatment of HSEO and Radiation

In the present study, discrete treatment of HSEO and radiation were effective in reducing the fungal growth (log CFU), production of DON and ZEA by *F. graminearum* in maize grains. A quantity of 5.79 ± 0.33 of log CFU/g, 6.24 ± 0.37 of DON (μg/g), and 8.67 ± 0.45 of ZEA (μg/g) were determined in the control sample. While, log CFU, DON, and ZEA content were reduced in test samples in a dose-dependent way with treatment of HSEO and radiation (**Tables 2**, **3**). The fungal growth, DON, and ZEA content were not detected at 3.15 mg/g

TABLE 1 | Chemical composition of *Hedychium spicatum* L. essential oil determined by GC-MS analysis.

S. No	Compound	RI*	RI#	Composition (%)
1	n-Nonane	903	900	0.18
2	Tricyclene	922	921	0.62
3	α-Thujene	925	924	0.05
4	α-Pinene	935	932	1.31
5	Camphene	948	946	0.19
6	Sabinene	970	969	0.34
7	β-Pinene	976	974	10.06
8	β-Myrcene	989	988	0.89
9	α-Phellandrene	1005	1002	2.27
10	α-Terpinene	1017	1014	3.81
11	p-Cymene	1022	1020	0.22
12	Limonene	1025	1024	2.29
13	1,8-Cineole	1028	1026	23.15
14	(E)-β-Ocimene	1044	1044	1.53
15	γ-Terpinene	1057	1054	8.16
16	Terpinolene	1091	1086	5.04
17	Linalool	1098	1095	12.82
18	n-Nonanal	1104	1100	0.22
19	Camphor	1144	1141	0.73
20	δ-Terpineol	1163	1162	2.89
21	Terpinen-4-ol	1175	1174	2.06
22	α-Terpineol	1188	1186	3.35
23	trans-Carveol	1218	1215	0.02
24	p-Cymen-7-ol	1291	1289	0.85
25	δ-Elemene	1337	1335	0.02
26	α-Terpinyl acetate	1348	1346	2.91
27	β-Elemene	1390	1389	0.87
28	Caryophyllene	1418	1417	0.16
29	Santalene	1450	1447	0.90
30	α-Humulene	1453	1452	0.34
31	γ-Muurolene	1481	1478	0.71
32	Germacrene-D	1486	1484	0.28
33	δ-Selinene	1495	1492	0.73
34	α-Muurolene	1502	1500	0.92
35	Germacrene A	1510	1508	0.06
36	γ-Cadinene	1513	1513	0.14
37	Cubebol	1515	1514	0.19
38	δ-Cadinene	1527	1522	0.07
39	cis-Sesquisabinene hydrate	1543	1542	0.53
40	Elemol	1554	1548	0.04
41	(E)-Nerolidol	1563	1561	0.39
42	Spathulenol	1578	1577	0.81
43	Caryophyllene oxide	1585	1582	0.60
44	10-epi-γ-Eudesmol	1625	1622	0.22
45	1-epi-Cubenol	1629	1627	0.37
46	γ-Eudesmol	1634	1630	0.98
47	α-Muurolol	1649	1644	0.70
48	α-Eudesmol	1655	1652	0.85
Total				96.84%

**Actual retention indices of compounds on DB-5 column. #Retention indices of compounds on DB-5 column in accordance to literature of Adams (2007).*

TABLE 2 | Discrete inhibitory effect of *H. spicatum* essential oil (HSEO) on growth rate (log CFU), production of deoxynivalenol (DON) and zearalenone (ZEA) by *F. graminearum* in maize grains.

S. No	Dose of HSEO (mg/g)	Log CFU/g	DON (μg/g)	ZEA (μg/g)
1	0 (control)	5.79 ± 0.33^{a}	6.24 ± 0.37^{a}	8.67 ± 0.45^{a}
2	0.5	5.38 ± 0.28^{ab}	6.03 ± 0.39^{ab}	7.95 ± 0.36^{b}
3	1	4.77 ± 0.37^{c}	4.92 ± 0.64^{c}	6.68 ± 0.28^{c}
4	1.5	3.36 ± 0.29^{d}	3.68 ± 0.45^{d}	4.60 ± 0.23^{d}
5	2	2.20 ± 0.26^{e}	2.33 ± 0.53^{e}	2.83 ± 0.29^{e}
6	2.5	1.04 ± 0.17^{f}	1.19 ± 0.21^{f}	1.58 ± 0.20^{f}
7	3	0.27 ± 0.11^{g}	0.43 ± 0.25^{g}	1.04 ± 0.13^{g}
8	3.15	0^{gh}	0^{gh}	0^{h}

The data was processed by one-way ANOVA following Tukey's test and the columns with same alphabetic letters were not significant ($p < 0.05$).

TABLE 3 | Discrete inhibitory effect of radiation treatment on growth rate (log CFU), production of deoxynivalenol (DON) and zearalenone (ZEA) by *F. graminearum* in maize grains.

S. No	Dose of radiation (kGy)	Log CFU/g	DON (μg/g)	ZEA (μg/g)
1	0 (control)	5.79 ± 0.33^{a}	6.24 ± 0.37^{a}	8.67 ± 0.45^{a}
2	1	5.26 ± 0.30^{ab}	5.89 ± 0.54^{ab}	7.64 ± 0.49^{b}
3	2	4.61 ± 0.40^{c}	4.92 ± 0.71^{bc}	6.42 ± 0.35^{c}
4	3	3.66 ± 0.38^{d}	3.73 ± 0.89^{d}	4.87 ± 0.41^{d}
5	4	2.39 ± 0.37^{e}	2.66 ± 0.79^{de}	3.70 ± 0.35^{e}
6	5	1.41 ± 0.46^{f}	1.58 ± 0.75^{ef}	1.84 ± 0.38^{f}
7	6	0^{g}	0^{g}	0^{g}

The data was processed by one-way ANOVA following Tukey's test and the columns with same alphabetic letters were not significant ($p < 0.05$).

of HSEO and 6 kGy of radiation. The linear regression curves for reductions of log CFU, DON, and ZEA content were constructed against different doses of HSEO and radiation (**Figures 1**, **2**). The obtained regression models exhibited the goodness of fit (R^2) close to 1 and found statistically significant ($p < 0.05$; **Tables 4**, **5**). The regression models confirmed that reductions of log CFU, DON, and ZEA content by HSEO and radiation were dose-dependent.

Best of our knowledge, till the date, the fungicidal activity of HSEO on *F. graminearum* was not reported, and this is the first report. On the other hand, Pawar and Thaker (2006) has determined the antifungal activity of HSEO on *Aspergillus niger* by a zone of inhibition assay as 8 mm. The growth inhibitory activity of radiation treatment on *F. graminearum* was less investigated. In support of our study, very few reports are available on the application of radiation treatment in the management of *F. graminearum* in food and feed matrices. Aziz et al. (2007) have reported the complete inhibition of *Fusarium* spp. growth at 4 kGy in barley and 6 kGy in wheat and maize grains. On the other hand, Ferreira-Castro et al. (2007) and Lima et al. (2011) have documented that high dose of 10 kGy radiation treatment was required for complete reduction of *Fusarium* spp. in maize and cowpea bean grains, respectively. The

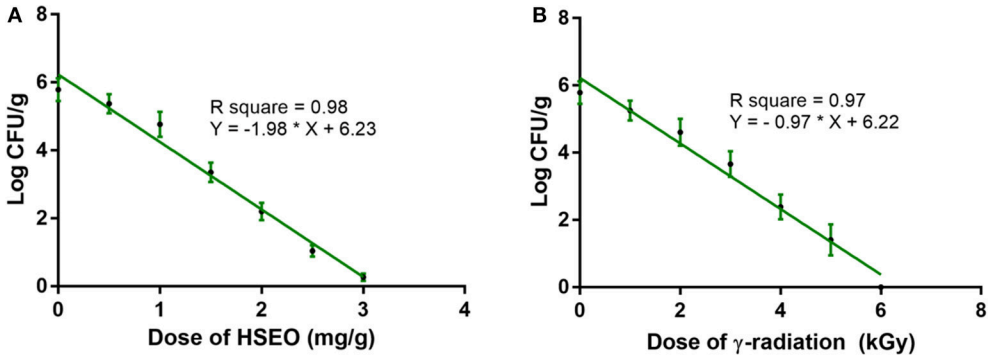


FIGURE 1 | Linear regression curve for growth inhibitory activity of different doses of **(A)** *H. spicatum* L. essential oil (HSEO) and **(B)** radiation treatments on *F. graminearum* in maize. The log CFU/g was declined with the dose of HSEO and radiation. The data was processed by one-way ANOVA following Tukey's test and value of $p < 0.05$ was considered significant.

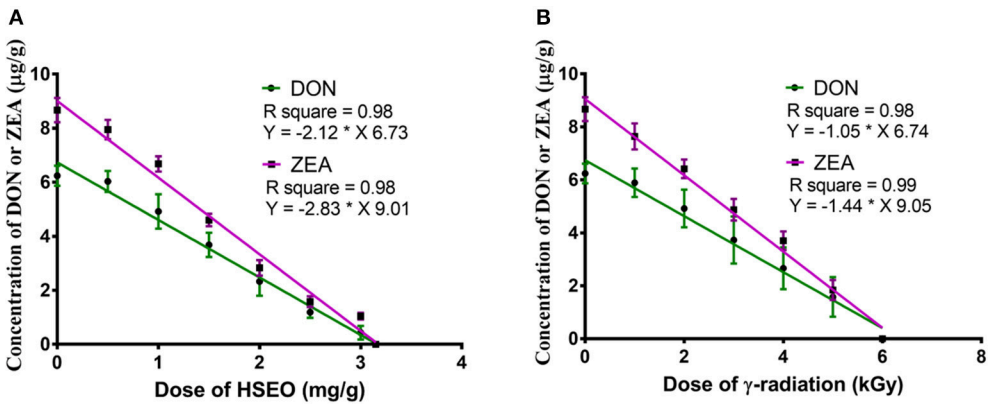


FIGURE 2 | Linear regression curve for dose-dependent effect of **(A)** *H. spicatum* L. essential oil (HSEO) and **(B)** radiation treatments on the reductions of deoxynivalenol (DON) and zearalenone (ZEA) content by *F. graminearum* in maize. The data was analyzed by one-way ANOVA following Tukey's test and value of $p < 0.05$ was considered significant.

radiation dose required for reduction of fungal growth depends on certain factors, such as the type of species, population number, intensity of pigmentation, water content, genetics, and induction of protective enzymes, which play a major role in the regaining of the damage induced by radiation (Jeong et al., 2015). In the present study, the background microbial flora of maize grains was removed by autoclave sterilization and which could affect the texture of the grains and have an impact on the results. Therefore, the differences in the radiation dose required for complete reduction of fungal growth were noticed in present and previous studies.

Combinational Treatment of HSEO and Radiation

Radiation process is measured as an ideal and beneficial decontamination technique at a low dose. High radiation dose produces intolerable features and toxic substances in food, such as structural and chemical changes, nutritional and sensory loss, undesirable odor and flavor, and rancidity (Calado et al., 2014). Therefore, minimally radiation processed foods are highly preferred and acceptable by consumer and regulatory bodies. In this context, food industry and food technologists have made many efforts through last decade to reduce the efficient radiation dosage rate and augment the decontamination efficiency of radiation. Currently, combining radiation with other decontamination agents, such as essential oils, chemical preservatives, and modified atmospheres is an effective and innovative way to improve the decontamination efficiency of radiation (Ghosh et al., 2017; Sirocchi et al., 2017; Wilson et al., 2017). The combinational approach reduces the possible means radiation dose required for decontamination of microbial flora and could promote food safety at low radiation dosage.

The combinational inhibitory effect of HSEO and radiation treatments on the fungal growth (log CFU), production of DON and ZEA by *F. graminearum* in maize grains were executed following the CCD of RSM statistical program (Wu et al., 2017). The RSM is a collection of statistical and mathematical techniques, and one of the widely used applications in the design, development, and invention of new process or products, and improvement of an existing process or product designs in food science and technology. The RSM requires only a minimal

TABLE 4 | Linear regression curve for discrete inhibitory effect of *H. spicatum* essential oil (HSEO) on growth rate (log CFU), production of deoxynivalenol (DON) and zearalenone (ZEA) by *F. graminearum* in maize grains.

	Log CFU/g	DON (μg/g)	ZEA (μg/g)
BEST-FIT VALUES ± SE			
Slope	−1.98 ± 0.12	−2.12 ± 0.10	−2.83 ± 0.14
Y-intercept	6.23 ± 0.22	6.73 ± 0.20	9.01 ± 0.29
X-intercept	3.14	3.16	3.17
1/slope	−0.50	−0.46	−0.35
95% CONFIDENCE INTERVALS			
Slope	−2.31 to −1.66	−2.37 to −1.88	−3.19 to −2.48
Y-intercept	5.65 to 6.82	6.23 to 7.23	8.29 to 9.72
X-intercept	2.86 to 3.50	2.97 to 3.39	2.96 to 3.43
GOODNESS OF FIT			
R square	0.98	0.98	0.98
Sy.x	0.33	0.30	0.44
IS SLOPE SIGNIFICANTLY NON-ZERO?			
F	248.3	446.8	379.8
DFn, DFd	1, 6	1, 6	1, 6
P-value	<0.005	<0.005	<0.005
Deviation from zero?	Significant	Significant	Significant
Equation	Y = −1.98*X + 6.23	Y = −2.12*X + 6.73	Y = −2.83*X + 9.01

TABLE 5 | Linear regression curve for discrete inhibitory effect of radiation treatment on growth rate (log CFU), production of deoxynivalenol (DON) and zearalenone (ZEA) by *F. graminearum* in maize grains.

	Log CFU/g	DON (μg/g)	ZEA (μg/g)
BEST-FIT VALUES ± SE			
Slope	−0.97 ± 0.06	−1.05 ± 0.06	−1.44 ± 0.06
Y-intercept	6.22 ± 0.23	6.74 ± 0.23	9.05 ± 0.2
X-intercept	6.38	6.38	6.28
1/slope	−1.02	−0.94	−0.69
95% CONFIDENCE INTERVALS			
Slope	−1.14 to −0.80	−1.22 to −0.88	−1.60 to −1.27
Y-intercept	5.63 to 6.82	6.13 to 7.35	8.46 to 9.64
X-intercept	5.80 to 7.17	5.82 to 7.10	5.88 to 6.77
GOODNESS OF FIT			
R square	0.97	0.98	0.99
Sy.x	0.34	0.34	0.33
IS SLOPE SIGNIFICANTLY NON-ZERO?			
F	229.5	260.4	515.4
DFn, DFd	1, 5	1, 5	1, 5
P-value	<0.005	<0.005	<0.005
Deviation from zero?	Significant	Significant	Significant
Equation	Y = −0.97*X + 6.22	Y = −1.05*X + 6.74	Y = −1.44*X + 9.05

number of experiments between input variables that potentially influence performance measures or quality characteristics of the product or process and allow to identify breakthrough productive information by means of reducing cost, errors, and disturbance. The relations between the input factors and responses were hypothesized or assessed by choosing a model (Whitcomb and Anderson, 2004).

The actual or obtained response of 13 CCD experiments of the present study was provided in **Table 6**. The data was analyzed by second-order polynomial regression analysis employing the software Design Expert (version 10.0.6) to optimize the response surface models.

The correlation matrix of regression coefficients of independent factors was correlated with one another on a

TABLE 6 | Central composite design (CCD) for combinational inhibitory action of *H. spicatum* essential oil (HSEO) and radiation treatments (independent factors) on the reductions of log CFU, DON, and ZEA (responses).

Run order	Independent factors		Actual responses		
	A: HSEO (mg/g)	B: radiation (kGy)	Log CFU/g	DON (μg/g)	ZEA (μg/g)
1	0.25 (−1)	5.52 (1)	1.02 ± 0.33	1.19 ± 0.23	1.56 ± 0.41
2	1.57 (0)	3(0)	1.07 ± 0.18	1.26 ± 0.47	1.71 ± 0.67
3	0.25 (−1)	0.47 (−1)	5.42 ± 0.37	5.97 ± 0.54	7.68 ± 0.84
4	1.57 (0)	3 (0)	0.84 ± 0.35	1.14 ± 0.34	1.32 ± 0.28
5	1.57 (0)	3 (0)	0.92 ± 0.49	1.31 ± 0.18	1.58 ± 0.55
6	1.57 (0)	3 (0)	0.94 ± 0.32	1.27 ± 0.48	1.83 ± 0.28
7	1.57 (0)	3 (0)	0.81 ± 0.20	1.02 ± 0.29	1.64 ± 0.40
8	1.57 (0)	6 (2)	0.00	0.00	0.00
9	2.89 (1)	0.47 (−1)	0.84 ± 0.31	1.12 ± 0.20	1.47 ± 0.25
10	0 (−2)	3 (0)	3.79 ± 0.58	4.12 ± 0.42	5.38 ± 0.45
11	2.89 (1)	5.52 (1)	0.00	0.00	0.00
12	3.15 (2)	3 (0)	0.00	0.00	0.00
13	1.57 (0)	0 (−2)	3.12 ± 0.66	3.48 ± 0.89	4.71 ± 0.38

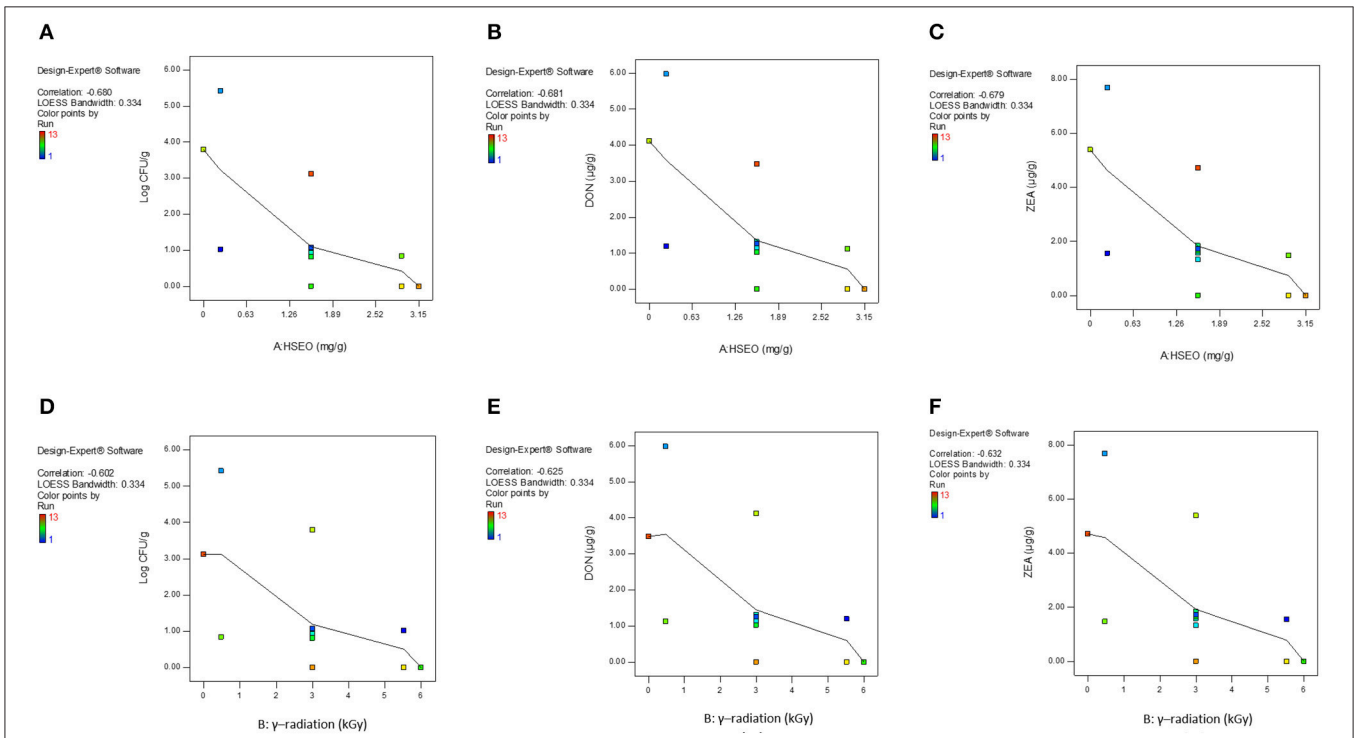


FIGURE 3 | Correlation matrix plots of HSEO **(A–C)** and radiation treatments **(D–F)** to accomplish their suitability for responses: log CFU, DON, and ZEA.

scale of perfect negative correlation (−1) to perfect positive correlation (+1) as per Pearson's correlation coefficients to conclude their appropriateness. The perfect negative correlated matrix was appropriate and agrees to the individual effect of independent factors on responses (Anderson and Whitcomb, 2016). In the present study, a perfect negative correlation matrix was observed for both independent factors (**Figure 3**). A perfect negative correlation matrix of − 0.680 and − 0.602 (log CFU), − 0.681 and − 0.625 (DON), and − 0.679 and − 0.632 (ZEA) were observed for HSEO and radiation treatments, respectively. It indicated that independent factors were independently effective in reducing the log CFU, DON, and ZEA. These results were in accordance with the assessment of the discrete inhibitory effect of HSEO and radiation treatments on the reductions of log CFU, DON, and ZEA content (**Figures 1**, **2**). Therefore, the combinational assessment of HSEO and radiation treatments for the aforementioned purposes were well appropriate and could reveal newer insights. Consequently, combinational treatments

of HSEO and radiation were highly effective in reducing the log CFU, DON, and ZEA content in maize grains. The complete reductions of fungal growth, DON, and ZEA content were noticed at combination of 1.89 mg/g of HSEO and 4.12 kGy of radiation treatments (**Table 11**). The dose of HSEO (1.89 mg/g) and radiation (4.12 kGy) required for complete reductions of fungal growth, DON, and ZEA content were much less in combinational treatment compared to discrete treatments of HSEO (3.15 mg/g) and radiation (6 kGy).

The obtained CCD results of the RSM study concluded that quadratic model was well appropriate for all the responses (log CFU, DON, and ZEA). The coefficient of independent variables in terms actual factors for second-order polynomial equation designed for the responses attained as below:

$$\begin{aligned}\text{Log CFU/g} = &+ 6.87 - 2.98 * \text{HSEO} - 1.27 * \text{radiation} \\ &+ 0.26 * \text{HSEO} * \text{radiation} + 0.34 * \text{HSEO}^2 \\ &+ 0.05 * \text{radiation}^2\end{aligned}$$

$$\begin{aligned}\text{DON } (\mu\text{g/g}) = &+ 7.42 - 3.03 * \text{HSEO} - 1.32 * \text{radiation} \\ &+ 0.27 * \text{HSEO} * \text{radiation} + 0.31 * \text{HSEO}^2 \\ &+ 0.05 * \text{radiation}^2\end{aligned}$$

$$\begin{aligned}\text{ZEA } (\mu\text{g/g}) = &+ 9.61 - 3.81 * \text{HSEO} - 1.72 * \text{radiation} \\ &+ 0.34 * \text{HSEO} * \text{radiation} + 0.38 * \text{HSEO}^2 \\ &+ 0.06 * \text{radiation}^2\end{aligned}$$

The significance of the second-order polynomial model was evaluated by analysis of variance (ANOVA) and coefficient of determination (R^2). The ANOVA results for the fitted quadratic polynomial models of log CFU, DON, and ZEA were presented in **Tables 7**, **8**, **9**, respectively. For any of the terms in the model, a greater *F*-value and smaller *p*-value would indicate a more significant effect on the respective response variables (Atkinson and Donev, 1992). The present model has larger *F*-value of 251.61 (log CFU), 335.88 (DON), and 229.45 (ZEA) and the associated *p*-value <0.0001 and implied that the optimized models were very significant. Furthermore, *F*-value and *p*-value of the lack of fit were 4.44 and 0.0918 for log CFU, 2.37 and 0.2120 for DON, 2.24 and 0.2255 for ZEA, respectively (**Table 10**). Which indicated that lack of fit was not significant and 9.18%, 21.20%, and 22.55% chance could occur due to noise in log CFU, DON, and ZEA, respectively. The goodness of fit of the models was judged by estimating the coefficient of determination (R^2). The value of R^2 for log CFU, DON, and ZEA were observed as 0.9945, 0.9958, and 0.9939, respectively. Which implies that 99.45%, 99.58%, and 99.39% of the variations could be explained by the fitted models of log CFU, DON, and ZEA, respectively. For a good statistical model, the predicted R-squared value should be close to adjusted R-squared value and the obtained differences in all the responses of the present study were appropriate (**Table 10**). Also, adequate precision should be >4.0 is desirable for the significant model and adequate precision of 52.343 (log CFU), 60.519 (DON), and 50.190 (ZEA) in the present study indicates an adequate signal and the obtained models were well appropriate to navigate the design space (**Table 10**).

TABLE 7 | ANOVA for log CFU/g response surface quadratic model.

Source	Sum of squares	Degree of freedom (df)	Mean square	*F*-value	*p*-value Prob > *F*
Model	32.18	5	6.44	251.61	0.0918 significant
A-HSEO	14.96	1	14.96	584.89	<0.0001
B-Radiation	11.73	1	11.73	458.67	<0.0001
AB	3.17	1	3.17	123.87	<0.0001
A^2	1.56	1	1.56	60.84	0.0001
B^2	0.57	1	0.57	22.11	0.0022
Residual	0.18	7	0.026		
Lack of fit	0.14	3	0.046	4.44	0.0918 not significant
Pure error	0.041	4	0.010		
Cor total	32.36	12			

TABLE 8 | ANOVA for DON (μg/g) response surface quadratic model.

Source	Sum of squares	Degree of freedom (df)	Mean square	*F*-value	*p*-value Prob > *F*
Model	37.67	5	7.53	335.88	<0.0001 significant
A-HSEO	17.53	1	17.53	781.33	<0.0001
B-Radiation	14.76	1	14.76	657.91	<0.0001
AB	3.35	1	3.35	149.30	<0.0001
A^2	1.38	1	1.38	61.36	0.0001
B^2	0.49	1	0.49	21.84	0.0023
Residual	0.16	7	0.022		
Lack of fit	0.10	3	0.033	2.37	0.2120 not significant
Pure error	0.057	4	0.014		
Cor total	37.83	12			

TABLE 9 | ANOVA for ZEA (μg/g) response surface quadratic model.

Source	Sum of squares	Degree of freedom (df)	Mean square	*F*-value	*p*-value Prob > *F*
Model	63.36	5	12.67	229.45	<0.0001 significant
A-HSEO	29.40	1	29.40	532.25	<0.0001
B-Radiation	25.48	1	25.48	461.39	<0.0001
AB	5.41	1	5.41	97.88	<0.0001
A^2	1.98	1	1.98	35.84	0.0005
B^2	0.83	1	0.83	15.04	0.0061
Residual	0.39	7	0.055		
Lack of fit	0.24	3	0.081	2.24	0.2255 not significant
Pure error	0.14	4	0.036		
Cor total	63.75	12			

TABLE 10 | Sequential model and regression coefficients of optimized designs.

Term model	Responses		
	Log CFU/g	DON (μg/g)	ZEA (μg/g)
F-Value	251.61	335.88	229.45
P > F	<0.0001	<0.0001	<0.0001
Mean	1.44	1.68	2.22
Standard deviation	0.16	0.15	0.24
C V %	11.08	8.90	10.58
R squared	0.9945	0.9958	0.9939
Adjusted R squared	0.9905	0.9929	0.9896
Predicted R squared	0.9664	0.9771	0.9673
Adequate precision	52.343	60.519	50.190
Model	Quadratic	Quadratic	Quadratic

The diagnostic and correlation plots were judged to endorse the obtained significance of ANOVA. The normal plot residuals of normal % probability vs. externally studentized residuals were constructed to illustrate the accuracy of optimized design (**Figure 4**). The distribution of externally studentized residuals was satisfactorily followed the normal distribution. Which indicated that residuals plots follow linear behavior and the predicted model is accurate (Myers et al., 2016). A Box-Cox plot for power transforms of responses were considered to determine the most appropriate power law transformation to fit the responses. The best recommended transform (λ) of 0.80, 0.94, and 0.91 were observed for log CFU, DON, and ZEA, respectively (**Figure 5**). The obtained λ values were close to the current value of 1 for none and which indicates that responses were well fitted by the optimized design (Box and Cox, 1964). The optimized designs were assessed to examine the correlation between the actual and predicted responses (**Figure 6**). The obtained data points were close to the straight line and presented a good correlation coefficient (R^2). The correlation curves suggested a high degree correlation of 0.9945, 0.9958, and 0.9939 between the actual and predicted values of the log CFU, DON, and ZEA, respectively and concluded that the fundamental assumptions of the analysis were well appropriate. The fitted polynomial equation was expressed as 3D surface plots to illustrate the interactive effects of the independent variables on the responses and to deduce optimum conditions (**Figure 7**). In conclusion, appropriateness of the optimized model for predicting the optimum responses was verified with suggested optimum conditions. The obtained actual values of suggested experiments were found in agreement with the predicted values (**Table 11**). The complete reductions of fungal growth (log CFU), DON, and ZEA content were noticed in maize grains at combinational treatment of 1.89 mg/g of HSEO and 4.12 kGy of radiation. These results suggested that the designed quadratic models for reductions of log CFU, DON, and ZEA content in maize grains were statistically significant and could adequately represent the real interdependence of factors chosen (HSEO and radiation).

The present study concluded that combinational exposure of HSEO and radiation on the reductions of fungal growth, DON, and ZEA content by *F. graminearum* in maize grains was highly efficient compared to their discrete exposure. In combinational approach, radiation dose required for complete reductions of fungi and mycotoxins were reduced from 6 kGy (discrete treatment) to 4.12 kGy. Which indicated that radiation treatment with the combination of HSEO was highly efficient and minimally radiation processed compared to unaccompanied treatment of radiation. However, structural, chemical and nutritional changes of maize grains were not determined in the present study. In support of our study, earlier reports demonstrated that essential oils with a combination of modified atmosphere packaging, mild heat treatment, and radiation were highly effective and safe in maintaining the microbiological safety of the food (Hossain et al., 2014). Several studies noticed that antifungal activity of essential oils is reliant on chemical constituents and their synergistic action (Hyldgaard et al., 2012; da Cruz Cabral et al., 2013). In our study, varieties of chemical compounds were noticed in HSEO (**Table 1**) and the attributed antifungal activity of HSEO against *F. graminearum* could be due to individual and combination of action of 1,8-cineole, linalool, β-pinene, limonene, germacrene-D, γ-terpinene, terpinolene, α-terpinene, and α-terpineol (Velluti et al., 2003; Van Vuuren and Viljoen, 2007; Deba et al., 2008; Chee et al., 2009; Vilela et al., 2009; Gao et al., 2016). These compounds could alter the integrity of cell membrane and disturb the membrane fluidity, osmotic balance, enzymatic functions, and leak cytoplasmic contents of the cell. These cascade of events stops the ATP synthesis, growth and proliferation of fungi, and activates the caspase-mediated apoptotic death by releasing the mitochondrial cytochrome c and death receptors (Ramsdale, 2008; Usta et al., 2009; Tian et al., 2012; Chen et al., 2014). In case of radiation treatment, radiolysis of cellular water of organisms produces positive-charged water radicals (H_2O^+) and negative-charged free electrons (e^-), and a series of cross-combination and recombination reactions generate highly reactive molecules (de Campos et al., 2004; Le Caër, 2011). These molecules attack and cleave hydrogen from sugar and bases of nuclear material, and destruct the other cellular components, i.e., protein and lipids and even damage the integrity of cellular membranes. This process inhibits spore germination and biomass production and promotes the death of fungi (Calado et al., 2014).

Henceforth, combined treatments of HSEO and radiation presented a superior antifungal efficiency on *F. graminearum* than the discrete treatment of HSEO or radiation alone. Moreover, many reports showed that the combinational treatment of essential oils with radiation up to a dose of 10 kGy do not produce any significant changes in the quality and quantity of essential oils and not produce any toxic residues (Chatterjee et al., 2000; Haddad et al., 2007). Consequently, functional features of essential oils do not effect on exposure to radiation and acceptable by Institute of Food Science & Technology (IFST), WHO, FAO, International Atomic Energy Agency (IAEA), and consumer (Lacroix and Ouattara, 2000; IAEA, 2001; Maherani et al., 2016). Henceforth, the proposed novel combinational exposure of HSEO and radiation could be highly acceptable and efficient for reductions of fungal growth and mycotoxins in food and feed matrices and thereby support in

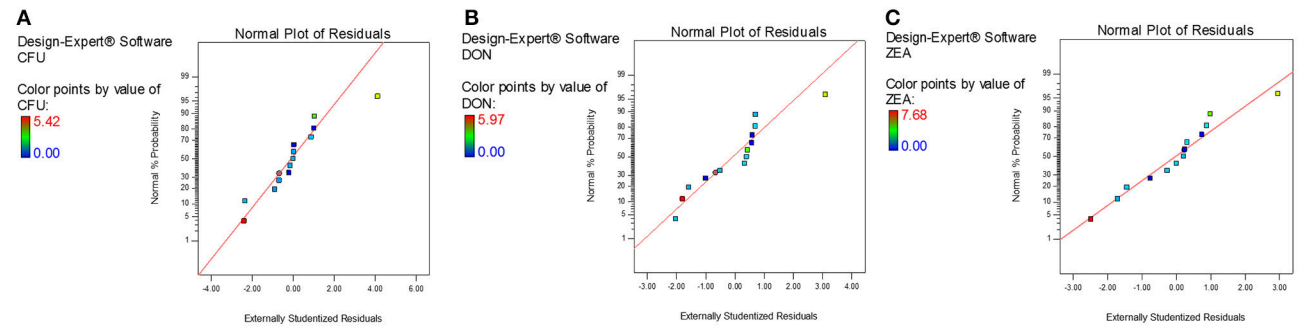


FIGURE 4 | Normal plot of residuals for combinational inhibitory effect of *H. spicatum* L. essential oil (HSEO) and radiation treatments on **(A)** log CFU/g (fungal growth), production of **(B)** DON (μg/g) and **(C)** ZEA (μg/g) by *F. graminearum* in maize grains.

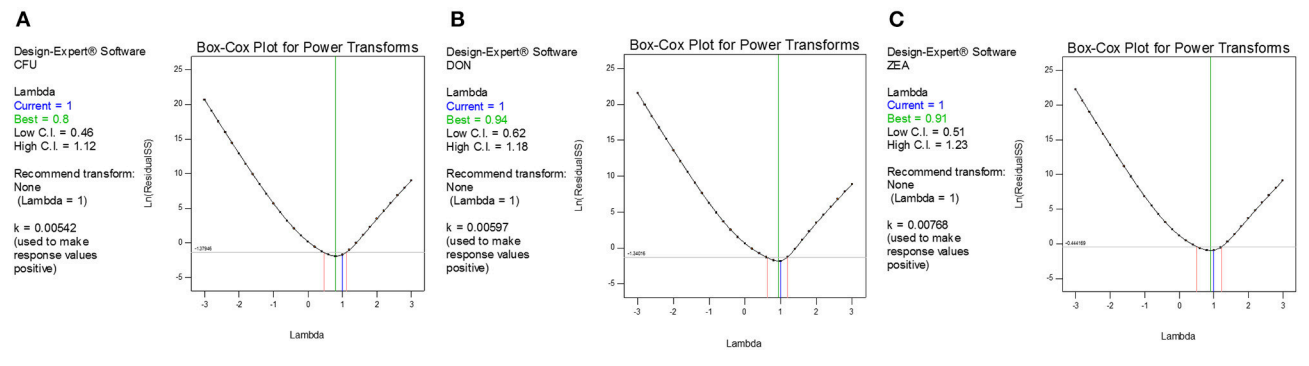


FIGURE 5 | Box-Cox plot for determination of the best power-transformed response surface model: **(A)** log CFU, **(B)** DON, and **(C)** ZEA.

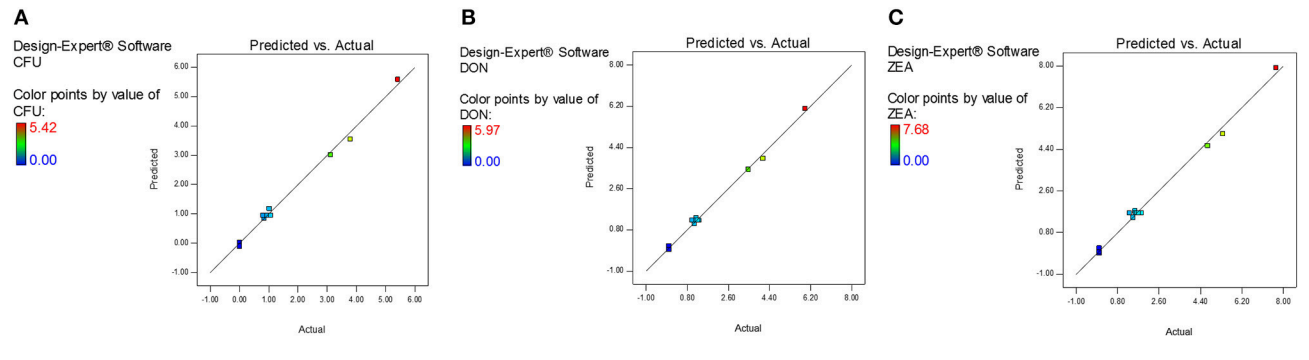


FIGURE 6 | Predicted vs. actual plots for combinational inhibitory effect of *H. spicatum* L. essential oil (HSEO) and radiation treatments on responses: **(A)** log CFU, **(B)** DON, and **(C)** ZEA.

obtaining the highly microbiological safe food products. Though, a greater understanding of the combinational antifungal activity of essential oils and radiation is required at the cellular and molecular level to exploit underlying the molecular death machinery.

CONCLUSION

In the present study, the discrete and combinational inhibitory effects of HSEO and radiation treatments on growth, production of DON and ZEA by *F. graminearum* in maize grains were assessed under laboratory set-ups. The GC-MS analysis of HSEO revealed the presence of 48 compounds constituting to 96.84% of total weight and major compounds were 1,8-cineole, linalool, and β-pinene. The simple linear regression analysis showed that HSEO and radiation treatments were discretely inhibited the fungal growth, DON, and ZEA content in maize grains by the dose-dependent way, and complete inhibition were noticed at 3.15 mg/g of HSEO and 6 kGy of radiation. The combinational inhibitory action of HSEO and

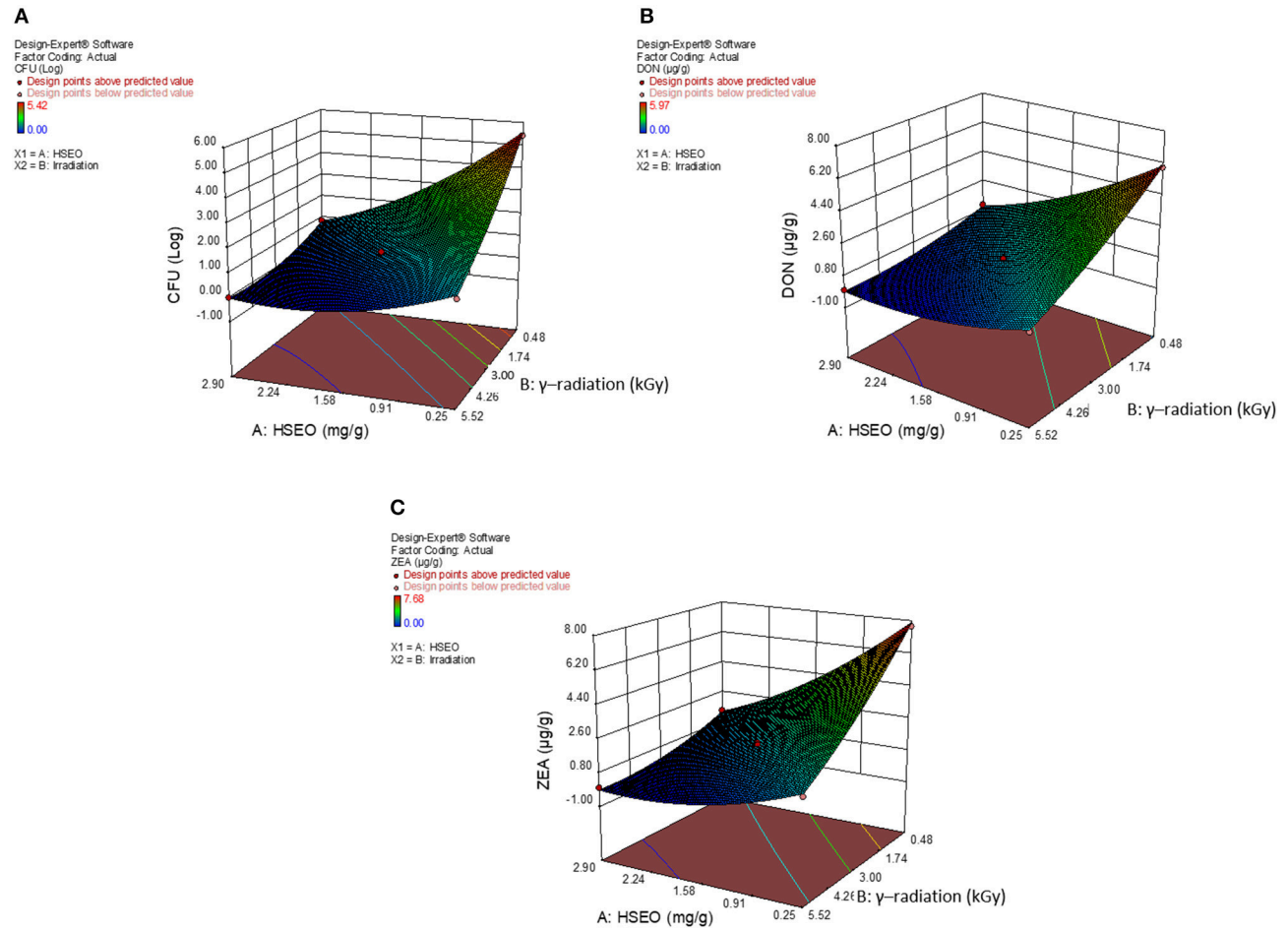


FIGURE 7 | 3D-response surface plot for combinational inhibitory effect of *H. spicatum* L. essential oil (HSEO) and radiation treatments on **(A)** log CFU/g (fungal growth), production of **(B)** DON (µg/g) and **(C)** ZEA (µg/g) by *F. graminearum* in maize grains.

TABLE 11 | Assessment of proposed predicted values of design with actual values of experiment to maximize the inhibition of growth rate, DON, and ZEA by *F. graminearum* in maize grains.

S. No	Independent factors		Responses					
			Predicted			Actual		
	A: HSEO (mg/g)	B: Radiation (kGy)	Log CFU/g	DON (µg/g)	ZEA (µg/g)	Log CFU/g	DON (µg/g)	ZEA (µg/g)
1	1.50	3.45	0.77 ± 0.26	1.01± 0.06	1.38 ± 0.10	0.84 ± 0.14	1.09 ± 0.22	1.41 ± 0.28
2	0.25	0.47	5.58 ± 0.50	6.10 ± 0.12	7.92 ± 0.19	5.71 ± 0.39	6.17 ± 0.53	8.08 ± 0.71
3	0.84	1.63	3.03 ± 0.27	3.44 ± 0.06	4.52 ± 0.10	2.92 ± 0.28	3.61 ± 0.41	4.29 ± 0.47
4	1.12	3.95	0.95 ± 0.25	1.20 ± 0.06	1.60 ± 0.10	0.81 ± 0.21	1.05 ± 0.34	1.22 ± 0.15
5	1.57	2.68	1.11 ± 0.26	1.40 ± 0.06	1.88 ± 0.10	1.19 ± 0.17	1.64 ± 0.22	1.79 ± 0.12
6	0.79	2.97	2.04 ± 0.26	2.37 ± 0.06	3.12 ± 0.10	1.87 ± 0.33	2.44 ± 0.39	3.51 ± 0.44
7	1.89	1.03	1.69 ± 0.29	2.03 ± 0.07	2.73 ± 0.11	1.80 ± 0.26	2.16 ± 0.41	2.98 ± 0.34
8	0.79	3.87	1.18 ± 0.25	1.44 ± 0.06	1.91 ± 0.10	1.24 ± 0.22	1.52 ± 0.24	1.87 ± 0.21
9	0.43	1.70	3.80 ± 0.32	4.23 ± 0.08	5.51 ± 0.12	3.92 ± 0.47	4.16 ± 0.32	5.67 ± 0.64
10	1.82	3.63	0.41 ± 0.25	0.62 ± 0.06	0.86 ± 0.10	0.58 ± 0.11	0.74 ± 0.12	1.03 ± 0.19
11	1.89	4.12	0	0	0	0	0	0

radiation treatments on growth, production of DON and ZEA by *F. graminearum* in maize grains was studied by CCD of RSM statistical program. The fungal growth, DON, and ZEA content were not detected at combinational treatment of 1.89 mg/g of HSEO and 4.12 kGy of radiation. The results revealed that combination of HSEO and radiation treatments could reduce the fungal growth, DON, and ZEA content at much lower concentration than their discrete inhibitory dose. The quadratic model was found well appropriate for reductions of fungal growth (log CFU), DON, and ZEA content in maize grains. The optimized design was found statistically significant ($p < 0.05$) with larger *F*-value and adequate precision, and a smaller *p*-value. The actual data points were close to the straight line and presented good correlation regression coefficients (R^2) for the responses. In addition, diagnostic and correlation plots, i.e., correlation matrix, normal plot residuals, Box-Cox, and actual vs. predicted plots were confirmed that optimized design was accurate and appropriate. The study concluded that optimized design could adequately represent the real interdependence of factors chosen (HSEO and radiation) for reductions of fungal growth, DON, and ZEA content. The present study suggests that combinational treatment of essential oil and radiation could be a novel promising approach for improving the shelf-life and safety of food and feed matrices. As well, study accomplishes that usage of natural antifungal agents with the combination of food-processing techniques is an appropriate and innovative strategy to improve decontamination efficiency of food-processing techniques.

AUTHOR CONTRIBUTIONS

NK, JK, CS, KK, and VM designed and interpreted data of the work. NK, JK, CS, VG, and VM have drafted the work. All authors have approved the final version to be published.

ACKNOWLEDGMENTS

The first author is thankful to the UGC, Government of India for providing Junior Research Fellowship [File no: F. 2-14/2012(SA-I)] to pursue Ph.D. Authors are also grateful to Dr. K. Jayathilakan and Dr. M.C. Pandey, Freeze Drying and Processing Technology Division, Defence Food Research Laboratory, Mysuru, India, for providing the γ–radiation facility. The authors are also thankful to Director, DFRL, and DRDO-BU-CLS for their kind support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01511/full#supplementary-material

REFERENCES

Adams, R. P. (2007). *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. Carol Stream, IL: Allured Publishing Corporation.

Aiyaz, M., Thimmappa Divakara, S., Mudili, V., George Moore, G., Kumar Gupta, V., Chandra Nayaka, S., et al. (2016). Molecular diversity of seed-borne *Fusarium* species associated with Maize in India. *Curr. Genom.* 17, 132–144. doi: 10.2174/1389202917666151116213056

Akocak, P. B., Churey, J. J., and Worobo, R. W. (2015). Antagonistic effect of chitinolytic *Pseudomonas* and *Bacillus* on growth of fungal hyphae and spores of aflatoxigenic *Aspergillus flavus*. *Food Biosci.* 10, 48–58. doi: 10.1016/j.fbio.2015.01.005

Anderson, M. J., and Whitcomb, P. J. (2016). *DOE Simplified: Practical Tools for Effective Experimentation*. Boca Raton, FL: CRC Press.

Annunziata, L., Stramenga, A., Visciano, P., Schirone, M., De Colli, L., Colagrande, M. N., et al. (2017). Simultaneous determination of aflatoxins, T-2 and HT-2 toxins, and fumonisins in cereal-derived products by QuEChERS extraction coupled with LC-MS/MS. *Anal. Bioanal. Chem.* 409, 5143–5155. doi: 10.1007/s00216-017-0462-z

Atkinson, A. C., and Donev, A. N. (1992). *Optimum Experimental Designs*. Oxford, UK: Oxford Science Publications.

Aziz, N. H., Ferial, M., Shahin, A. A., and Roushy, S. M. (2007). Control of *Fusarium* moulds and fumonisin B 1 in seeds by gamma-irradiation. *Food Control*. 18, 1337–1342. doi: 10.1016/j.foodcont.2005.12.013

Bernhoft, A., Torp, M., Clasen, P. E., Løes, A. K., and Kristoffersen, A. B. (2012). Influence of agronomic and climatic factors on *Fusarium* infestation and mycotoxin contamination of cereals in Norway. *Food Addit. Contaminants* 29, 1129–1140. doi: 10.1080/19440049.2012.672476

Box, G. E. P., and, Cox, D. R. (1964). An analysis of transformation (with discussion). *J. R. Statist. Soc. B*26, 211–252.

Calado, T., Venâncio, A., and Abrunhosa, L. (2014). Irradiation for mold and mycotoxin control: a review. *Comprehens. Rev. Food Sci. Food Safety* 13, 1049–1061. doi: 10.1111/1541-4337.12095

CAST (Council for Agricultural Science and Technology) (2003). *Mycotoxins: Risks in Plant, Animal and Human Systems.* Task Force Report No. 139, CAST, Ames, IA. 199 pp.

Chatterjee, S., Variyar, P. S., Gholap, A. S., Padwal-Desai, S. R., and Bongirwar, D. R. (2000). Effect of γ-irradiation on the volatile oil constituents of turmeric *(Curcuma longa). Food Res. Int.* 33, 103–106. doi: 10.1016/S0963-9969(00)00012-0

Chee, H. Y., Kim, H., and Lee, M. H. (2009). *In vitro* antifungal activity of limonene against *Trichophyton rubrum*. *Mycobiology* 37, 243–246. doi: 10.4489/MYCO.2009.37.3.243

Chen, Y., Zeng, H., Tian, J., Ban, X., Ma, B., and Wang, Y. (2014). Dill (*Anethum graveolens* L.) seed essential oil induces *Candida albicans* apoptosis in a metacaspase-dependent manner. *Fungal Biol.* 118, 394–401. doi: 10.1016/j.funbio.2014.02.004

Council of Europe. (1997). "*Methods of pharmacognosy*" in European. *Pharmacopoeia* 3, 121–122.

da Cruz Cabral, L., Pinto, V. F., and Patriarca, A. (2013). Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. *Int. J. Food Microbiol.* 166, 1–14. doi: 10.1016/j.ijfoodmicro.2013.05.026

Deba, F., Xuan, T. D., Yasuda, M., and Tawata, S. (2008). Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidens pilosa* Linn. *var. Radiata. Food Control* 19, 346–352. doi: 10.1016/j.foodcont.2007.04.011

de Campos, S. X., Sanches, S. M., Falone, S. Z., and Vieira, E. M. (2004). Influence of rate dose in the degradation of the 2, 4 dichlorophenoxyacetic acid (2, 4-D) herbicide in water and methanol by gamma radiation from cobalto-60. *Eclética Química* 29, 41–46. doi: 10.1590/S0100-46702004000100005

Divakara, S. T., Santosh, P., Aiyaz, M., Venkata Ramana, M., Hariprasad, P., Nayaka, S. C., et al. (2014). Molecular identification and characterization of *Fusarium* spp. associated with sorghum seeds. *J. Sci. Food Agric.* 94, 1132–1139. doi: 10.1002/jsfa.6380

Du, M., Liu, Y., and Zhang, G. (2017). Interaction of aflatoxin B1 and fumonisin B1 in HepG2 cell apoptosis. *Food Biosci.* 20, 131–140. doi: 10.1016/j.fbio.2017.09.003

Ferreira-Castro, F. L., Aquino, S., Greiner, R., Ribeiro, D. H. B., Reis, T. A., and Correa, B. (2007). Effects of gamma radiation on maize samples contaminated with *Fusarium verticillioides*. *Appl. Radiat. Isotopes* 65, 927–933. doi: 10.1016/j.apradiso.2007.03.011

Gao, T., Zhou, H., Zhou, W., Hu, L., Chen, J., and Shi, Z. (2016). The fungicidal activity of thymol against *Fusarium graminearum* via inducing lipid peroxidation and disrupting ergosterol biosynthesis. *Molecules* 21:E770. doi: 10.3390/molecules21060770

George, E., Kasipandi, M., Vekataramana, M., Kumar, K. N., Allen, J. A., Parimelazhagan, T., et al. (2016). *In vitro* anti-oxidant and cytotoxic analysis of *Pogostemon mollis* Benth. *Bangladesh J. Pharmacol.* 11, 148–158. doi: 10.3329/bjp.v11i1.24157

Ghosh, P. K., Bhattacharjee, P., and Das, S. (2017). Extension of shelf life of tuberose flowers using a combination of gamma irradiation and generally regarded as safe (GRAS) preservatives and assessment of antimicrobial potency of senesced flowers. *J. Hortic. Sci. Biotechnol.* 92, 130–145. doi: 10.1080/14620316.2016.1234340

Gobbo-Neto, L., and Lopes, N. P. (2007). Medicinal plants: factors of influence on the content of secondary metabolites. *Quimica Nova* 30, 374–381. doi: 10.1590/S0100-40422007000200026

Gonçalves, R. A., Navarro-Guillén, C., Gilannejad, N., Dias, J., Schatzmayr, D., Bichl, G., et al. (2018). Impact of deoxynivalenol on rainbow trout: growth performance, digestibility, key gene expression regulation and metabolism. *Aquaculture* 490, 362–372. doi: 10.1016/j.aquaculture.2018.03.001

Haddad, M., Herent, M. F., Tilquin, B., and Quetin-Leclercq, J. (2007). Effect of gamma and e-beam radiation on the essential oils of *Thymus vulgaris thymoliferum*, *Eucalyptus radiata*, and *Lavandula angustifolia*. *J. Agric. Food Chem.* 55, 6082–6086. doi: 10.1021/jf063540+

Hossain, F., Follett, P., Vu, K. D., Salmieri, S., Senoussi, C., and Lacroix, M. (2014). Radiosensitization of *Aspergillus niger* and *Penicillium chrysogenum* using basil essential oil and ionizing radiation for food decontamination. *Food Control* 45, 156–162. doi: 10.1016/j.foodcont.2014.04.022

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* 3:12. doi: 10.3389/fmicb.2012.00012

IARC. (1999). *Overall Evaluations of Carcinogenicity to Humans. International Agency for Research on Cancer*. IARC Monographs 1–73, 1–36.

IAEA. (2001). "Consumer acceptance and market development of irradiated food in Asia and the Pacific. IAEA TECDOC-1219," in *Proceedings of the Final Research Co-Ordination Meeting* (Leuven), 24–28.

Iram, W., Anjum, T., Iqbal, M., Ghaffar, A., and Abbas, M. (2016). Structural elucidation and toxicity assessment of degraded products of Aflatoxin B1 and B2 by aqueous extracts of *Trachyspermum ammi*. *Front. Microbiol.* 7:346. doi: 10.3389/fmicb.2016.00346

Jeong, R. D., Chu, E. H., Shin, E. J., Lee, E. S., Kwak, Y. S., and Park, H. J. (2015). Antifungal effect of gamma irradiation and sodium dichloroisocyanurate against *Penicillium expansum* on pears. *Lett. Appl. Microbiol.* 61, 437–445. doi: 10.1111/lam.12466

Ji, F., Xu, J., Liu, X., Yin, X., and Shi, J. (2014). Natural occurrence of deoxynivalenol and zearalenone in wheat from jiangsu province, China. *Food Chem.* 157, 393–397. doi: 10.1016/j.foodchem.2014.02.058

Joshi, S., Chanotiya, C. S., Agarwal, G., Prakash, O., Pant, A. K., and Mathela, C. S. (2008). Terpenoid compositions, and antioxidant and antimicrobial properties of the rhizome essential oils of different *Hedychium* species. *Chem. Biodivers.* 5, 299–309. doi: 10.1002/cbdv.200890027

Kalagatur, N. K., Ghosh, O. S. N., Sundararaj, N., and Mudili, V. (2018a). Antifungal activity of chitosan nanoparticles encapsulated with *Cymbopogon martinii* essential oil on plant pathogenic fungi *Fusarium graminearum*. *Front. Pharmacol.* 9:610. doi: 10.3389/fphar.2018.00610

Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., and Sreepathi, M. H. (2018b). Effect of high pressure processing on growth and mycotoxin production of *Fusarium graminearum* in maize. *Food Biosci.* 21, 53–59. doi: 10.1016/j.fbio.2017.11.005

Kalagatur, N. K., Karthick, K., Allen, J. A., Nirmal Ghosh, O. S., Chandranayaka, S., Gupta, V. K., et al. (2017). Application of activated carbon derived from seed shells of jatropha curcas for decontamination of zearalenone mycotoxin. *Front. Pharmacol.* 8:760. doi: 10.3389/fphar.2017.00760

Kalagatur, N. K., Mudili, V., Siddaiah, C., Gupta, V. K., Natarajan, G., Sreepathi, M. H., et al. (2015). Antagonistic activity of *Ocimum sanctum* L. essential oil on growth and zearalenone production by *Fusarium graminearum* in maize grains. *Front. Microbiol.* 6:892. doi: 10.3389/fmicb.2015.00892.

Kanapitsas, A., Batrinou, A., Aravantinos, A., Sflomos, C., and Markaki, P. (2016). Gamma radiation inhibits the production of Ochratoxin A by Aspergillus carbonarius. Development of a method for OTA determination in raisins. *Food Biosci.* 15, 42–48. doi: 10.1016/j.fbio.2016.05.001

Koul, S., Raina, V., and Sharma, S. K. (2005). Conservation and propagation of high altitude medicinal and aromatic plant: *Hedychium spicatum*. *J. Plant Biochem. Biotechnol.* 14, 57–59. doi: 10.1007/BF03263227

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A. S., Fillinger, S., Mernke, D., et al. (2009). Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathogens* 5:e1000696. doi: 10.1371/journal.ppat.1000696

Kumar, K. N., Venkataramana, M., Allen, J. A., Chandranayaka, S., Murali, H. S., and Batra, H. V. (2016). Role of *Curcuma longa* L. essential oil in controlling the growth and zearalenone production of *Fusarium graminearum*. *LWT-Food Sci. Technol.* 69, 522–528. doi: 10.1016/j.lwt.2016.02.005

Lacroix, M., and Ouattara, B. (2000). Combined industrial processes with irradiation to assure innocuity and preservation of food products—a review. *Food Res. Int.* 33, 719–724. doi: 10.1016/S0963-9969(00)00085-5

Le Caër, S. (2011). Water radiolysis: influence of oxide surfaces on H_2 production under ionizing radiation. *Water* 3, 235–253. doi: 10.3390/w3010235

Lima, K. D. S. C., e Souza, L. B., de Oliveira Godoy, R. L., França, T. C. C., and dos Santos Lima, A. L. (2011). Effect of gamma irradiation and cooking on cowpea bean grains (*Vigna unguiculata* L. *Walp). Radiat. Phys. Chem.* 80, 983–989. doi: 10.1016/j.radphyschem.2011.04.011

Maherani, B., Hossain, F., Criado, P., Ben-Fadhel, Y., Salmieri, S., and Lacroix, M. (2016). World market development and consumer acceptance of irradiation technology. *Foods* 5:79. doi: 10.3390/foods5040079

Mallmann, C. A., Dilkin, P., Mallmann, A. O., Oliveira, M. S., Adaniya, Z. N. C., and Tonini, C. (2017). Prevalence and levels of deoxynivalenol and zearalenone in commercial barley and wheat grain produced in Southern Brazil: an eight-year (2008 to 2015) summary. *Trop. Plant Pathol.* 42, 146–152. doi: 10.1007/s40858-017-0152-6

Mishra, S., Ansari, K. M., Dwivedi, P. D., Pandey, H. P., and Das, M. (2013). Occurrence of deoxynivalenol in cereals and exposure risk assessment in Indian population. *Food Control* 30, 549–555. doi: 10.1016/j.foodcont.2012.07.041

Mudili, V., Siddaih, C. N., Nagesh, M., Garapati, P., Naveen Kumar, K., Murali, H. S., et al. (2014). Mould incidence and mycotoxin contamination in freshly harvested maize kernels originated from India. *J. Sci. Food Agric.* 94, 2674–2683. doi: 10.1002/jsfa.6608

Muniyandi, K., George, E., Mudili, V., Kalagatur, N. K., Anthuvan, A. J., Krishna, K., et al. (2017). Antioxidant and anticancer activities of *Plectranthus stocksii* Hook. f. leaf and stem extracts. *Agric. Nat. Res.* 51, 63–73. doi: 10.1016/j.anres.2016.07.007

Muthulakshmi, S., Maharajan, K., Habibi, H. R., Kadirvelu, K., and Venkataramana, M. (2018). Zearalenone induced embryo and neurotoxicity in zebrafish model (Danio rerio): role of oxidative stress revealed by a multi biomarker study. *Chemosphere* 198, 111–121. doi: 10.1016/j.chemosphere.2018.01.141

Myers, R. H., Montgomery, D. C., and Anderson-Cook, C. M. (2016). *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*. Hoboken, NJ: John Wiley & Sons.

Pasquali, M., Beyer, M., Logrieco, A., Audenaert, K., Balmas, V., Basler, R., et al. (2016). A European database of *Fusarium graminearum* and *F. culmorum* trichothecene genotypes. *Front. Microbiol.* 7:406. doi: 10.3389/fmicb.2016.00406

Pawar, V. C., and Thaker, V. S. (2006). *In vitro* efficacy of 75 essential oils against *Aspergillus niger*. *Mycoses* 49, 316–323. doi: 10.1111/j.1439-0507.2006.01241.x

Pitt, J. I., and Hockings, A. D. (2009). *Fungi and Food spoilage, 3rd Edn*. New York, NY: Springer.

Ramana, M. V., Balakrishna, K., Murali, H. C. S., and Batra, H. V. (2011). Multiplex PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in rice and fingermillet collected from southern India. *J. Sci. Food Agric.* 91, 1666–1673. doi: 10.1002/jsfa.4365

Ramsdale, M. (2008). Programmed cell death in pathogenic fungi. *Biochim. Biophys. Acta* 1783, 1369–1380. doi: 10.1016/j.bbamcr.2008.01.021

Reddy, K. E., Lee, W., young Jeong, J., Lee, Y., Lee, H. J., Kim, M. S., et al. (2018). Effects of deoxynivalenol-and zearalenone-contaminated feed on the gene expression profiles in the kidneys of piglets. *Asian-australas. J. Anim. Sci.* 31, 138–148. doi: 10.5713/ajas.17.0454

Reddy, K. J., Jayathilakan, K., and Pandey, M. C. (2015). Effect of ionizing radiation on the protein and lipid quality characteristics of mutton kheema treated with rice bran oil and sunflower oil. *Radiat. Phys. Chem.* 117, 217–224. doi: 10.1016/j.radphyschem.2015.09.002

Sabulal, B., George, V., Dan, M., and Pradeep, N. S. (2007). Chemical composition and antimicrobial activities of the essential oils from the rhizomes of four *Hedychium* species from South India. *J. Essent. Oil Res.* 19, 93–97. doi: 10.1080/10412905.2007.9699237

Schirone, M., Visciano, P., Tofalo, R., and Suzzi, G. (2016). Biological hazards in food. *Front. Microbiol.* 7:2154. doi: 10.3389/fmicb.2016.02154

Schumann, B., Winkler, J., Mickenautsch, N., Warnken, T., and Dänicke, S. (2016). Effects of deoxynivalenol (DON), zearalenone (ZEN), and related metabolites on equine peripheral blood mononuclear cells (PBMC) *in vitro* and background occurrence of these toxins in horses. *Mycotoxin Res.* 32, 153–161. doi: 10.1007/s12550-016-0250-1

Sellamani, M., Kalagatur, N. K., Siddaiah, C., Mudili, V., Krishna, K., Natarajan, G., et al. (2016). Antifungal and zearalenone inhibitory activity of *Pediococcus pentosaceus* isolated from dairy products on *Fusarium graminearum*. *Front. Microbiol.* 7:890. doi: 10.3389/fmicb.2016.00890

Sirocchi, V., Devlieghere, F., Peelman, N., Sagratini, G., Maggi, F., Vittori, S., et al. (2017). Effect of *Rosmarinus officinalis* L. essential oil combined with different packaging conditions to extend the shelf life of refrigerated beef meat. *Food Chem.* 221, 1069–1076. doi: 10.1016/j.foodchem.2016.11.054

Tian, J., Ban, X., Zeng, H., He, J., Chen, Y., and Wang, Y. (2012). The mechanism of antifungal action of essential oil from dill (*Anethum graveolens* L.) on *Aspergillus flavus*. *PLoS ONE* 7:e30147. doi: 10.1371/journal.pone.0030147

Tima, H., Berkics, A., Hannig, Z., Ittzés, A., Kecskésné Nagy, E., Mohácsi-Farkas, C., et al. (2018). Deoxynivalenol in wheat, maize, wheat flour and pasta: surveys in Hungary in 2008–2015. *Food Addit. Contamin.* 11, 37–42. doi: 10.1080/19393210.2017.1397061

Tralamazza, S. M., Bemvenuti, R. H., Zorzete, P., de Souza Garcia, F., and Corrêa, B. (2016). Fungal diversity and natural occurrence of deoxynivalenol and zearalenone in freshly harvested wheat grains from Brazil. *Food Chem.* 196, 445–450. doi: 10.1016/j.foodchem.2015.09.063

Udomkun, P., Wiredu, A. N., Nagle, M., Bandyopadhyay, R., Müller, J., and Vanlauwe, B. (2017). Mycotoxins in Sub-Saharan Africa: present situation, socio-economic impact, awareness, and outlook. *Food Control* 72, 110–122. doi: 10.1016/j.foodcont.2016.07.039

Usta, J., Kreydiyyeh, S., Knio, K., Barnabe, P., Bou-Moughlabay, Y., and Dagher, S. (2009). Linalool decreases HepG2 viability by inhibiting mitochondrial complexes I and II, increasing reactive oxygen species and decreasing ATP and GSH levels. *Chem. Biol. Interact.* 180, 39–46. doi: 10.1016/j.cbi.2009.02.012

Van Vuuren, S. F., and Viljoen, A. M. (2007). Antimicrobial activity of limonene enantiomers and 1, 8-cineole alone and in combination. *Flavour Fragr. J.* 22, 540–544. doi: 10.1002/ffj.1843

Velluti, A., Sanchis, V., Ramos, A. J., Egido, J., and Mari, S. (2003). Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarose essential oils on growth and fumonisin B 1 production by *Fusarium proliferatum* in maize grain. *Int. J. Food Microbiol.* 89, 145–154. doi: 10.1016/S0168-1605(03)00116-8

Venkataramana, M., Nayaka, S. C., Anand, T., Rajesh, R., Aiyaz, M., Divakara, S. T., et al. (2014). Zearalenone induced toxicity in SHSY-5Y cells: the role of oxidative stress evidenced by N-acetyl cysteine. *Food Chem. Toxicol.* 65, 335–342. doi: 10.1016/j.fct.2013.12.042

Vilela, G. R., de Almeida, G. S., D'Arce, M. A. B. R., Moraes, M. H. D., Brito, J. O., da Silva, M. F. D. G., et al. (2009). Activity of essential oil and its major compound, 1, 8-cineole, from *Eucalyptus globulus* Labill. against the storage fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. *J. Stored Prod. Res.* 45, 108–111. doi: 10.1016/j.jspr.2008.10.006

Whitcomb, P. J., and Anderson, M. J. (2004). *RSM Simplified: Optimizing Processes Using Response Surface Methods for Design of Experiments*. Boca Raton, FL: CRC Press.

Wilson, M. D., Stanley, R. A., Eyles, A., and Ross, T. (2017). Innovative processes and technologies for modified atmosphere packaging of fresh and fresh-cut fruits and vegetables. *Crit. Rev. Food Sci. Nutr.* 1–12. doi: 10.1080/10408398.2017.1375892

Wilson, W., Dahl, B., and Nganje, W. (2018). Economic costs of fusarium head blight, scab and deoxynivalenol. *World Mycotoxin J.* 11, 291–302. doi: 10.3920/WMJ2017.2204

Wu, L., Qiu, L., Zhang, H., Sun, J., Hu, X., and Wang, B. (2017). Optimization for the production of deoxynivalenol and zearalenone by *Fusarium graminearum* using response surface methodology. *Toxins* 9:57. doi: 10.3390/toxins9020057

Xu, L. L., Wen, Y. Q., Liu, Y. L., and Ma, Y. X. (2018). Occurrence of deoxynivalenol in maize germs from North China Plain and the distribution of deoxynivalenol in the processed products of maize germs. *Food Chem.* 198, 111–121. doi: 10.1016/j.foodchem.2018.05.111

Zinedine, A., Soriano, J. M., Molto, J. C., and Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food Chem. Toxicol.* 45, 1–18. doi: 10.1016/j.fct.2006.07.030

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Kalagatur, Kamasani, Siddaiah, Gupta, Krishna and Mudili. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 July 2018
doi: 10.3389/fmicb.2018.01708

Cronobacter sakazakii and Microbiological Parameters in Dairy Formulas Associated With a Food Alert in Chile

Julio Parra-Flores[1], Fabiola Cerda-Leal[1], Alejandra Contreras[1], Nicole Valenzuela-Riffo[1], Alejandra Rodríguez[1] and Juan Aguirre[2]*

[1] Molecular Microbiology Laboratory, Faculty of Health and Food Sciences, Universidad del Bío-Bío, Chillán, Chile,
[2] Department of Agricultural Industry and Enology, Universidad de Chile, Santiago, Chile

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università di Teramo, Italy

Reviewed by:
Chiara Montanari,
Università degli Studi di Bologna, Italy
Antonio Martínez,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

***Correspondence:**
Julio Parra-Flores
juparra@ubiobio.cl;
juparraf@gmail.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 16 April 2018
Accepted: 09 July 2018
Published: 31 July 2018

Citation:
Parra-Flores J, Cerda-Leal F, Contreras A, Valenzuela-Riffo N, Rodríguez A and Aguirre J (2018) Cronobacter sakazakii and Microbiological Parameters in Dairy Formulas Associated With a Food Alert in Chile. Front. Microbiol. 9:1708. doi: 10.3389/fmicb.2018.01708

The objective of this study was to evaluate the presence of *Cronobacter sakazakii* and microbiological parameters in dairy products associated with a food alert. Ninety dairy product samples were analyzed, including seven commercial brands and two product types (liquid and powdered) from four countries. Aerobic plate count (APC) and *Enterobacteriaceae* count were performed according to Chilean standards. *Cronobacter* spp. and *C. sakazakii* were identified by polymerase chain reaction real time amplification of *rpoB* and *cgcA* genes and the genotype by multilocus sequence typing. Eighty-eight percent of dairy products showed APC higher than the detection limit. Fifty percent of liquid commercial brand samples contained APC: 2.6, 2.3, 1.1, and 2.9 CFU/mL in brands A, C, E, and G, respectively. Results for powdered commercial brands were 3.0, 3.6, and 5.7 CFU/g in brands B, D, and F, respectively. Maximum count (5.7 CFU/g) occurred in brand F dairy product manufactured in Chile. *Enterobacteriaceae* were found in 55% of the samples, 64% in liquid and 51% in powdered commercial brands. In 50% of brands B, D, and E, samples contained 2.9, 2.8, and 2.7 log CFU/g, respectively. Only liquid commercial brands from the United States had *Enterobacteriaceae* values between 0.1 and 4.5 CFU/mL. Seventeen suspicious strains were isolated and nine were identified as *Enterobacter* spp. Only eight suspicious strains from four powdered commercial brands (Chile and Singapore) were confirmed as *C. sakazakii* by *rpoB* and *cgcA* gene amplification and *fusA* sequencing. *C. sakazakii* prevalence in the analyzed samples was 8.8%. There were 11% of powdered milk brands that contained APC between 4.0 and 4.7 log CFU/g and 55% of the samples contained *Enterobacteriaceae*. *C. sakazakii* was found in dairy products manufactured in Chile and Singapore. On the basis of this information, the Chilean Ministry of Health (RSA) decreed a national and international food alert and recalled all the product batches that resulted positive in the present study from supermarkets and pharmacies.

Keywords: *Cronobacter sakazakii*, food alert, microbiological parameters, powdered infant formula, liquid dairy formula

INTRODUCTION

On June 2, 2017 the Chilean Ministry of Health issued a national and international food alert as a result of the presence of *Cronobacter sakazakii* in two powdered formula samples intended for children under 10. Researchers from the Universidad del Bío-Bío conducted a study which led to the food alert. This preventive measure was adopted because of the risk of disease associated with *Cronobacter* spp. and *C. sakazakii* in hypersensitive groups of the population (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2008; Jason, 2015).

Cronobacter spp. was initially defined as the new bacterial species *Enterobacter sakazakii* by Farmer et al. (1980); it was later classified by Iversen et al. (2008) and Joseph et al. (2012a,b) as *Cronobacter* spp. and included seven species: *C. sakazakii, C. malonaticus, C. universalis, C. turicensis, C. muytjensii, C. dublinensis,* and *C. condimenti*.

Cronobacter spp. is considered as an emerging pathogen that is especially aggressive in hypersensitive individuals, such as children and the elderly (Fanning and Forsythe, 2008; Hunter and Bean, 2013). Newborns and the elderly are the population groups that are most affected by *C. sakazakii*, although the highest incidence and severity occurs in preterm infants (Hariri et al., 2013). Outbreaks have generally been the most common cause of infection (Jason, 2012). Clinical symptoms are mainly found in meningitis, septicemia, or necrotizing enteritis in infants (Block et al., 2002; Bowen and Braden, 2006), but diarrhea, urinary tract infection, and septicemia have also been observed. Mortality rates are associated with general infection (42–80%) and neonatal meningitis and septicemia (15–25%) (Holý and Forsythe, 2014).

The disease is associated with the consumption of rehydrated milk as a carrier of the pathogen, as well as the eventual involvement of utensils and equipment as reservoirs (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2008; Kalyantanda et al., 2015). Since it is widespread, it can be isolated in powdered infant formula (PIF), rehydrated milk (R-PIF), infant cereals, various foods, water, surfaces, homes, and hospitals (Baumgartner et al., 2009). Even when the source of primary contamination is unclear (Norberg et al., 2012), some researchers suggest that the natural habitat is PIF manufacturing plants. This situation has been reported by Jacobs et al. (2011) and Li et al. (2014), who identified *Cronobacter* spp. in different parts of PIF plants in both China and Australia. It has also been strongly associated with by-products used in its formulation, which are also probable carriers (Jongenburger et al., 2011). The control of this pathogen in the first stages of PIF production is the most important step to reduce its incidence in the final product (Yan et al., 2012; Fei et al., 2015) because viable *Cronobacter* spp. strains have been found 2 years after the product was packaged (Caubilla-Barron and Forsythe, 2007).

Although updated detection and identification techniques are being used, there are still cases of disease and mortality every year (Norberg et al., 2012). It is therefore necessary to improve hygiene and the production process to reduce the impact of *C. sakazakii.* Biochemical tests (API 20E, RAPID, BIOLOG microarray), molecular confirmation of the *Cronobacter* spp. genus by polymerase chain reaction (PCR) (Lehner et al., 2004; Cetinkaya et al., 2012), and especially multilocus sequence typing (MLST) have been used to complement its identification. These techniques have allowed advances in correctly identifying it, and thus decreasing the possibility of false negatives (Baldwin et al., 2009; Joseph and Forsythe, 2011; Yan et al., 2015; Ogrodzki and Forsythe, 2017). Several primers have been generated to detect *Cronobacter* spp. by amplifying specific sequences of variable and conserved regions of 16S ribosomal rDNA of the bacterium (Lehner et al., 2004; Hassan et al., 2007), *OmpA* (Mohan-Nair and Venkitanarayanan, 2006), as well as others that are more specific, which detect *C. sakazakii* by *rpoB* (Stoop et al., 2009; Lehner et al., 2012; Li et al., 2016) and *cgcA* gene amplification (Carter et al., 2013).

Studies of *Cronobacter* spp. incidence in powdered milk have demonstrated a positivity range between 3 and 30% (Chap et al., 2009; Siqueira-Santos et al., 2013; Fei et al., 2017). Sáez et al. (2012) found 5% positivity of *Cronobacter* spp. in 80 PIF samples from a dairy processing plant in the Los Lagos Region in Chile. Parra-Flores et al. (2015a) found an incidence of 9.5% *C. sakazakii* in an exploratory study with a limited number of samples using MLST in PIF manufactured in Chile in 2014. If PIF samples manufactured in other countries are considered, incidence was 2.7% in all the analyzed PIF samples.

Given the need to ensure safety in PIFs, the FAO/WHO have held expert meetings to study cases of diseases related to its consumption, whether epidemiologically or microbiologically. Three categories of microorganisms were identified based on the soundness of evidence of a causal relationship between their presence in food and the disease: (A) microorganisms with clear causality evidence, enteric *Salmonella,* and *Cronobacter* spp. (*E. sakazakii*); (B) microorganisms in which causality is possible but has not yet been demonstrated, primarily from the *Enterobacteriaceae* family; and (C) microorganisms in which causality is less probable or has not yet been demonstrated, and have not been identified in PIF (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2006; Jackson et al., 2015). The WHO therefore recommended the absence of *Cronobacter* spp., *Salmonella*, and *Enterobacteriaceae* in dairy products (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2004, 2006).

Given that PIFs are not sterile foods, the determination of microbial indicators, such as aerobic plate count (APC) and *Enterobacteriaceae* (ENT), provides useful information about the hygienic conditions of their preparation or post-process contamination (Friedemann, 2009; Parra-Flores et al., 2015a; Heperkan et al., 2017).

Cronobacter spp. was not considered in the Chilean Food Sanitary Regulations (Reglamento Sanitario de los Alimentos, RSA) when the Chilean Ministry of Health decreed the food alert. The decision was taken due to the risk of disease associated with the pathogen described in the scientific literature (Jason, 2015), factors that affect PIF contamination (Parra-Flores et al., 2015b), and the variability in its cellular response (Parra-Flores et al., 2016). The PIFs associated with this alert

are not only commercialized in Chile but throughout the Americas.

Therefore, the objective of this study was to evaluate the presence of *C. sakazakii* and the microbiological parameters of APC and ENT in dairy products associated with a food alert in Chile in June 2017.

MATERIALS AND METHODS

Food Samples

Sampling was conducted from August 2016 to May 2017. Ninety samples were collected in four countries (United States, Singapore, Chile, and Holland), from three manufacturers (1, 2, and 3), seven commercial dairy brands (A, B, C, D, E, F, G) of which B, D, and F were powdered and A,C, E, and G were liquid products sold in supermarkets and pharmacies in Chile. All the analyses were performed in duplicate. The sampling criteria used as a reference were the standards of the Chilean RSA and CAC/RPC 66 of the Codex Alimentarius.

Microbiological Quantification

The APC of the mesophilic microorganisms and ENT count were used. Quantification of both microbial groups and identification of isolated enterobacteria (including suspicious strains of *Salmonella* or *Escherichia coli*) were performed in the Accredited Food Testing and Certification Laboratory (LECYCA-UBB) and the Molecular Epidemiology and Microbiology Laboratory of the Universidad del Bío-Bío. References are NCh 2659 (2002) for AMC, NCh 2676 (2002) for ENT, NCh 2636 (2002) for *E. coli*, and NCh 2675 (2002) to isolate *Salmonella*.

Isolation of *Cronobacter* spp.

The technique described by Parra-Flores et al. (2015a) was applied. For each sample, 225 mL of buffered peptone water (BPW) were added to 25 g of powdered infant formula (PIF) or dairy product (DP) and then homogenized in a stomacher at a mean velocity for 60 s. Liquid products in their original container were directly incubated at 37°C. Then 10 mL of each sample was inoculated after incubation at 37°C for 24 h in 90 mL *Enterobacteriaceae* enrichment broth (BD Difco, Sparks, MD, United States). A loop was extracted from the culture suspension and striated in Brilliance Chromogenic Agar CM 1035 (Oxoid Thermo Fisher, United Kingdom) at 37°C for 20 h. Five strains, presumed to be colonies of *Cronobacter* spp. (green or blue), were striated in trypticase soy agar (BD Difco, Sparks, MD, United States) to verify their purity prior to future analyses. The isolated strains were maintained in a strain collection and stored at −80°C.

Identification of *Cronobacter* spp. and *Cronobacter sakazakii*

Genomic DNA of the suspicious strains was extracted and purified with the Ultra Clean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States). The strains were confirmed as *Cronobacter* spp. by *OmpA* gene amplification (Mohan-Nair and Venkitanarayanan, 2006) and later identified as *C. sakazakii* by qPCR amplification of *rpoB* and *cgcA* genes (Stoop et al., 2009; Carter et al., 2013) (**Table 1**) in the Stratagene Mx3000P qPCR System equipment (Agilent Technologies).

Sequencing of *fusA* Gene to Identify Species of *Cronobacter* spp.

The methodology described by Baldwin et al. (2009) was followed using PCR CORE Kit QIAGEN (Cat No. 201225) solutions. Amplified products were sent to MACROGEN in Korea for sequencing. Identification was performed with the free access online database https://pubmlst.org/cronobacter/ and BLASTn (NCBI).

Bioinformatic and Statistical Analyses

The sequenced products were analyzed with the Gentle software and later aligned with the ClustalW software. A phylogenetic tree was constructed using the maximum likelihood method with the MEGA7 software. Statistical description included measures of central tendency, dispersion, and position for quantitative variables, while absolute frequencies and percentages were used for qualitative variables. The Mann–Whitney and Kruskal–Wallis tests were used for comparison purposes with the STATA 14 software at the significance level $\alpha = 0.05$.

RESULTS

Of the 90 analyzed Chilean and foreign samples, 79 had APC. When analyzing APC for each DP commercial brand, no

TABLE 1 | Polymerase chain reaction (PCR) primers used in the study.

Identification	Gene	Primer sequence	Reference	Annealing temperature
Cronobacter spp.	*16S*	F: GCT YTG CTG ACG AGT GGC GG R: ATC TCT GCA GGA TTC TCT GG	Lehner et al., 2004	60°C
Cronobacter spp.	*OmpA*	F: GGATTTAACCGTGAACTTTTCC R: CGCCAGCGATGTTAGAAGA	Mohan-Nair and Venkitanarayanan, 2006	60°C
Cronobacter sakazakii	*rpoB*	F: ACG CCA AGC CTA TCT CCG CG R: ACG GTT GGC GTC ATC GTG	Stoop et al., 2009	67°C
Cronobacter sakazakii	*cgcA*	F: GTGGCSGGGTATGACAAAGAC R: GGCGGACGAAGCCTCAGAGAGT	Carter et al., 2013	62°C
Cronobacter sakazakii	*fusA*	F: GAAACCGTATGGCGTCAG' R: AGAACCGAAGTGCAGACG	Baldwin et al., 2009.	58°C

significant statistical differences were found ($p > 0.05$). However, half of the liquid DP commercial brands contained 2.6 log CFU/mL, 2.3 log CFU/mL, 1.1 log CFU/mL, and 2.9 log CFU/mL for brands A, C, E, and G, respectively. The powdered DP (including PIF) commercial brands had values of 3.0 log CFU/g, 3.6 log CFU/g, and 5.7 log CFU/g for brands B, D, and F, respectively. Positivity for ENT was found in all the evaluated brands. In the liquid DP brands A, C, E, and G, the sample means were 1.8, 0.8, <0.1, and <0.1 log UFC/mL, respectively. In half of powdered DP brands B, D, and E, samples contained 2.9, 2.8, and 2.7 log CFU/g, respectively. Statistical differences were only found in the ENT counts ($p = 0.012$). For DP manufacturers, company 2 had the highest count with 5.9 log CFU/g. *Enterobacteriaceae* counts were found in 55% of the total analyzed samples. Company 1 obtained the highest counts with 3.5 log CFU/g (**Table 2**).

Regarding the country of origin (**Table 3**), Chile exhibited the highest APC count means with 5.0 log CFU/g and the United States showed the lowest count with 2.1 log CFU/g. The DP produced in Holland had the highest ENT counts followed by Singapore with 3.6 and 3.2 log CFU/g, respectively. The lowest count was obtained in the US with 0.1 log CFU/g. No significant differences existed in the APC and ENT counts for country of origin ($p > 0.05$). As for the type of DP, 100% of the liquid DP brands contained APC and 55% ENT counts. Only the liquid DP brands produced in the US had ENT counts with values between 0 and 4.5 log CFU/mL. The highest count in powdered DPs was obtained in Holland with 4.4 log CFU/g.

Of the total analyzed samples, 17 suspicious strains were isolated from the chromogenic agar. Nine were identified as *E. cloacae, Klebsiella pneumoniae, E. hormaechei*, and *Enterobacter* spp., whereas *Salmonella* spp. was not isolated in any of the samples. However, *E. coli* was identified in one powdered milk (PM) product manufactured in Chile.

TABLE 2 | Aerobic plate count (APC) and *Enterobacteriaceae* (ENT) count for each commercial brand and manufacturer.

	Counts	
	APC log CFU/g Median (minimum–maximum)	**ENT log CFU/g Median (minimum–maximum)**
Commercial brand		
A	2.6 (1.7–5.7)	1.8 (1.6–4.5)
B	3.0 (2.3–4.1)	2.9 (1.0–3.6)
C	2.3 (2.0–5.8)	0.85 (0–2.9)
D	3.6 (1.3–5.9)	2.8 (1.0–4.4)
E	1.1 (0.9–5.8)	<0.1 (0.1–4.5)
F	5.7 (1.3–6.7)	2.7 (1.0–3.0)
G	2.9 (2.6–5.7)	<0.1
p*-value	ns	0.0121
Manufacturer		
1	3.0 (1.3–5.9)	2.6 (0–4.5)
2	5.7 (0.9–6.8)	2.7 (1.0–3.0)
3	2.9 (2.6–5.7)	<0.1
p*-value	ns	0.0107

**p-value according to Kruskal–Wallis test. Brands A, C, E, and G: liquid dairy products; Brands B, D, and F: powdered milk products; ns: non-significant.*

TABLE 3 | Aerobic plate count (APC) and *Enterobacteriaceae* (ENT) count for each country.

	Counts	
	APC log CFU Median (minimum–maximum)	**ENT log CFU Median (minimum–maximum)**
Country		
Chile	5.0 (1.3–6.8)	2.7 (1.0–3.0)
Holland	3.6 (1.3–5.9)	2.8 (1.0–4.4)
Singapore	3.0 (2.3–4.1)	2.9 (1.0–3.6)
United States	2.1 (0.9–5.8)	1.7 (0–4.5)
p*-value	ns	ns

**p-value according to Kruskal–Wallis test; **p-value according to Mann–Whitney test. ns, non-significant.*

Only eight suspicious strains from the PM from Chile and Singapore were confirmed as *Cronobacter* spp. by amplifying the *ompA* gene. These strains were subsequently confirmed as *C. sakazakii* through the amplification of the gene products for *rpoB* and *cgcA* by PCR in real time. One of the PM products in which *C. sakazakii* was isolated was intended for consumption by infants under 2 years (CH84), and another from Singapore was intended for consumption by children older than 1 year (CH65).

Furthermore, six more strains were confirmed, which were not part of the food alert because of their expiry date and were PM products manufactured in Chile (CH42, CH43, CH44, CH45, CH50, and CH85) (**Table 4**). Two more samples were also detected by real-time PCR with *Cronobacter* spp. from PIFs manufactured in Holland, but it was not possible to recover the pathogen from the samples.

Cronobacter sakazakii incidence in the total evaluated samples was 8.8% (**Table 5**).

All the *C. sakazakii* strains were genotyped by sequencing the *fusA* gene using MLST in the database https://pubmlst.org/cronobacter/ and BLASTn (NCBI). The information of the sequences was later used to construct a phylogenetic tree (**Figure 1**).

DISCUSSION

The PIFs analyzed in the present study are commercialized throughout the Americas. Therefore, evaluating their microbiological quality allows determining aspects such as the hygienic conditions in which they were prepared, as well as identifying microbial hazards from probable recontamination occurring when they are supplemented with nutrients after pasteurization (Kent et al., 2015).

For APC, 72% of all analyzed powdered and liquid DP samples contained less than 3 log CFU. There were 11% of PM brands that contained between 4.0 and 4.7 log CFU/g originating only from the United States and Singapore; two products manufactured in Chile had values of 6.7 log CFU/g. Liquid DPs revealed five

TABLE 4 | Identification of *Cronobacter* spp. and *Cronobacter sakazakii* by molecular amplification.

Strain	Gene amplification					Identification	
	16S	*OmpA*	*rpoB*	*cgcA*	*fusA* sequencing allelle	MLST database	BLAST
CH42	+	+	+	+	1	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH43	+	+	+	+	1	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH44	+	+	+	+	1	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH45	+	+	+	+	16	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH50	+	+	+	+	16	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH65	+	+	+	+	1	*Cronobacter sakazakii*	*Cronobacter sakazakii*
CH84	+	+	+	+	71	*Cronobacter* spp.	*Cronobacter sakazakii*
CH85	+	+	+	+	71	*Cronobacter* spp.	*Cronobacter sakazakii*

TABLE 5 | Positivity of *Cronobacter sakazakii* for country of origin and product type.

Positivity of *Cronobacter sakazakii* in dairy product (DP) samples					
Country	*n*	Powdered infant formula	Liquid DP	Total	
		(+)	(+)	(+)	%
Chile	20	7	0	7	35
United States	25	NE	0	0	0
Holland	35	0	NE	0	0
Singapore	10	1	NE	1	10
Total	90	8	0	8	8.8

NE, not evaluated.

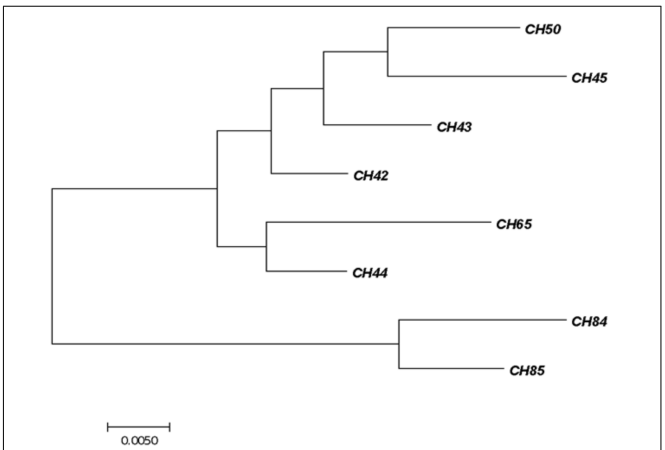


FIGURE 1 | Phylogenetic tree of fusA sequencing identified as *Cronobacter sakazakii*. The tree with the highest log likelihood (–1450.2262) is shown. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms. The analysis involved 24 nucleotide sequences. There were 401 positions in the final dataset. Evolutionary analyses were conducted with the MEGA7 software program.

samples that ranged between 5.6 and 5.8 log CFU/g, four of which were produced in the United States and one in Chile. These APC values are within ranges reported by other authors (Iversen et al., 2004; Kim et al., 2011; Parra-Flores et al., 2015a). However, it was a concern to find counts greater than 5 log CFU/g in PM; these values were very high compared to results reported by Chap et al. (2009), who found 2% in this range. Heperkan et al. (2017) found values between 1.7 log for PIF and 4.9 log CFU/g for PM in a study of 80 PM samples; counts were similar to those determined in the present study. There is an evident need to control the contamination sources of DM products due to the wide range of microorganisms present in the APC and the higher susceptibility of infection in children of different ages who consume DPs (Chap et al., 2009).

Positivity was found for ENT in 55% of all analyzed samples, and there was a significant statistical relationship in the counts for commercial brand ($p = 0.012$) and manufacturer ($p = 0.010$). Eight PM and two liquid DPs obtained count means of 2 log CFU and 11 samples had values from 3.4 to 4.5 log CFU. Muytjens et al. (1988) encountered ENT in 52% of 141 evaluated formulas from 35 countries. On the other hand, ENT was found in 47% of PIF manufactured in Indonesia and Malaysia (Estuningsih et al., 2006), 22.5% in Ivory Coast (Yao et al., 2012), and 100% in Chile (Parra-Flores et al., 2015a). All these ENT counts are much higher than the values permitted according to the international standard of Codex Alimentarius Commission (2008), which requires the absence of this indicator in 10 g. In the present study, the high ENT positivity is compatible with the presence of several opportunistic microorganisms and pathogens associated with disease in infants reported in different publications (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2006; Kent et al., 2015). Therefore, these findings should be analyzed in terms of risk associated with the consumption of PM by infants, the lack of control by manufacturers, and health authorities responsible for inspection in Chile. Although the association between the risk of falling ill with the consumption of ENT-contaminated PM has not yet been established with certainty, its absence in PM provides additional protection for newborns, especially the preterm, immunocompromised, and those with low (<2,500 g) and very low (<1,500 g) birth weight during the preparation, storage, and administration of infant feeding (Abdullah Sani et al., 2013).

Other microorganisms belonging to this family were also identified in the present study. *E. cloacae, K. pneumoniae*,

and *E. hormaechei* were found. This situation does not seem altogether exceptional considering that other authors have also found these microorganisms of the ENT family in PIF (Iversen et al., 2004; Giammanco et al., 2011; Kim et al., 2011; Abdullah Sani et al., 2013), and especially for the risk associated with its ability to maintain itself for at least 8 months under desiccation conditions (Caubilla-Barron and Forsythe, 2007; Juma et al., 2016). Furthermore, the Food and Agriculture Organization and World Health Organization [FAO/WHO] (2004, 2006) also recognized that other ENT can be recovered from PIF and could present a risk to infants, although no reported cases had been confirmed at that time. Jackson et al. (2015) re-evaluated a reported *C. sakazakii* outbreak through the consumption of contaminated reconstituted PIF in Mexico. Using DNA sequencing, they demonstrated that the causative agents were misidentified strains of *E. hormaechei* and *Enterobacter* spp. Meanwhile, *E. hormaechei* has been shown to have clinical significance with the report of several outbreaks of sepsis in neonatal intensive care units in Brazil and the United States (Wenger et al., 1997; Campos et al., 2007; Townsend et al., 2008).

The high APC and ENT values can indicate a non-strict adherence to hygienic practices recommended for the preparation of PIF, which has been mentioned by other authors (Mullane et al., 2007; Jongenburger et al., 2011). This can imply a permanent risk for populations that usually consume this product.

The *C. sakazakii* incidence was 8.8% in the total of evaluated samples, particularly in 10 and 35% of samples produced in Singapore and Chile, respectively. This high positivity should give rise to greater control by the manufacturers and health authorities because of its high lethality, related neurological sequela, and risk of falling ill by *C. sakazakii* (Lai, 2001; Holý and Forsythe, 2014). An infection rate of 1 in 100,000 newborns has been estimated in the United States; this rate increases to 8.7 in 100,000 in infants weighing less than 1500 g, and 1 in 10,660 preterm infants with low birth weight (Hunter and Bean, 2013). In Holland, *Cronobacter* spp. causes from 0.5 to 0.7% of all the cases of meningitis in infants, with a probable range of infection of 0.00062 to 0.62 cases per year. When this probability is adjusted with all the cases that have occurred in the last 30 years, the projected probability is 0.53 cases of infection per year with a rate of 1 in 100,000 infants (Reij et al., 2009). There is no doubt that these values are greatly underestimated (Kucerova et al., 2011; Jason, 2015). Patrick et al. (2014) stated that the median age in adults is 59 for disease caused by *Cronobacter* spp., this value has been widely referred to by the lay press and the representatives of formula manufacturers. Parra-Flores et al. (2016) evaluated cell response variability of *C. sakazakii* after mild heat treatments using stochastic approaches and reported that these can better describe microbial single cell response than deterministic models. They found that the mean probability of illness from the initial inoculum size of 1 cell was less than 0.2 in all cases, while the mean probability of illness was greater than 0.7 in most cases for the inoculum size of 50 cells.

A principal aspect of our study was the correct identification of *Cronobacter* spp. and *C. sakazakii* species by several methods described in the literature (**Table 1**). When comparing the methods, a very good correlation was found for these methods by using different primers with *fusA* gene sequencing, which today enables the most accurate speciation because it follows the whole genome phylogeny and adjusts to taxonomic changes (Forsythe et al., 2014; Jackson et al., 2014; Xu et al., 2014; Alsonosi et al., 2015; Vojkovska et al., 2016). Therefore, the information generated when using molecular techniques can improve the confidence level in the identification and confirmation of presumptive strains even when molecular tests provide the best identification and phenotyping methods (Joseph et al., 2013; Jackson and Forsythe, 2016).

The robustness of the results was a primary facet in our decision to declare the national and international food alert and the massive recall of the products involved. There was another important point health authorities needed to consider, that is, the 2007 WHO recommendation to use water at >70°C to rehydrate PM to limit the risk of infection by *Cronobacter* spp. (World Health Organization [WHO], 2007). It was also recommended that rehydrated PM for children be administered within 2 h of its preparation or conservation under refrigeration at <4°C. This was the main idea of the publicity campaign launched by the authorities for the Chilean population.

Unfortunately, there are situations that warn us that microbiological control cannot be relaxed in food products consumed by hypersensitive populations, such as children and the elderly. For example, the case of the recall of *C. sakazakii*-contaminated PM destined for children in Argentina in 2015, and the recent contamination of Lactalis milk with *Salmonella* spp., which affected 83 European countries. However, recent studies demonstrate that the microbiological quality of these products is still inadequate even when we know that milk and DPs for child feeding are not sterile (Parra-Flores et al., 2015a).

In summary, an inadequate microbiological quality of powdered and liquid PM consumed by children under 10 was found in the present study. The presence of ENT and *C. sakazakii* was also identified, which is a wake-up call to manufacturers and public health regulatory authorities in Chile and throughout the Americas. It is therefore necessary to establish greater control of hygienic conditions in PM production and microbiological vigilance to prevent unnecessary risks for the child population that massively consumes these products (Koletzko et al., 2012). Disease caused in children as a consequence of any pathogen present in the PM they consume, requires manufacturers and health authorities to ensure the highest possible level of food safety (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2006; Jason, 2015; Kent et al., 2015).

CONCLUSION

Powdered infant milk formulas (PIF) are not sterile products; according to the specifications established by the Codex Alimentarius, this type of product should be treated as a possible

food safety issue for high risk populations, such infants and neonates, due to the presence of the *C. sakazakii* pathogen. A total of 11% of the powdered milk brands contained APCs between 4.0 and 4.7 log CFU/g, which is considered as the rejection level by the updated Chilean Food Sanitary Regulations (RSA). Of all the samples, 55% contained *Enterobacteriaceae*; *E. cloacae*, *E. hormaechei*, and *K. pneumoniae* were identified. The overall incidence of *C. sakazakii* was 8.8%, which was found in samples produced either in Chile or Singapore.

Based on this information, the Chilean Ministry of Health decreed a national and international food alert and recalled all the product batches from supermarkets and pharmacies that tested positive in the study. After the first survey conducted for PIF contaminated with *Cronobacter* spp., it was pointed out that this microorganism was present and represented a risk that was not considered in the Chilean food safety standards. The RSA therefore included a new regulation for *Cronobacter* spp. in PIF in November 2017 because of social media pressure and the scientific results provided by our team.

AUTHOR CONTRIBUTIONS

JP-F conceived the experiments. JP-F, FC-L, and JA designed the experiments. NV-R, AC, and JP-F conducted the laboratory work. JP-F, NV-R, AR, and FC-L drafted the manuscript. All authors reviewed and approved the final manuscript.

FUNDING

This study was provided by the Research Directorate of the Universidad del Bío-Bío, Projects 161720 3/R, 091824/R, and GI 171220/EF.

ACKNOWLEDGMENTS

We wish to thank the Chilean Ministry of Health, the Chilean Institute of Public Health, and the Research Directorate of the Universidad del Bío-Bío.

REFERENCES

Abdullah Sani, N., Hartantyo, S., and Forsythe, S. (2013). Microbiological assessment and evaluation of rehydration instructions on powdered infant formulas, follow-up formulas and infant foods in Malaysia. *J. Dairy Sci.* 96, 1–8. doi: 10.3168/jds.2012-5409

Alsonosi, A., Hariri, S., Kajsik, M., Orieskova, M., Hanulik, V., Roderova, M., et al. (2015). The speciation and genotyping of *Cronobacter* isolates from hospitalised patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 1979–1988. doi: 10.1007/s10096-015-2440-8

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., et al. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiol.* 9:223. doi: 10.1186/1471-2180-9-223

Baumgartner, A., Grand, M., Liniger, M., and Iversen, C. (2009). Detection and frequency of *Cronobacter* spp. (*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula. *Int. J. Food Microbiol.* 136, 189–192. doi: 10.1016/j.ijfoodmicro.2009.04.009

Block, C., Peleg, O., Minster, N., Bar-Oz, B., Simhon, A., Arad, I., et al. (2002). Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. *Eur. J. Clin. Microbiol. Infect. Dis.* 21, 613–616. doi: 10.1007/s10096-002-0774-5

Bowen, A., and Braden, C. (2006). Invasive *Enterobacter sakazakii* disease in infants. *Emerg. Infect. Dis.* 12, 1185–1189. doi: 10.3201/eid1208.051509

Campos, L., Lobianco, L., Seki, L., Santos, R., and Asensi, M. (2007). Outbreak of *Enterobacter hormaechei* septicaemia in newborns caused by contaminated parenteral nutrition in Brazil. *J. Hosp. Infect.* 66, 95–97. doi: 10.1016/j.jhin.2007.02.013

Carter, L., Lindsey, L. A., Grim, C. J., Sathyamoorthy, V., Jarvis, K. G., Gopinath, G., et al. (2013). Multiplex PCR assay targeting a diguanylate cyclase-encoding gene, *cgcA*, to differentiate species within the genus *Cronobacter*. *Appl. Environ. Microbiol.* 79, 734–737. doi: 10.1128/AEM.02898-12

Caubilla-Barron, J., and Forsythe, S. J. (2007). Dry stress and survival time of *Enterobacter sakazakii* and other *Enterobacteriaceae* in dehydrated powdered infant formula. *J. Food Prot.* 70, 2111–2117. doi: 10.4315/0362-028X-70.9.2111

Cetinkaya, E., Joseph, S., Ayhan, K., and Forsythe, S. (2012). Comparison of methods for the microbiological identification and profiling of *Cronobacter* species from ingredients used in the preparation of infant formula. *Mol. Cell. Probes* 27, 60–64. doi: 10.1016/j.mcp.2012.10.003

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., et al. (2009). International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. *Int. J. Food Microbiol.* 136, 185–188. doi: 10.1016/j.ijfoodmicro.2009.08.005

Codex Alimentarius Commission (2008). *CAC/RCP 66 – 2008. Code of Hygienic Practice for Powdered Formulae for Infants and Young Children. Codex Web site*. Available at: www.fao.org/input/download/standards/11026/CXP_066e.pdf

Estuningsih, S., Kress, C., Hassan, A., Akineden, O., Schneider, E., and Usleber, E. (2006). *Enterobacteriaceae* in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. *J. Food Prot.* 69, 3013–3017. doi: 10.4315/0362-028X-69.12.3013

Fanning, S., and Forsythe, S. (2008). "Isolation and identification of *Enterobacter sakazakii*," in *Enterobacter sakazakii. Emerging Issues in Food Safety Series*, eds J. M. Farber and S. J. Forsythe (Washington, DC: ASM Press), 27–59.

Farmer, J. J., Asbury, M. A., Hickman, F., Brenner, D., and The Enterobacteriaceae Study Group (1980). *Enterobacter sakazakii*, new species of *Enterobacteriaceae* isolated from clinical specimens. *Int. J. Syst. Bacteriol.* 30, 569–584. doi: 10.1099/00207713-30-3-569

Fei, P., Jiang, Y., Feng, J., Forsythe, S. J., Li, R., Zhou, Y., et al. (2017). Antibiotic and desiccation resistance of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and processing environments. *Front. Microbiol.* 8:316. doi: 10.3389/fmicb.2017.00316

Fei, P., Man, C., Lou, B., Forsythe, S., Chai, Y., Li, R., et al. (2015). Genotyping and source tracking of the *Cronobacter sakazakii* and *C. malonaticus* isolated from powdered infant formula and an infant formula production factory in China. *Appl. Environ. Microbiol.* 81, 5430–5439. doi: 10.1128/AEM.01390-15

Food and Agriculture Organization and World Health Organization [FAO/WHO] (2004). "Enterobacter sakazakii and Other Microorganisms in Powdered Infant Formula: Meeting Report. Microbiological Risk Assessment. Series No. 6. Geneva: WHO.

Food and Agriculture Organization and World Health Organization [FAO/WHO] (2006). *Enterobacter sakazakii and Salmonella Powdered Infant Formula. Microbiological Risk Assessment.* Series No. 10. Rome: FAO.

Food and Agriculture Organization and World Health Organization [FAO/WHO] (2008). *Enterobacter sakazakii (Cronobacter spp.) in Powdered Follow-Up Formulae. Microbiological Risk Assessment.* Series No. 15. Rome: FAO.

Forsythe, S. J., Dickins, B., and Jolley, K. A. (2014). *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. *BMC Genomics* 15:1121. doi: 10.1186/1471-2164-15-1121

Friedemann, M. (2009). Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 28, 1297–1304. doi: 10.1007/s10096-009-0779-4

Giammanco, G., Aleo, A., Guida, I., and Mammina, C. (2011). Molecular epidemiological survey of *Citrobacter freundii* misidentified as *Cronobacter* spp. (*Enterobacter sakazakii*) and *Enterobacter hormaechei* isolated from powdered infant milk formula. *Foodborne Pathog. Dis.* 8, 517–525. doi: 10.1089/fpd.2010.0719

Hariri, S., Joseph, S., and Forsythe, S. J. (2013). *Cronobacter sakazakii* ST4 strains and neonatal meningitis, United States. *Emerg. Infect. Dis.* 19, 175–177. doi: 10.3201/eid1901.120649

Hassan, A., Akineden, A., Kress, C., Estuningsih, S., Schneider, E., and Usleber, E. (2007). Characterization of the gene encoding the 16S rRNA of *Enterobacter sakazakii* and development of a species specific PCR method. *Int. J. Food Microbiol.* 116, 214–220. doi: 10.1016/j.ijfoodmicro.2006.12.011

Heperkan, D., Dalkilic-Kaya, G., and Juneja, V. (2017). *Cronobacter sakazakii* in baby foods and baby food ingredients of dairy origin and microbiological profile of positive samples. *LWT Food Sci. Technol.* 75, 402–407. doi: 10.1016/j.lwt.2016.09.013

Holý, O., and Forsythe, S. J. (2014). *Cronobacter* species as emerging causes of healthcare-associated infection. *J. Hosp. Infect.* 86, 169–177. doi: 10.1016/j.jhin.2013.09.011

Hunter, C. J., and Bean, J. F. (2013). *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *J. Perinatol.* 33, 581–585. doi: 10.1038/jp.2013.26

Iversen, C., Lane, M., and Forsythe, S. J. (2004). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Lett. Appl. Microbiol.* 38, 378–382. doi: 10.1111/j.1472-765X.2004.01507.x

Iversen, C., Mullane, N., Mc Cardell, B., Tall, B. D., Lehner, A., Fanning, S., et al. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. muytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. dublinensis subsp. nov., *C. dublinensis* sp. nov. subsp. lausannensis subsp. nov., and *C. dublinensis* sp. nov. subsp. lactaridi subsp. nov. *Int. J. Syst. Evol. Microbiol.* 58, 1442–1447. doi: 10.1099/ijs.0.65577-0

Jackson, E., and Forsythe, S. J. (2016). Comparative study of *Cronobacter* identification according to phenotyping methods. *BMC Microbiol.* 16:146. doi: 10.1186/s12866-016-0768-6

Jackson, E., Parra-Flores, J., Fernandez-Escartin, E., and Forsythe, S. J. (2015). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *J. Food Prot.* 2015 78, 1191–1196. doi: 10.4315/0362-028X.JFP-14-563

Jackson, E., Sonbol, H., Masood, N., and Forsythe, S. (2014). Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *Cronobacter helveticus, Cronobacter pulveris*, and *Cronobacter zurichensis*. *Food Microbiol.* 44, 226–235. doi: 10.1016/j.fm.2014.06.013

Jacobs, C., Braun, P., and Hammer, P. (2011). Reservoir and routes of transmission of *Enterobacter sakazakii* (*Cronobacter* spp.) in a milk powder-producing plant. *J. Dairy Sci.* 94, 3801–3810. doi: 10.3168/jds.2011-4318

Jason, J. (2012). Prevention of invasive *Cronobacter* infection in young infants fed powdered infant formulas. *Pediatrics* 130, 1076–1084. doi: 10.1542/peds.2011-3855

Jason, J. (2015). The roles of epidemiologists, laboratorians, and public health agencies in preventing invasive *Cronobacter* infection. *Front. Pediatr.* 3:110. doi: 10.3389/fped.2015.00110

Jongenburger, I., Reij, M., Boer, E., Gorris, L., and Zwietering, M. (2011). Actual distribution of *Cronobacter* spp in industrial batches of powdered infant formula and consequences for performance of sampling strategies. *Int. J. Food Microbiol.* 151, 62–69. doi: 10.1016/j.ijfoodmicro.2011.08.003

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., and Forsythe, S. J. (2012a). *Cronobacter condimenti* sp. Nov., isolated from spiced meat, and *Cronobacter universalis* sp. Nov., a species designation for *Cronobacter* sp. Geneomoespecies 1, recovered from a leg infection, water and food ingredients. *Int. J. Syst. Evol. Microbiol.* 62, 1277–1283. doi: 10.1099/ijs.0.032292-0

Joseph, S., and Forsythe, S. (2011). Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerg. Infect. Dis.* 17, 1713–1715. doi: 10.3201/eid1709.110260

Joseph, S., Hariri, S., and Forsythe, S. (2013). Lack of continuity between *Cronobacter* biotypes and species as determined using multilocus sequence typing. *Mol. Cell. Probes* 27, 137–139. doi: 10.1016/j.mcp.2013.02.002

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., and Forsythe, S. (2012b). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. *J. Clin. Microbiol.* 50, 3031–3039. doi: 10.1128/JCM.00905-12

Juma, N., Manning, G., and Forsythe, S. (2016). Desiccation survival of *Acinetobacter* spp. in infant formula. *Food Control* 68, 162–166. doi: 10.1016/j.foodcont.2016.03.043

Kalyantanda, G., Shumyak, L., and Archibald, L. K. (2015). *Cronobacter* species contamination of powdered infant formula and the implications for neonatal health. *Front. Pediatr.* 3:56. doi: 10.3389/fped.2015.00056

Kent, R., Fitzgerald, G., Hill, C., Stanton, C., and Ross, R. (2015). Novel approaches to improve the intrinsic microbiological safety of powdered infant milk formula. *Nutrients* 7, 1217–1244. doi: 10.3390/nu7021217

Kim, S., Oh, S., Lee, Y., Imm, J., Hwang, I., Kang, D., et al. (2011). Microbial contamination of food products consumed by infants and babies in Korea. *Lett. Appl. Microbiol.* 53, 532–538. doi: 10.1111/j.1472-765X.2011.03142.x

Koletzko, B., Shamir, R., and Ashwell, M. (2012). Quality and safety aspects of infant nutrition. *Ann. Nutr. Metab.* 60, 179–184. doi: 10.1159/000338803

Kucerova, E., Joseph, S., and Forsythe, S. (2011). The *Cronobacter* genus: ubiquity and diversity. *Qual. Assur. Safety Crops Foods* 3, 104–122. doi: 10.1111/j.1757-837X.2011.00104.x

Lai, K. K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and review of the literature. *Medicine* 80, 113–122. doi: 10.1097/00005792-200103000-00004

Lehner, A., Fricker-Feer, C., and Stephan, R. (2012). Identification of the recently described *Cronobacter condimenti* by an rpoB-gene-based PCR system. *J. Med. Microbiol.* 61, 1034–1035. doi: 10.1099/jmm.0.042903-0

Lehner, A., Tasara, T., and Stephan, R. (2004). 16S rRNA gene based analysis of *Enterobacter sakazakii* strains from different sources and development of a PCR assay for identification. *BMC Microbiol.* 4:43. doi: 10.1186/1471-2180-4-43

Li, Y., Chen, Q., Zhao, J., Lu, F., Bie, X., and Lu, Z. (2014). Isolation, identification and antimicrobial resistance of *Cronobacter* spp. iolated from various foods in China. *Food Control* 37, 109–114. doi: 10.1016/j.foodcont.2013.09.017

Li, Z., Ge, W., Li, K., Gan, J., Zhang, Y., Zhang, Q., et al. (2016). Prevalence and Characterization of *Cronobacter sakazakii* in retail milk-based infant and baby foods in Shaanxi, China. *Foodborne Pathog. Dis.* 13, 1–7. doi: 10.1089/fpd.2015.2074

Mohan-Nair, M. K., and Venkitanarayanan, K. S. (2006). Cloning and sequencing of the *ompA* gene of *Enterobacter sakazakii* and development of an *ompA*-targeted PCR for rapid detection of *Enterobacter sakazakii* in infant formula. *Appl. Environ. Microbiol.* 72, 2539–2546. doi: 10.1128/AEM.72.4.2539-2546.2006

Mullane, N., Whyte, P., Wall, P., Quinn, T., and Fanning, S. (2007). Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *Int. J. Food Microbiol.* 116, 73–81. doi: 10.1016/j.ijfoodmicro.2006.12.036

Muytjens, H. L., Zanen, H. C., Sonderkamp, H. J., Kollee, L. A., Wachsmuth, I. K., and Farmer, J. J. (1988). Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. *J. Clin. Microbiol.* 18, 115–120.

Norberg, S., Stanton, C., Ross, R., Hill, C., Fitzgerald, G., and Cotter, P. (2012). *Cronobacter* spp. in powdered infant formula. *J. Food Prot.* 75, 607–620. doi: 10.4315/0362-028X.JFP-11-285

Ogrodzki, P., and Forsythe, S. J. (2017). DNA-sequence based typing of the *Cronobacter* genus using MLST, CRISPR-cas array and capsular profiling. *Front. Microbiol.* 8:1875. doi: 10.3389/fmicb.2017.01875

Parra-Flores, J., Juneja, V., Garcia de Fernando, G., and Aguirre, J. (2016). Variability in cell response of *Cronobacter sakazakii* after mild-heat treatments and its impact on food safety. *Front. Microbiol.* 7:535. doi: 10.3389/fmicb.00535

Parra-Flores, J., Oliveras, L., Rodriguez, A., Riffo, F., Jackson, E., and Forsythe, S. (2015a). Risk of *Cronobacter sakazakii* contamination in powdered milk

for infant nutrition. *Rev. Chil. Nutr.* 42, 83–89. doi: 10.4067/S0717-75182015000100011

Parra-Flores, J., Rodriguez, A., Riffo, F., Arvizu-Medrano, S. M., Arias-Rios, E. V., and Aguirre, J. (2015b). Investigation on the factors affecting *Cronobacter sakazakii* contamination levels in reconstituted powdered infant formula. *Front. Pediatr.* 3:72. doi: 10.3389/fped.2015.00072

Patrick, M., Mahon, B., Greene, S., Rounds, J., Conquist, A., Wymore, K., et al. (2014). Incidence of *Cronobacter* spp. infections, United States, 2003–2009. *Emerg. Infect. Dis.* 20, 1520–1523. doi: 10.3201/eid2009.140545

Reij, M., Jongerburger, I., Gkogka, E., Gorris, L., and Zwietering, M. (2009). Perspective on the risk to infants in the Netherlands associated with *Cronobacter* spp. occurring in powdered infant formula. *Int. J. Food Microbiol.* 136, 232–237. doi: 10.1016/j.ijfoodmicro.2009.07.011

Sáez, M., Llanos, S., and Tamayo, R. (2012). Primer aislamiento de *Cronobacter* spp. *(Enterobacter sakazakii)* en fórmula láctea en polvo producida en Chile. *Rev. Chil. Salud Púb.* 16, 11–15. doi: 10.5354/0719-5281.2012.18607

Siqueira-Santos, R. F. S., da Silva, N., Junqueira, V. C. A., Kajsik, M., Forsythe, S., and Pereira, J. L. (2013). Screening for *Cronobacter* species in powdered and reconstituted infant formulas and from equipment used in formula preparation in maternity hospitals. *Ann. Nutr. Metab.* 63, 62–68. doi: 10.1159/000353137

Stoop, B., Lenher, A., Iversen, C., and Fanning, S. (2009). Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int. J. Food Microbiol.* 136, 165–168. doi: 10.1016/j.ijfoodmicro.2009.04.023

Townsend, S., Hurrell, E., and Forsythe, S. (2008). Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. *BMC Microbiol* 8:64. doi: 10.1186/1471-2180-8-64

Vojkovska, H., Karpiskova, R., Orieskova, M., and Drahovska, H. (2016). Characterization of *Cronobacter* spp. isolated from food of plant origin and environmental samples collected from farms and from supermarkets in the Czech Republic. *Int. J. Food Microbiol.* 217, 130–136. doi: 10.1016/j.ijfoodmicro.2015.10.017

Wenger, P., Tokars, J. I., Brennan, P., Samel, C., Bland, L., Miller, M., et al. (1997). An Outbreak of *Enterobacter hormaechei* infection and colonization in an intensive care nursery. *Clin. Infect. Dis.* 24, 1243–1244. doi: 10.1086/513650

World Health Organization [WHO] (2007). *Safe Preparation, Storage and Handling of Powdered Infant Formula: Guidelines*. Geneva: WHO.

Xu, X., Qingping, W., Jumei, Z., Yingwang, Y., Xiaojuan, Y., and Xiaohui, D. (2014). Occurrence and characterization of *Cronobacter* spp. in powdered formula from Chinese retail markets. *Foodborne Pathog. Dis.* 11, 307–312. doi: 10.1089/fpd.2013.1657

Yan, Q., Condell, O., Power, K., Buttler, F., Tall, B., and Fanning, S. (2012). *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium. *J. Appl. Microbiol.* 113, 1–15. doi: 10.1111/j.1365-2672.2012.05281.x

Yan, Q., Wang, J., Gangiredla, J., Cao, Y., Martins, M., Gopinath, G., et al. (2015). Comparative genotypic and phenotypic analysis of *Cronobacter* species cultured from four powdered infant formula production facilities: indication of pathoadaptation along the food chain. *Appl. Environ. Microbiol.* 81, 4388–4402. doi: 10.1128/AEM.00359-15

Yao, K., Zinzendorf, N., Bohoua, G., Kouassi, K., Koua, A., Kouadio, L., et al. (2012). Occurrence of *Enterobacter sakazakii* (Cronobacter) and other *Enterobacteriaceae* in commercial powdered infant formula in Abidjan, Ivory Coast. *Food Nutr. Sci.* 3, 822–826. doi: 10.4236/fns.2012.36110

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Parra-Flores, Cerda-Leal, Contreras, Valenzuela-Riffo, Rodríguez and Aguirre. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 August 2018
doi: 10.3389/fmicb.2018.01568

Exploration of the Regulatory Mechanism of Secondary Metabolism by Comparative Transcriptomics in *Aspergillus flavus*

*Guangshan Yao, Yuewei Yue, Yishi Fu, Zhou Fang, Zhangling Xu, Genli Ma and Shihua Wang**

Fujian Key Laboratory of Pathogenic Fungi and Mycotoxins, Key Laboratory of Biopesticide and Chemical Biology of Ministry of Education, School of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, China

Mycotoxins cause a huge threaten to agriculture, food safety, and human and animal life. Among them, aflatoxins (AFs) have always been considered the most potent carcinogens, and filamentous fungi from *Aspergillus* genus are their major producers, especially *A. flavus*. Although the biosynthesis path of these chemicals had been well-identified, the regulatory mechanisms controlling expression of AF gene cluster were poorly understood. In this report, genome-wide transcriptome profiles of *A. flavus* from AF conducing [yeast sucrose media (YES)] and non-conducing [yeast peptone media (YEP)] conditions were compared by using deep RNA sequencing (RNA-seq), and the results revealed that AF biosynthesis pathway and biosynthesis of amino acids were significantly upregulated in YES vs. YEP. Further, a novel LaeA-like methyltransferase AFLA_121330 (Lael1) was identified for the first time, to play a specific role in the regulation of AF biosynthesis. Contrary to LaeA, which gene deletion reduced the level, *lael1* deletion resulted in a significant increase in AF production. Further, co-expression network analysis revealed that mitochondrial pyruvate transport and signal peptide processing were potentially involved in AF synthesis for the first time, as well as biological processes of ribosome, branched-chain amino acid biosynthetic process and translation were co-regulated by AfRafA and AfStuA. To sum up, our analyses could provide novel insights into the molecular mechanism for controlling the AF and other secondary metabolite synthesis, adding novel targets for plant breeding and making fungicides.

Keywords: ***Aspergillus flavus*, aflatoxin, transcriptome, LaeA-like methyltransferase, RNA-seq**

OPEN ACCESS

Edited by:
Pierina Visciano,
Università degli Studi di Teramo, Italy

Reviewed by:
Gang Liu,
Institute of Microbiology (CAS), China
Massimo Reverberi,
Sapienza Università di Roma, Italy

***Correspondence:**
Shihua Wang
wshyyl@sina.com

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *04 January 2018*
Accepted: *25 June 2018*
Published: *07 August 2018*

Citation:
Yao G, Yue Y, Fu Y, Fang Z, Xu Z, Ma G and Wang S (2018) Exploration of the Regulatory Mechanism of Secondary Metabolism by Comparative Transcriptomics in Aspergillus flavus. Front. Microbiol. 9:1568. doi: 10.3389/fmicb.2018.01568

INTRODUCTION

Aflatoxins (AFs) has always been labeled as the most potent carcinogens (Squire, 1981; Amaike and Keller, 2011). In general, AF and its major producer *Aspergillus flavus* have been frequently detected in oil-enriched seeds such as peanuts, maize seeds, and walnuts (Bhatnagar-Mathur et al., 2015). However, a recent fear was that aflatoxin B1 (AFB1) or aflatoxin M1(AFM1) were widely detected in our daily food on a global scale, including their presence in rice and rice products in Pakistan (Iqbal et al., 2016), sugarcane juice in Egypt (Abdallah et al., 2016), sausages in Croatia

(Kabak and Dobson, 2017), milk in Europe (Kumar et al., 2017b), vegetable oil (Sun et al., 2011) and Chinese traditional medicines in China (Zhao et al., 2016), and even human breast milk in Turkey (Kiliç Altun et al., 2017). An increasing body of evidences demonstrated that exposure to or ingestion of AF severely impaired human or animal health (Kumar et al., 2017b). Long time ingestion of AF-containing food or food adducts are associated with high rates in hepatocellular carcinoma (Pitt and Miller, 2017). Even worse, several lines of evidences suggested that AF uptake and hepatitis B virus synergistically induced the development of liver cancer (Kew, 2003; Aydin et al., 2015; Chu et al., 2017). Therefore, an improved understanding of AF synthesis and metabolism, and where its regulatory mechanism is urgently required, which would greatly contribute to the development of new and effective long-term management strategies to avoid the severe effects caused by these toxic chemicals.

Until now, the AF biosynthetic pathway had been essentially clarified (Roze et al., 2013), however, the regulatory mechanism that orchestrating the AF cluster gene expression remains largely unknown. Nutrient significantly impact the synthesis of AF, and yeast sucrose media (YES) containing high concentration sucrose could induce the formation of AFs, but yeast peptone media (YEP) could not (Fountain et al., 2015). Paradoxically, an earlier report found that addition of peptone in a chemical defined media increased AFs (Reddy et al., 1979). Compared to other nitrogen source, glutamine was reported to be the best one to induce the toxin production (Wang et al., 2017). As the secondary metabolism from *Aspergillus*, biosynthesis of AFs was affected and governed by various environmental cues and a number of proteins at multiple levels, including chromosome, transcription, and post-translation modifications *in vivo* (Amaike and Keller, 2011; Amare and Keller, 2014; Calvo and Cary, 2015). A screen of sterigmatocystin (the precursor of AFs) mutants in *A. nidulans* lead to the identification of an essential regulator LaeA (Bok and Keller, 2004). It is well-known that LaeA, containing a methyltransferase domain, functions as a global regulator of secondary metabolism in various filamentous fungi (Bok and Keller, 2004; Kale et al., 2008; Kosalkova et al., 2009; Chettri and Bradshaw, 2016; Kumar et al., 2017a), especially toxicogenic *A. flavus* (Kale et al., 2008). And, a systematic transcriptome analysis revealed that LaeA affected expression of 26 of 55 secondary metabolite biosynthetic clusters in *A. flavus* (Georgianna et al., 2010). Recently, LlmF, a laeA-like protein, was reported to negatively regulate the production of sterigmatocystin production in *A. nidulans* by modulating the nuclear import of VeA in *A. nidulans* (Palmer et al., 2013). As in *A. nidulans*, a number of novel proteins containing a methyltransferases domain showed high similarity to LaeA in *A. flavus*. Until now, only the role of LaeA in AF synthesis had been confirmed (Bok and Keller, 2004; Kale et al., 2008), but other LaeA-like methyltransferases remains unidentified in *A. flavus*.

Pathway-specific and global transcription factors (TFs) were also reported to critically involve in the regulation of AF gene cluster expression. AflR, as the AF pathway-specific TF, is absolutely required for expression of most of the genes in the AF cluster (Ehrlich et al., 1999). And, AflS (previously named as AflJ) was reported to interact with, and activate AflR to exert its regulatory role (Chang, 2003). It was now well known that fungal development was closely associated with biosynthesis of secondary metabolism (Amare and Keller, 2014; Calvo and Cary, 2015). It was recently found that several TFs co-regulated the conidiation, sclerotial development and AF formation in *A. flavus*, including MtfA (Zhuang et al., 2016), RtfA (Lohmar et al., 2016), and NsdC (Gilbert et al., 2016). More recently, we had identified two APSES TFs AfRafA and AfStuA to be required for fungal conidial and sclerotial development, and colonization of plants. Further, loss of AfStuA completely inhibited the biosynthesis of mycotoxins including AFs and cyclopiazonic acid, and Δ*AfRafA* reduced AF, but enhanced the cyclopiazonic acid biosynthesis (Yao et al., 2017). Therefore, it is reasonable to speculate that AfRafA and AfStuA mediate a complex regulatory network with crucial roles in multiple cellular processes in *A. flavus*. Undoubtedly, comprehensively identifying their downstream genes or pathways contributes to obtain novel regulators for toxin biosynthesis or fungal pathogenicity.

In the present report, we comprehensively compared the transcriptomes of *A. flavus* WT strains culturing in AF inducing vs. non-inducing conditions, Δ*AfRafA* vs. WT, and Δ*AfStuA* vs. WT. Our results revealed that amino acid biosynthesis and metabolism were significantly activated under YES relative to YEP and subjected to regulation of both AfRafA and AfStuA. Weighted correlation network analysis of differentially expressed genes reveal that AfRafA and AfStuA coordinately control novel cellular processes with potential roles in AF biosynthesis. Further, many AF regulators were found to be regulated by AfRafA or AfStuA, or both, and activated in YES vs. YEP. In addition, our comparative transcriptome suggested that expression of many SM gene clusters differentially response to AfRafA and AfStuA. Most importantly, a novel LaeA-like protein Lael1 was demonstrated to have a specific role in regulation of the AF expression.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains used in this study including WT, Δ*AfRafA*, and Δ*AfStuA* were stored in our lab constructed previously (Yao et al., 2017). The strain PTSΔku70ΔpyrG (Chang et al., 2010) was used as the recipient strain to generate the deletion mutants for gene *AFLA_121330*. All *A. flavus* strains were cultured onto Potato dextrose agar (PDA, BD Difco, United States) to obtain mycelia and conidia, and then stored in 30% glycerol solution at −70°C. When compared AF synthesis, YES (2% yeast extract, 15% sucrose, and 0.1% $MgSO_4$) and GMM (Glucose 10 g/L, $NaNO_3$ 6 g, KCl 0.52 g/L, $MgSO_4.7H_2O$ 0.52 g/L, KH_2PO_4 1.52 g/L, and added 1 mL trace elements solution per liter) with 5 mM glutamine (Shimizu

and Keller, 2001), and YEP (2% yeast extract, 15% peptone) represents the AF conducing and non-inducing conditions, respectively.

Determination of AF Production via TLC and HPLC

Extraction and determination of AFs were performed as previously described (Yao et al., 2017). The AFs were extracted by using chloroform. TLC analysis of AFB1 was performed with the acetone:chloroform (1:9, v/v) solvent system, and AFB1 spots were displayed under ultraviolet activation at 365 nm. HPLC analysis of AFB1 was conducted by using the Waters HPLC 1525 system (Waters, United States) equipped with a MYCOTOX^{TM} reversed-phase C18 column (5 μm, 4.6 mm × 150 mm) and a fluorescent detector (λ_{ex} = 365 nm, λ_{em} = 430 nm). Firstly, the column was equilibrated in the mobile phase (water:methanol:acetonitrile, 56:22:22) at 42°C for 1 h. Each chloroform extract was re-dissolved in methanol, filtered through a 0.22 μm nylon filter membrane, and then separated in a 100% mobile phase at a flow rate of 1.0 mL/min. The AFB1 concentration of each sample was counted by using a calibration curves and the AFB1 standard (HPLC grade) were purchased from Sigma (Sigma, Germany).

Gene Deletion and Complementation

Extraction of genomic DNA of *A. flavus* and standard PCR were performed as previously described (Yu et al., 2004). The AfRafA and AfStuA deletion strains used were constructed previously (Yao et al., 2017) and the *Lael1* deletion strains was generated in the present study. Double-joint PCR was used to construct a gene deletion cassette, using the *pyrG* gene amplified from *A. fumigatus* as the selectable marker. All primers that were used to amplify the 5′- and 3′-flanks were listed in **Supplementary Table S2** with the *A. flavus* gDNA as template. The entire gene deletion cassette was amplified with specific primers, using the 5′- and 3′-flanks for gene *AFLA_121330*, and *pyrG* mix as template. The PCR products were transformed into the protoplasts of PTSΔ*ku70*Δ*pyrG*. Protoplast preparation and PEG-mediated fungal transformation were performed following previously described methods (Chang, 2008). Diagnostic PCR and RT-PCR were used to identify the positive transformants. To construct the complementation strain, an expression cassette containing promoter, coding region, and terminator was amplified by using high-fidelity pfu DNA polymerase (TransGen, Beijing, China). Purified expression cassette together with plasmid pTRI with pyrithiamine (*ptrA*) as the selectable marker (Chang et al., 2010), were co-transformed into the protoplast of the deleted strain. All primers used are listed in **Table 1**.

RNA Extraction

Aspergillus flavus strains were inoculated into YES liquid media and pre-cultured for 24 h, and then transferred into fresh YES media or YEP for stationary culture at 29°C for 48 h. The collected mycelia were frozen in liquid nitrogen, and stored under −80°C conditions. RNA extraction was performed using an RNA reagent Kit (TRIzol reagent, Biomarker Technologies, China) and following the protocols of the manufacture. The quality and integrity of RNA samples were determined using Nanodrop and Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), respectively, while the quantity was determined with a Qubit RNA assay kit (Life Invitrogen, United States).

TABLE 1 | Primers used in this study.

Primer name	Sequence 5′–3′
AFLA_121330F1	AATGGAGCACCCACTTTGAC
AFLA_121330F2	ATGGACGATAAATCAAACGA
AFLA_121330R1	ATCGCAGATAGTTTAGCACC
AFLA_121330R2	AGAATACGAGGCGAACAATA
AFLA121330pyrGR	GGGTGAAGAGCATTGTTTGAG GCTCTTGTTCAAGAATTGCGGA
AFLA121330pyrGF	GCATCAGTGCCTCCTCTCAGA CCTGCCTACCGATGATCGATA
PyrGF	GCCTCAAACAATGCTCTTCACCC
PyrGR	GTCTGAGAGGAGGCACTGATGC
AFLA_121330RTF	TCAACTGCTTCTCCGACGAT
AFLA_121331RTR	CAATTCCTTCCGCATACCTG

RNA-seq and Enrichment Analysis of Differentially Expressed Genes

The total RNA of three biological replicates for Δ*AfRafA*, Δ*AfStuA*, and WT grown in YES and YEP was sequenced. Libraries were prepared according to standard protocols from Illumina Inc. (San Diego, CA, United States) and sequenced on a HiSeq 2000 platform (Novogene, Beijing, China). Low-quality reads (Phred ≤ 20) and adaptor sequences were filtered out, and the Q20, Q30, and GC content of the clean data were calculated (**Table 2**). Sequenced clean reads were mapped against predicted transcripts of the *A. flavus* NRRL 3357 genome[1] using Tophat v2.0.4 (Trapnell et al., 2009), and only unique matches were allowed. Transcript abundance (i.e., FPKM) were estimated using the HTSeq package. Differentially expressed genes were analyzed with the DESeq package (Anders and Huber, 2010), and both a twofold change cut-off and an adjusted *p*-value of ≤0.05 were established as thresholds. An enrichment analysis of differential expression was performed using the GOSeq R package (Young et al., 2010). GO terms (including cellular component, molecular function, and biological process) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were classified as significantly enriched among differentially expressed genes only when their Benjamini adjusted *p*-values were ≤0.05. All the RNA-seq data had been stored in GEO database with an ID of GSE107025.

Real Time Quantitative PCR

cDNA was synthesized from above mRNA sample by using the RevertAid RT Reverse Transcription Kit (Thermo Scientific, United States) following the manufacture's instruction. Real time quantitative PCR (qPCR) was performed on a PikoReal Real-Time PCR System (Thermo Scientific, Inc.). All utilized primers

[1]http://fungi.ensembl.org/Aspergillus_flavus/Info/Index

TABLE 2 | Summary of RNA-Seq data.

Sample	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
WTYES	30430096	28722480	4.31G	0.02	95.62	89.42	52.3
WTYES	26018734	25164896	3.77G	0.02	95.41	88.94	52.18
WTYES	33291592	32246484	4.84G	0.01	97.45	93.38	52.38
DAfRafA	27344356	26437012	3.97G	0.02	95.36	88.9	52.15
DAfRafA	27222764	26268644	3.94G	0.02	95.39	88.94	52.3
DAfRafA	25355708	24273186	3.64G	0.03	95.04	88.26	52.36
DAfStuA	23060848	22148982	3.32G	0.02	95.32	88.83	52.75
DAfStuA	28387068	27318602	4.1G	0.01	97.47	93.41	52.53
DAfStuA	30533304	29493780	4.42G	0.01	97.41	93.33	52.75
WTYEP	23429420	21992086	3.3G	0.02	96.67	91.59	51.64
WTYEP	23791918	22660542	3.4G	0.02	97.2	92.76	52.18
WTYEP	23052088	21889226	3.28G	0.02	97.11	92.62	52.32

were listed in **Table 1**. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method and the expression level of actin encoding gene was used as the internal control.

Gene Co-expression Network

Co-expression network were constructed using a WGCNA R package (Langfelder and Horvath, 2008). The weighted matrix of pair-wise connection strengths (module) was built and genes were grouped into modules by hierarchical clustering. The power β was used to calculate the correlation coefficients and β = 14 was set as the saturation level for a soft threshold. These networks were then visualized using Cytoscape software.[2]

Statistical Analysis

The significance of the data was tested using the Student's *t*-test. A *p*-value of $\leq$0.05 was considered as significantly different.

RESULTS

Comparison of Transcriptome of *A. flavus* Between AF Inducing and Non-inducing Conditions

Previous studies reported that YES strongly stimulate the biosynthesis of AF but YEP could not (Fountain et al., 2015). Since then, YES and YEP were defined as the AF conducing and non-conducing condition, respectively. In this study, we cultured the *A. flavus* WT strains in YES and YEP for 7 days, and the AF production was determined. As shown in **Figure 1**, YES induce a significant production of AF (11.5 ± 0.5 mg/10 mL cultures). However, no AF could be detectable in YEP, which was consistent with the previous report (Fountain et al., 2015), and confirmed that YES and YEP were AF conducing and non-conducing conditions, respectively. To understand the molecular mechanism underlying that YES but not YEP stimulates the AF biosynthesis, the transcriptome profiles of *A. flavus* cultured in both YES and YEP were comprehensively explored. The 48 h RNA was extracted and analyzed via high-throughput sequencing. High reproducible RNA-seq data were obtained from three biological replicates per culturing condition with Pearson correlation coefficients above 0.96 and 0.88 for YES and YEP, respectively (**Figure 2A**). In total, we obtained >3.3G clean bases with error rate $\leq$0.02 for each biological repeat of both two samples via deep sequencing. Quantifying the expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM), it was found that 2374 genes were differential expressed (DGE) with fold changes $\geq$2 as a threshold by comparing the transcriptomes between in YES and YEP. Among them, expression of 1336 genes were up-regulated, and 1038 genes were down-regulated in YES relative to in YEP (**Figure 2B** and **Supplementary Table S1**). Considering that seventeen percentage of *A. flavus* genome showed differential expression in YES vs. YEP, implying that these two media activate significantly different transcriptomes.

Genome-Wide Identification of Gene Functions and Metabolic Pathways That Are Responsive to YES Induction, Regulation by AfRafA and AfStuA

Recently, we identified two novel APSES transcription factors (TFs), AfRafA and AfStuA, as important and essential activators for AF biosynthesis, respectively (Yao et al., 2017). To comprehensively understand their function in *A. flavus*, it is necessary to systematically identify both gene function and cellular processes they target. RNA-seq was performed to illustrate the gene landscapes regulated by AfRafA and AfStuA. The 48 h RNA samples of Δ*AfRafA* and Δ*AfStuA* under the same culture conditions as WT in YES were extracted and analyzed via sequencing. High reproducible RNA-seq data were obtained from three biological replicates per strain with Pearson correlation coefficients above 0.97 and 0.92 for Δ*AfRafA* and Δ*AfStuA*, respectively (**Figure 2A**). In total, we obtained >3.5G clean bases with error rate $\leq$0.02 and Q20 > 95% for each biological repeat of any strain via deep sequencing. Furthermore, by quantifying the FPKM, we found that 1401 genes were differential expressed when the transcriptomes were compared between Δ*AfRafA* and WT (**Supplementary Table S2**).

[2]http://cytoscape.org/

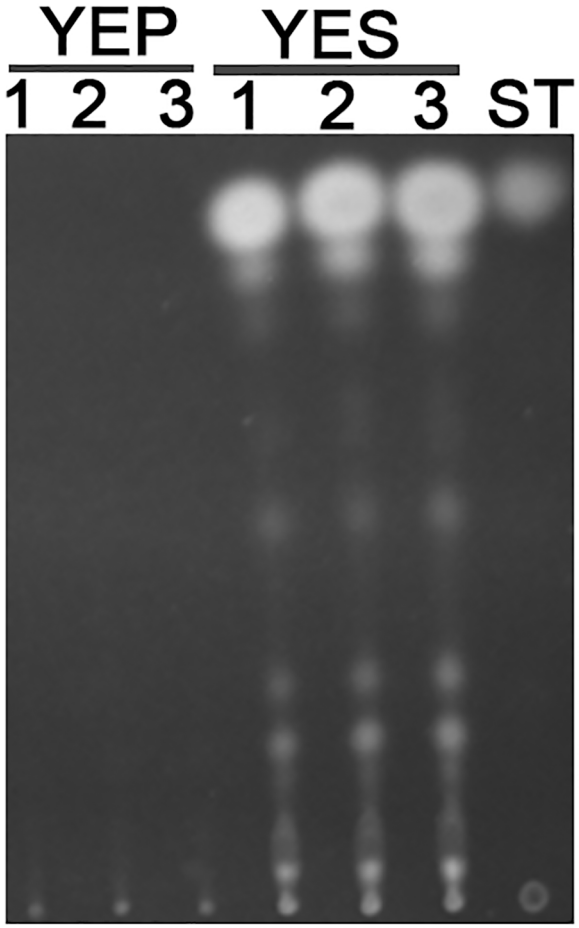


FIGURE 1 | Aflatoxin production in YEP and YES. Conidia of *Aspergillus flavus* WT strain were grown in YEP and YES liquid for 7 days, and then 1.5 mL cultures were extracted by chloroform. TLC analysis of extracts from both YES and YEP, along with an AFB1 standard.

These include 996 upregulated and 405 downregulated genes (**Figure 2C**). At the same time, a total of only 823 differentially expressed genes (DGE) were identified in Δ*AfStuA* vs. WT with identical threshold described above, including 356 upregulated and 467 downregulated genes (**Figure 2D** and **Supplementary Table S3**), suggesting that compared to AfStuA, AfRafA might play a more wide-range regulatory role in *A. flavus*. The Venn diagram showed that AfRafA and AfStuA shared 262 differential expression genes (**Figure 3A**), suggesting that these two proteins play overlapping roles in cellular regulation, which is further supported by their similar roles in AF and pathogenesis described previously (Yao et al., 2017). However, 1139 genes were subjected to AfRafA-specific regulation, while AfStuA uniquely controlled 561 genes (**Figure 3A**). It was also found that 1497 DGEs only showed increased or decreased expression in YES vs. YEP, unaffected by AfRaf or AfStuA. Functional enrichment of KEGG pathway of the DGEs between the deletion and WT strains, as well as AF-conducing vs. non-conducing conditions were performed, and the results were displayed in **Figures 3B–D**. Enriched analyses of the upregulated DGEs in YES uncovered that AF biosynthesis (afv00254) was significantly enriched, which accorded with our expectation (**Figure 3B**). Furthermore, DNA replication, purine metabolism, ribosome, and biosynthesis of amino acids were also significantly activated in YES (**Figure 3B**), which implying that they might have roles in AF synthesis. In addition, the downregulated DGEs were significantly enriched in valine, leucine and isoleucine degradation, penicillin and cephalosporin biosynthesis, and alpha-linolenic acid metabolism. As expected, the most downregulated genes in both Δ*AfRafA* vs. WT and Δ*AfStuA* vs. WT were also responsible for the pathway of AF biosynthesis (**Figures 3C,D**), further confirming that these two TFs function as key regulators for activating this toxin biosynthesis. These results are in well agreement with our previous AF production assays (Yao et al., 2017). Likewise, a number of metabolic processes, including valine, leucine and isoleucine biosynthesis, 2-oxocarboxylic acid metabolism, and pantothenate and CoA biosynthesis, which might relate to AF and other SMs biosynthesis were downregulated in Δ*AfRafA* vs. WT (**Figure 3C**). Correspondingly, the degradation of valine, leucine, and isoleucine was significantly upregulated in Δ*AfRafA* vs. WT. In addition, non-homologous end-joining and homologous recombination were markedly activated in Δ*AfRafA*, suggesting that AfRafA possibly involve in the DNA repair process. More interestingly, only AF biosynthesis was enriched in downregulated DGEs in Δ*AfStuA* vs. WT (**Figure 3D**), confirming that AfStuA functioned as essential AF-specific regulator. On the contrary, biosynthesis of other secondary metabolites showed upregulation in Δ*AfStuA* vs. WT, indicating a block of AF synthesis in Δ*AfStuA* would promote the redirection of metabolic flux to synthesize other SMs.

Co-expression Network Analysis

To explore the relationship between AfRafA and AfStuA regulons, a network analysis was determined by WGCNA (Langfelder and Horvath, 2008). According to the level of AFB1 production, we set phenotype of WT, Δ*AfRafA*, and Δ*AfStuA* as 1, 0.5, and 0, respectively. A gene set with differential expression were clustered into several modules labeling as distinct colors. Two modules (MEblack and MEgreenyellow) showed the highest correction with phenotype (R^2 = 0.86 and R^2 = 0.89). Three hundred and twelve genes of MEblack module were interacted to form the network **Figure 4B**. GO enrichment analysis of these genes showed that ribosome (GO:0005840), branched-chain amino acid biosynthetic process (GO:0009082) and translation (GO:0006412) were identified. Network from genes from MEgreenyellow module was shown in **Figure 4C** and no GO term was significantly enriched. No AF could be detected in YEP-cultured WT and YES-cultured Δ*AfStuA*,it is interesting to uncover general mechanism for aflatoxigenic phenotype. Analysis of all RNA-seq data of WT_YES, WT_YEP, and Δ*AfStuA* were cluster into 21 modules. One module (colored as MEmagenta) had a significant correction with phenotype (**Figure 4D**). Two hundred and eighty-seven genes from this module generated a gene co-expression network (**Figure 4E**). Among these genes, branched-chain amino acid biosynthetic process (GO:0009082), mitochondrial pyruvate

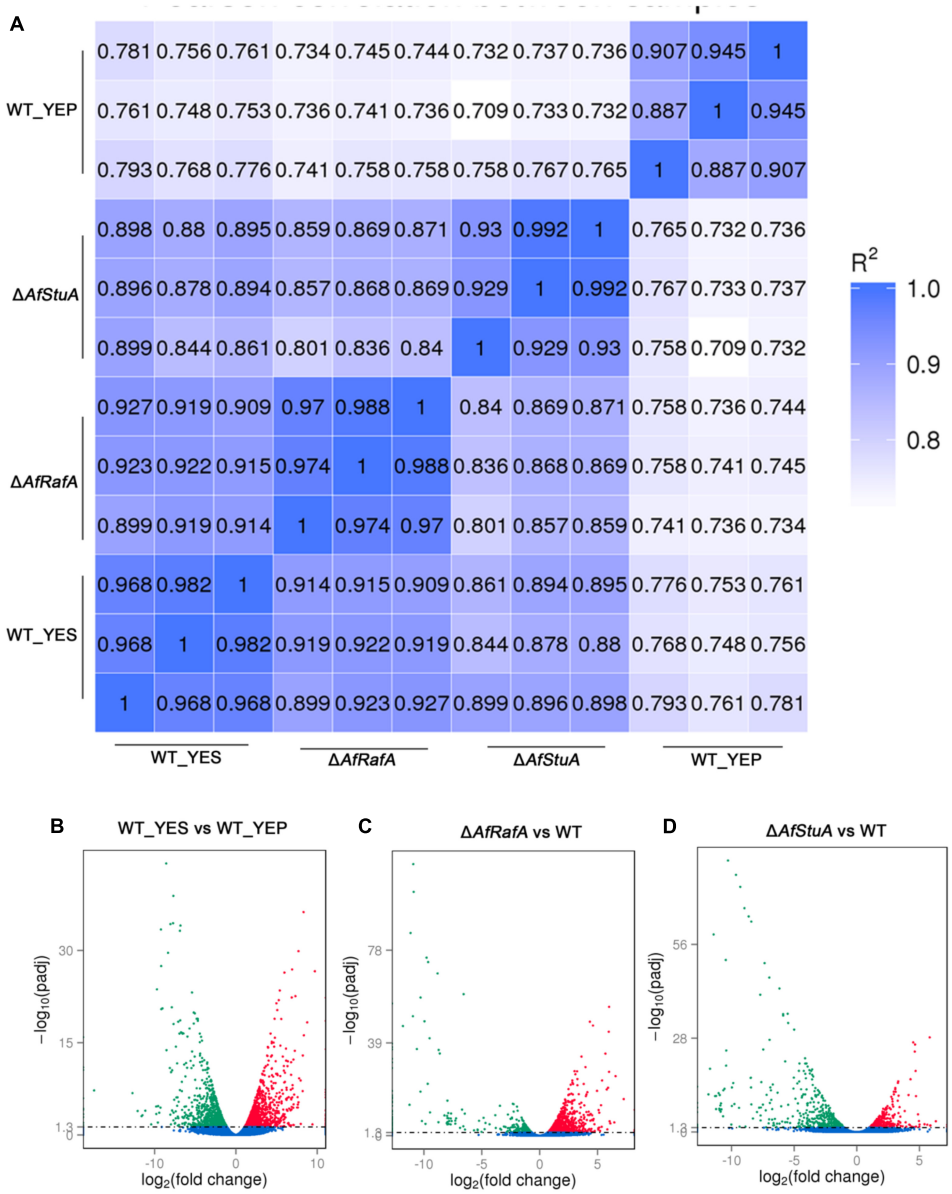


FIGURE 2 | Analysis of differentially expressed genes in different conditions and various strains. **(A)** The Pearson correlation index among three biological repeats was calculated using R software. **(B)** Number of genes showed upregulated and downregulated expression of WT strain cultured in YEP vs. in YES; **(C)** Number of genes showed upregulated and downregulated expression in Δ*AfRafA* vs. WT; **(D)** Number of genes showed upregulated and downregulated expression in Δ*AfStuA* vs. WT.

transport (GO:0006850), signal peptide processing (GO:0006465) were enriched. It was worth to note that mitochondrial pyruvate transport and signal peptide processing potentially implicated in AF biosynthesis was proposed for the first time.

Most Up-regulated Genes in YES vs. YEP Involve in Carbon and Nitrogen Metabolism

Analyses of the top twenty in upregulated DGEs in YES vs. YEP showed that most of them have roles in the nitrogen and carbon metabolism. Two ammonium transporters genes (*AFLA_108260* and *AFLA_130040*) were increased by above fivefold and sixfold under AF conducing condition compared with growth in non-conducing one. Likewise, the glutamate synthase gene (AFLA_022340), glutamate/phenylalanine/leucine/valine dehydrogenase gene (AFLA_113320), the amino acid transporter gene (*AFLA_073030*) were also up-regulated above fivefold in the AF conducing condition. Three most upregulated genes (7- ∼8-fold) were involved in carbon metabolism including alcohol dehydrogenase (*AFLA_097820*), carbohydrate kinase (*AFLA_097830*), glyceraldehyde 3-phosphate dehydrogenase

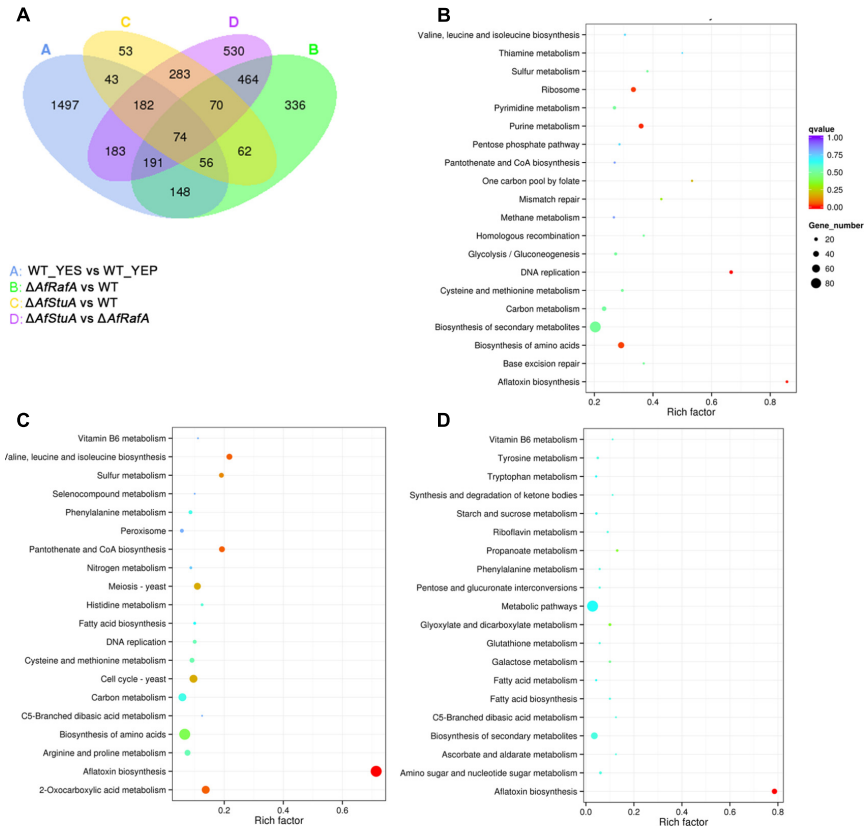


FIGURE 3 | Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differential expression genes. **(A)** Venn diagram of differential expression genes. **(B)** Enrichment analysis of genes upregulated above twofold in WT strain cultured in YES vs. in YEP; **(C)** Enrichment analysis of genes upregulated above twofold in Δ*AfRafA* vs. WT; **(D)** Enrichment analysis of genes upregulated above twofold in Δ*AfStuA* vs. WT.

(*AFLA_042390*). In addition, lipopolysaccharide-modifying protein (*AFLA_002000*), centromere protein V (*AFLA_096180*), isoprenoid synthase (*AFLA_042370*), O-methyltransferase (*AFLA_016120*), a TF with winged helix-turn-helix DNA-binding domain (*AFLA_016130*), antibiotic biosynthesis monooxygenase (*AFLA_121090*), phosphoesterase (*AFLA_050610*), and five genes with unknown function were identified, their roles in AF biosynthesis required further characterization. To summarize briefly, gene expression profiles of *A. flavus* strain cultures from YES and YEP were compared, and confirmed that AF biosynthesis, and genes related with carbon and nitrogen metabolism were significantly up-regulated in YES vs. YEP.

Monooxygenase Subject to Specific Regulation of AfStuA

Intriguingly, the monooxygenase activity (GO:0004497) was significantly enriched when comparing Δ*AfStuA* and WT transcriptomes. In fact, 33 of 116 genes that encode cytochrome P450 monooxygenase showed differential expression (**Supplementary Table S4**). Among these, 25 genes were down-regulated, and four genes were up-regulated in Δ*AfStuA*. Remarkably, nine monooxygenase genes (*aflX, aflW, aflV, aflQ, aflI, aflL, aflG, aflN,* and *aflCa*) from the AF cluster were concurrently and strongly downregulated in Δ*AfStuA*. These results suggest a possibility that a variety of monooxygenases targeted by AfStuA, might contribute to the defective phenotype of Δ*AfStuA*. Consistent with this result is a recent study, which reported cytochrome P450 monooxygenases to be widely involved in various cellular processes, including fungal development, secondary metabolism, and virulence in the plant pathogen *Fusarium graminearum* (Shin et al., 2017). In summary, these data demonstrated that AfStuA exerted extensive regulatory roles in cellular primary and secondary metabolism, possibly by modulating the expression of various monooxygenases.

Co-regulated Genes by AfRafA and AfStuA Are Mainly Enzymes Functioning at Early and Middle Stages in AF Biosynthesis

To fully understand the role of AfRafA and AfStuA in the activation of the AF gene cluster, the expression of 29 genes that were responsible for the generation of AF were selected out and compared as illustrated in **Figure 4**. A previous study reported that the enzymatic reactions, which are responsible for AF synthesis could be divided into three stages: early,

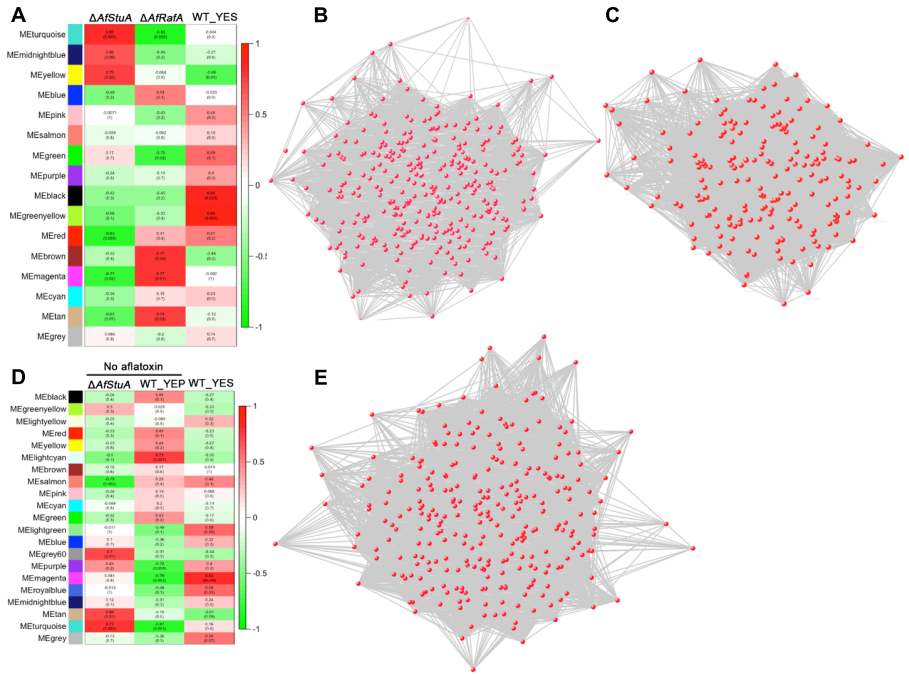


FIGURE 4 | Weighted co-expression gene network. **(A)** Relationship between phenotype and consensus module eigengenes obtained by WGCNA for differentially expressed genes in Δ*AfRafA* vs. WT and Δ*AfStuA* vs. WT. **(B)** Gene network of MEblack module. **(C)** Gene network of MEgreenyellow module. **(D)** Relationship between phenotype and consensus module eigengenes obtained by WGCNA for differentially expressed genes in YES vs. YEP and Δ*AfStuA* vs. WT. **(E)** Gene network of MEmagenta module.

middle, and late stage (**Figure 5**). AflD, AflM, and AflP in *A. flavus*, as well as NorA, Ver-1, and OmtA in *A. nidulans* were representatives in the three stages, respectively (Chanda et al., 2009). Interestingly, genes encoding early enzymes showed a more severe downregulation of their transcription in both deletion strains relative to those decoding enzymes that functioning at middle and late stages, which was reflected by the almost undetectable mRNA of most of these early genes in Δ*AfStuA*. As expected, almost all AF genes (not including *AflR*) were significantly induced in YES in comparison to in YEP (**Figure 5**). And, most of the AF cluster genes in this group conformed an expression pattern that was highest in WT, lower in Δ*AfRafA*, and even lower or completely block in Δ*AfStuA*, indicating that AfStuA played a more significant regulatory role in AF biosynthesis relative to AfRafA. However, the three late enzymes encoding genes *aflW*, *aflX*, and *aflY* showed a WT-level expression in Δ*AfRafA* and Δ*AfStuA*. In summary, AfStuA and AfRafA played key roles in activating the AF gene cluster at the initial phase, also demonstrating that expression of the early-stage enzyme genes for AF synthesis were co-regulated by these two TFs.

Expression of Multiple AF Regulators Are Responsive to YES, or AfRafA and AfStuA Regulation

Interestingly, 12 known AF regulators were identified by our comparative transcriptome analysis, because their expression pattern showed differential response to culturing condition, and AfRafA and AfStuA regulation. Two transcription regulators MeaB and RtfA, was previously identified as negative regulators (Amaike et al., 2013; Lohmar et al., 2016), and their expression levels were significantly downregulated in YES when compared with in YEP, but seems not to be regulated by AfRafA or AfStuA in this study (**Figures 6A,B**). Unexpectedly, the bZIP TF AtfB, positively regulating AF biosynthesis (Wee et al., 2017), its transcription level was downregulated by twofold in YES vs. in YEP, whereas upregulated by threefold in Δ*AfStuA* mutant in compassion to WT (**Figure 6C**), implying that AfStuA regulates AF in an AtfB-independent way. G protein-coupled receptor gene *gprP* was up-regulated in all strains used in YES compared with WT cultured in YEP (**Figure 6D**), and its encoding protein negatively control the AF biosynthesis (Affeldt et al., 2014). On the contrary, expression of two phosphodiesterase genes *pdeH* and *pedL* were downregulated significantly in YES in each mutant strain relative to YEP (**Figures 6E,F**), which was consistent with their negative roles in toxin formation (Yang et al., 2017). More recently, the novel protein LaeB was demonstrated to be crucially required for both sterigmatocystin and AFs biosynthesis in *A. nidulans* and *A. flavus*, respectively (Pfannenstiel et al., 2017). Unexpectedly, it was showed that expression of laeB was markedly upregulated in YES, and upregulated by AfStuA without statistical significance, but not by AfRafA (**Figure 6G**). Oxylipin-generating dioxygenases include PpoC, were reported to involve in the AF synthesis (Brown et al., 2009). In accordance with the expression pattern of

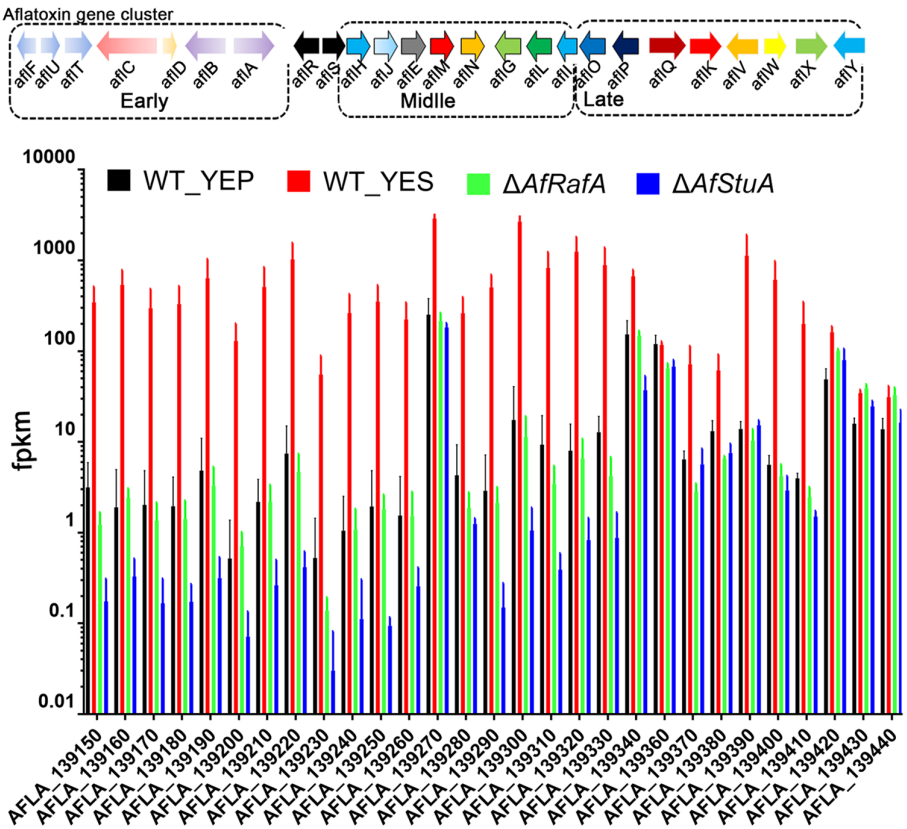


FIGURE 5 | Differential roles for AfRafA and AfStuA in the regulation expression of different stage aflatoxin genes. Expression levels (fpkm) for all 29 aflatoxin genes in Δ*AfRafA* and in Δ*AfStuA*, as well as WT under YES and YEP were compared and grouped into three stages according to the roles of their protein product in making aflatoxin.

AF cluster, expression of *ppoC* was the highest in WT under YES media, lower in YEP or Δ*AfRafA*, and further lower in Δ*AfStuA* (**Figure 6H**). Recently, it was reported that genes responsible for hyphal anastomosis regulated the biosynthesis of AF in the LaeA-dependent manner. Especially, deletion of *hamF*, *hamG*, *hamH*, or *hamI* resulted in an almost abolish of AF biosynthesis, similar to the phenotype of Δ*laeA* (Zhao et al., 2017). Interestingly, our data suggested a role of these proteins in the induction of AF in an AfRafA or AfStuA, or both dependent way. As for *hamE*, YES have negligible impact on its expression, but its expression level was greatly decreased in Δ*AfRafA* or Δ*AfStuA* (**Figure 6I**). Expression of *hamF* was not affected by YES induction or AfStuA activation, showing a specific downregulation in Δ*AfRafA* (**Figure 6J**). *hamG* was expressed at the highest level in WT under YES, decreased threefold in WT under YEP, and further downregulated in Δ*AfRafA* (sixfold) and Δ*AfStuA* (above 10-fold) (**Figure 6K**). Expression of *hamH* was responsive to the regulation of AfRafA and AfStuA, regardless of media used (**Figure 6L**). All together, these observations indicated that AfRafA and AfStuA played a divergent role in the control expression of hyphal anastomosis genes, and their mediated AF biosynthesis. Totally, 12 important AF regulators were identified by the comparative transcriptome analysis, indicating that our comparative transcriptomic strategy is efficient to distinguish the potential targets with roles in AF synthesis.

A Novel LaeA-Like Methyltransferase Involves in the Control of AF Biosynthesis

Beyond known AF regulators, a number of novel candidate genes that might involve in AF were also identified in our transcriptomic analysis (see Discussion). Intriguingly, it was suggested that expression of one LaeA-like methyltransferases genes *AFLA_121330* (thereafter named as *lael1*) was significantly downregulated in YES relative to in YEP, and further subjected to regulation of both AfRafA and AfStuA (**Figure 7A**). In addition, regulated expression pattern of *lael1* by media or regulators of AfRafA and AfStuA was further confirmed by qPCR analyses (**Figure 7A**), implying that it might have roles in the induction of biosynthesis of AFs. To examine the role of Lael1 in AF, we generated the gene knockout mutant strain for gene *lael1*. After two rounds of genetic transformation, three deletion transformants were obtained and verified (**Supplementary Figure S1**). And then, the phenotype and the AF production of these mutants were determined. Deletion of *lael1* did not affect colony extension or conidiation when growth either on PDA or

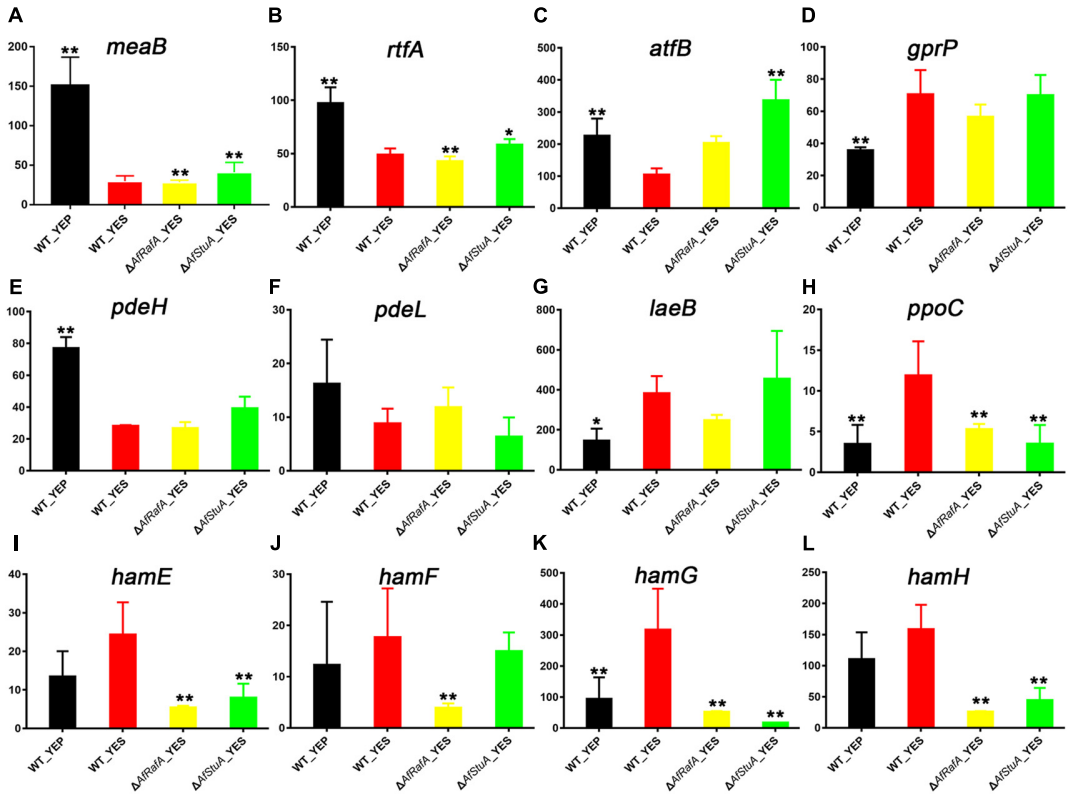


FIGURE 6 | Expression of known aflatoxin regulators in various strains. Expression level (fpkm) for 12 aflatoxin regulator genes in Δ*AfRafA* and in Δ*AfStuA*, as well as WT under YES and YEP were compared, including *meaB* **(A)**, *rtfA* **(B)**, *atfB* **(C)**, *gprP* **(D)**, *pdeH* **(E)**, *pdeL* **(F)**, *laeB* **(G)**, *ppoC* **(H)**, *hamE* **(L)**, *hamF* **(M)**, *hamG* **(N)**, and *hamH* **(L)**. $*p \leq 0.05$, $**p \leq 0.01$.

GMM agar plate supplemented with glutamine (data not shown), suggesting that Lael1 did not involve in the regulation of fungal growth and development. However, the AF titers of Δ*lael1* were significantly increased in our assays (**Figures 7B,C**). Especially, Δ*lael1* produced threefold more AFB1 than that of WT when growth in YES liquid media. To confirm the effect of Lael1 on AF biosynthesis, the complementation strain (Lael1com) was constructed by re-introduction the expression cassette to Δ*lael1*. As expected, the complementation strain restored the AF production to wild-type level (data not shown). As expected, only trace AF could be detected in Δ*AfStuA*, and significantly reduced AF synthesis was detected in Δ*AfRafA* (**Figure 7C**). In addition, conidia of Δ*lael1* was more pigmented than those of other mutant and WT strains. Combined above, it was confirmed that Lael1 played crucial roles in suppression of the biosynthesis of AF, and its expression were activated by YEP but not YES, and further positively regulated by both AfRafA and AfStuA.

Both AfRafA and AfStuA Function as Global Regulators and Affect the Expression of Multiple SMs Clusters

In addition to AF, the *A. flavus* genome harbors additional 54 SM clusters, however, most of their chemical nature remains unclear. To clarify the roles of AfRafA and AfStuA in other SMs, the expression pattern of SM core enzyme encoding genes were examined between in YEP and in YES, as well as in gene knockout mutants and in WT, and the results are hierarchically clustered as showed in **Figure 8**. In total, 19 gene clusters, each of which was responsible for the synthesis of at least one class of SMs, was expressed in at least one sample. All the SM core genes were clustered into three groups based on their mRNA abundance in Δ*AfRafA*, Δ*AfStuA*, and WT. One group containing four SM gene clusters displayed the highest expression level in WT, lower expression in Δ*AfRafA*, and much lower or even non-existent expression in Δ*AfStuA*. Among these, only SM54 had been identified and was an AF gene cluster. The core enzymes of SM13, SM16, and SM17 were non-ribosomal peptide synthetase (NRPS), L-ornithine-N5-oxygenase (SidA), and polyketide synthase (PKS)-like, respectively, implying that they might produce non-ribosomal peptides, siderophore, and unknown chemicals, respectively. Interestingly, the second group of SM genes shared an expression pattern that showed higher expression in Δ*AfRafA*, but lower or even a complete lack of expression in Δ*AfStuA* when compared to WT. This group includes SM9, SM10, SM20, SM37, and SM55. Among these, the product of SM55 has recently been characterized as cyclopiazonic acids, which was agreed with our previous results that AfRafA negatively and AfStuA positively regulated the biosynthesis of cyclopiazonic acid (Yao et al., 2017). The

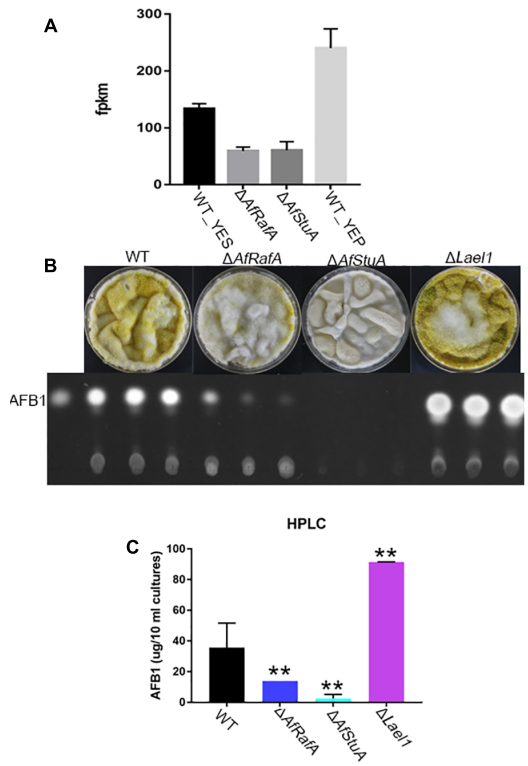


FIGURE 7 | A novel LaeA-like methyltransferase Lael1 controls aflatoxin biosynthesis. **(A)** Expression level of methyltransferase gene *lael1* when cultured in YES and YEP and in Δ*AfRafA* and Δ*AfStuA*; **(B)** TLC analysis of aflatoxin of Δ*lael1*, Δ*AfRafA*, Δ*AfStuA*, and WT strains induced by YES; **(C)** HPLC analysis of aflatoxin titers of Δ*lael1*, Δ*AfRafA*, Δ*AfStuA*, and WT strains, $^{**}p \leq 0.01$.

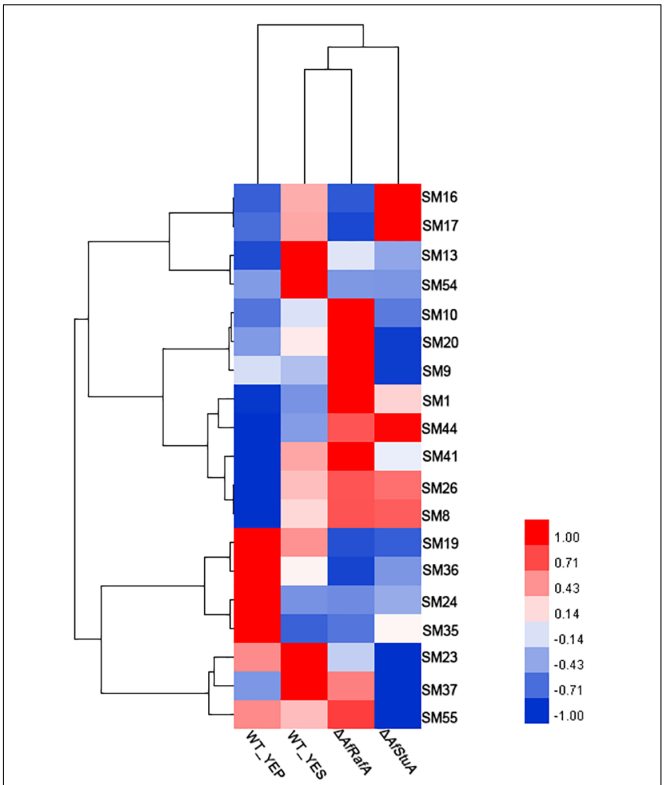


FIGURE 8 | Heatmap of expression levels of secondary metabolism core genes in various strains. Expression level (fpkm) for 19 genes encoding the core enzymes for secondary metabolisms in Δ*AfRafA* and in Δ*AfStuA*, as well as WT under YES and YEP were clustered into three patterns and showed in the heatmap.

enzymes for synthesizing the backbone of SM9 and SM37, SM10, and SM20 were the NRPS, scytalone dehydratase, and PKS, respectively. Four SM clusters were specifically induced in YES relative to in YEP, including SM19, SM24, SM35, and SM36, harboring the core enzymes encoding dimethylallyl tryptophan synthase, NRPS, NRPS-like enzyme, and PKS-like enzyme, respectively. Markedly, most of the SMs showed lowest or even non-existent expression in Δ*AfStuA* vs. WT, suggesting that *AfStuA* likely function as a global activator of secondary metabolism. On the contrary, AfRafA played a negative role in the biosynthesis of many SMs, except for AF gene cluster. The knowledge obtained in this study would pave a novel way for activating the internally silent SM by over-expressing AfStuA or deleting AfRafA in our future natural product discovery in *A. flavus*.

DISCUSSION

Nitrogen metabolism links with AF biosynthesis (Reddy et al., 1979; Payne and Hagler, 1983; Bhatnagar et al., 1986; Ehrlich and Cotty, 2002; Wilkinson et al., 2007; Georgianna and Payne, 2009; Tudzynski, 2014). Payne and Hagler (1983) reported that casein strongly repress AF in both *A. flavus* and *Aspergillus parasiticus*. By analogy, in this study casein-derived peptone has a similar effect. Both casein and peptone enriched in amino acids were preferentially utilized as favored nitrogen resource. Metabolism of these favored nitrogen triggers *n*itrogen *m*etabolite *r*epression (NMR) results in expression of genes for metabolizing non-favored nitrogen are downregulated (Tudzynski, 2014). An increasing evidence demonstrated a regulatory role of NMR in the biosynthesis of secondary metabolism, including AF in filamentous fungi. Two TFs AreA and NmrA are central regulators in NMR. Chang et al. (2000) reported that AreA binds with the intergenic region between *aflR* and *aflS* to regulate *aflS* expression. Recently, NmrA was shown to strongly affect AF synthesis on glutamine and alanine media (Han et al., 2016). Relative to YEP, YES is poor in amino acids. Therefore, it was proposed that YEP mediates the nitrogen repression effect, but YES resulted in depression of AF gene cluster. In fact, YES induces a similar transcriptional response as shortage of amino acid, reflected by organonitrogen compound biosynthetic process (GO:1901566, Corrected *p*-value = 1.29E−11) and cellular amino acid biosynthetic process (GO:0008652, Corrected *p*-value = 9.72E−07) were significantly upregulated. Accordingly, metabolism of valine, leucine and isoleucine (*p*-value = 6.03E−05) and phenylalanine (*p*-value = 0.014072269) were downregulated.

Further analysis indicated that three genes specially involved in proline biosynthesis (*AFLA_014050*, *AFLA_047450*, and *AFLA_047280*) were upregulated in YES. Aspartate has been shown to stimulate more AF biosynthesis than other nitrogen (Payne and Hagler, 1983). Bhatnagar et al. (1986) suggested that glutamine synthetase played an important regulatory role in biosynthesis of AFs by modulating glutamine level. Our recent results further supported that glutamine induces more AF production than other inorganic nitrogen and amino acids (Wang et al., 2017). Combined, it was proposed that glutamine is converted into acetate through alpha-ketoglutarate and then incorporated into AFs. Indeed, both glutamine synthetase gene *AFLA_022340* (converting glutamine into glutamate) and aminotransferase gene *AFLA_026470* (converting glutamate into 2-oxoglutarate) was upregulated 10- and 8-fold in YES vs. YEP, respectively. Interestingly, tryptophan metabolism mediated regulation of AF synthesis was different between in *A. flavus* and in *A. parasiticus* (Wilkinson et al., 2007). In addition, amino acid variety in crops could also influence severity of crop AF contamination (Senyuva et al., 2008). Altogether, nitrogen resource exerts a complex effect on AF biosynthesis, further characterization of nitrogen metabolism and regulatory genes in *A. flavus* are required.

Biosynthesis of AF is a highly regulated process; a hierarchy and interconnected network is involved. In the network, AflR function as a pathway-specific regulator, function at the basal level to activate AFB. Expression of *aflR* was downregulated in Δ*AfRafA*, or even completely inhibited in Δ*AfStuA* by our RNA-seq analysis, according well with our previous qPCR results (Yao et al., 2017), supporting that AfRafA and AfStuA function as a global regulator functioning upstream of AflR. In the middle, Lael1, as a methyltransferase, might activate *aflR* transcription by modification of chromatin structure, just as LaeA (Bok et al., 2006). In addition, our results support that expression of *Lael1* was subjected to regulation of AfStuA and AfRafA. Co-expression network analysis demonstrated that AfStuA and AfRafA control both the same and divergent cellular processes, suggesting a cross-talk between AfRafA regulon and AfStuA regulon.

One novel LaeA-like proteins Lael1 were demonstrated to have crucial roles in AF production, but appear not to affect other cellular processes, suggesting it might serve as an AF-specific regulator. Previously, as the SAM-dependent methyltransferase, LaeA positively regulated the biosynthesis of mycotoxin in all most filamentous fungi (Bok and Keller, 2004; Wu et al., 2012; Crespo-Sempere et al., 2013; Kim et al., 2013; Estiarte et al., 2016). However, our data supported a negative regulatory role of Lael1 in the biosynthesis of AFs. Likewise, LlmF, a LaeA-like methyltransferase, negatively control the sterigmatocystin synthesis by mediating the cellular location of VeA (Palmer et al., 2013). Genetic screen of *A. nidulans* LaeA-like methyltransferase proteins did not suggest a regulatory role for Lael1 ortholog in sterigmatocystin production (Palmer et al., 2013). It was widely accepted that biosynthesis of AF in *A. flavus* shares a same regulatory network to sterigmatocystin (the penultimate precursor of AF) in *A. nidulans*. However, our data highlighted a caution that knowledge obtained in sterigmatocystin biosynthesis in *A. nidulans* may not absolutely applied to biosynthesis of AF in *A. flavus*. Actually, this hypothesis was supported by several reports. Loss of the upstream development regulator FluG lead to a block of biosynthesis of sterigmatocystin in *A. nidulans* but not in *A. flavus* (Chang et al., 2012). Unlike in *A. nidulans*, the C_2H_2 TF RsrA do not impact the biosynthesis of secondary metabolism in *A. flavus* (Bok et al., 2014). In addition, extracellular pH causes a divergent effect on the biosynthesis of AF in *A. flavus* and *A. parasiticus*, and sterigmatocystin in *A. nidulans*, respectively (Ehrlich et al., 2005; Delgado-Virgen and Guzman-de-Pena, 2009).

The first step in the biosynthesis of AF is the formation of a hexanoyl starter unit from acetyl-CoA and malonyl-CoA (Minto and Townsend, 1997). In fact, pantothenate and CoA biosynthesis (afv00770) was more strongly expressed in AF-inducing condition than in non-inducing, on the contrary, significantly downregulated in the transcriptional profiles of Δ*AfRafA* and Δ*AfStuA*. It had been demonstrated that initiation of AF biosynthesis correlated with the activation of acetyl-CoA carboxylase by calmodulin or oxidative stress (Rao and Subramanyam, 2000; Narasaiah et al., 2006). Therefore, combined above results might inspired us that increasing biotechnologically the building block could be an efficient way to improve the production of polyketide-derived secondary metabolism.

By comparative transcriptomics, we identified a number of known and novel regulators required for AF biosynthesis. Comparing the composition, high concentration sucrose and $MgSO_4$ in YES were replaced with peptone in YEP, implying sucrose and magnesium ion might facilitate AF synthesis. Indeed, metal ion, including magnesium showed an effect on the AF biosynthesis (Tiwari et al., 1986). To support the above hypothesis, sucrose had been shown to induce the AF synthesis in the concentration-dependent way (Llewellyn et al., 1980; Wilkinson et al., 2007). It would be more important to note that novel proteins with an identical expression pattern to AF cluster, potentially involve in the toxin biosynthesis. Eighteen candidate genes satisfy the criteria in our comparative transcriptome analysis, including two TFs – AFLA_084720 and NosA, five transporters – allantoate permease (AFLA_019420), ZIP Zinc transporter (AFLA_081920), pantothenate transporter (AFLA_108250), ammonium transporter (AFLA_108260), and efflux pump (AFLA_131810), two monooxygenase (AFLA_019430 and AFLA_138920), forkhead domain protein (AFLA_132980), NADH-cytochrome B5 reductase (AFLA_039650), pyridoxamine phosphate oxidase (AFLA_108810), fucose-specific lectin (AFLA_065960), neuroligin (AFLA_013540), and four genes with unknown function. Interestingly, Zhao et al. (2017) reported that NosA regulates *ham* genes and heterokaryon, virulence and AF production, while the function of these novel genes in *A. flavus* remain unidentified. Defining their possible roles in AF biosynthesis warrant further investigation. In conclusion, comparative transcriptome established here was an efficient strategy to identify the potential regulators for AF and other SMs biosynthesis.

AUTHOR CONTRIBUTIONS

GY and SW conceived and designed the work. GY, YF, YY, GM, ZF, and ZX performed the experiments, analyzed the data, and wrote the manuscript. SW revised the manuscript.

FUNDING

This study was funded by grants from the National Natural Science Foundation of China (No. 31172297), China Postdoctoral Science Foundation (2017M612105), and the Research Foundation of FAFU (132300227).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01568/full#supplementary-material

FIGURE S1 | Verification of gene deletion of *Lael1*. **(A)** Scheme of gene deletion of *AFLA_121330*; **(B)** PCR verification of locus of knockout cassette; **(C)** RT-PCR verification gene loss of *AFLA_121330*.

TABLE S1 | Differential expression genes in YES vs. YEP.

TABLE S2 | Differential expression genes in Δ*AfRafA* vs. WT.

TABLE S3 | Differential expression genes in Δ*AfStuA* vs. WT.

TABLE S4 | Expression level of monooxygenase genes.

REFERENCES

Abdallah, M. F., Krska, R., and Sulyok, M. (2016). Mycotoxin contamination in sugarcane grass and juice: first report on detection of multiple mycotoxins and exposure assessment for aflatoxins B_1 and G_1 in humans. *Toxins (Basel)* 8:E343. doi: 10.3390/toxins8110343

Affeldt, K. J., Carrig, J., Amare, M., and Keller, N. P. (2014). Global survey of canonical *Aspergillus flavus* G protein-coupled receptors. *mBio* 5:e01501-14. doi: 10.1128/mBio.01501-14

Amaike, S., Affeldt, K. J., Yin, W. B., Franke, S., Choithani, A., and Keller, N. P. (2013). The bZIP protein MeaB mediates virulence attributes in *Aspergillus flavus*. *PLoS One* 8:e74030. doi: 10.1371/journal.pone.0074030

Amaike, S., and Keller, N. P. (2011). *Aspergillus flavus*. *Annu. Rev. Phytopathol.* 49, 107–133. doi: 10.1146/annurev-phyto-072910-095221

Amare, M. G., and Keller, N. P. (2014). Molecular mechanisms of *Aspergillus flavus* secondary metabolism and development. *Fungal Genet. Biol.* 66, 11–18. doi: 10.1016/j.fgb.2014.02.008

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi: 10.1186/gb-2010-11-10-r106

Aydin, M., Aydin, S., Bacanli, M., and Basaran, N. (2015). Aflatoxin levels in chronic hepatitis B patients with cirrhosis or hepatocellular carcinoma in Balikesir, Turkey. *J. Viral Hepat.* 22, 926–935. doi: 10.1111/jvh.12410

Bhatnagar-Mathur, P., Sunkara, S., Bhatnagar-Panwar, M., Waliyar, F., and Sharma, K. K. (2015). Biotechnological advances for combating *Aspergillus flavus* and aflatoxin contamination in crops. *Plant Sci.* 234, 119–132. doi: 10.1016/j.plantsci.2015.02.009

Bhatnagar, R. K., Ahmad, S., Mukerji, K. G., and Venkitasubramanian, T. A. (1986). Nitrogen metabolism in *Aspergillus parasiticus* NRRL 3240 and *A. flavus* NRRL 3537 in relation to aflatoxin production. *J. Appl. Bacteriol.* 60, 203–211. doi: 10.1111/j.1365-2672.1986.tb01074.x

Bok, J. W., and Keller, N. P. (2004). LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot. Cell* 3, 527–535. doi: 10.1128/EC.3.2.527-535.2004

Bok, J. W., Noordermeer, D., Kale, S. P., and Keller, N. P. (2006). Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Mol. Microbiol.* 61, 1636–1645. doi: 10.1111/j.1365-2958.2006.05330.x

Bok, J. W., Wiemann, P., Garvey, G. S., Lim, F. Y., Haas, B., Wortman, J., et al. (2014). Illumina identification of RsrA, a conserved C2H2 transcription factor coordinating the NapA mediated oxidative stress signaling pathway in *Aspergillus*. *BMC Genomics* 15:1011. doi: 10.1186/1471-2164-15-1011

Brown, S. H., Scott, J. B., Bhaheetharan, J., Sharpee, W. C., Milde, L., Wilson, R. A., et al. (2009). Oxygenase coordination is required for morphological transition and the host-fungus interaction of *Aspergillus flavus*. *Mol. Plant Microbe Interact.* 22, 882–894. doi: 10.1094/MPMI-22-7-0882

Calvo, A. M., and Cary, J. W. (2015). Association of fungal secondary metabolism and sclerotial biology. *Front. Microbiol.* 6:62. doi: 10.3389/fmicb.2015.00062

Chanda, A., Roze, L. V., Pastor, A., Frame, M. K., and Linz, J. E. (2009). Purification of a vesicle-vacuole fraction functionally linked to aflatoxin synthesis in *Aspergillus parasiticus*. *J. Microbiol. Methods* 78, 28–33. doi: 10.1016/j.mimet.2009.03.014

Chang, P. K. (2003). The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. *Mol. Genet. Genomics* 268, 711–719.

Chang, P. K. (2008). A highly efficient gene-targeting system for *Aspergillus parasiticus*. *Lett. Appl. Microbiol.* 46, 587–592. doi: 10.1111/j.1472-765X.2008.02345.x

Chang, P. K., Scharfenstein, L. L., Mack, B., and Ehrlich, K. C. (2012). Deletion of the *Aspergillus flavus* orthologue of *A. nidulans* fluG reduces conidiation and promotes production of sclerotia but does not abolish aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 78, 7557–7563. doi: 10.1128/AEM.01241-12

Chang, P. K., Scharfenstein, L. L., Wei, Q., and Bhatnagar, D. (2010). Development and refinement of a high-efficiency gene-targeting system for *Aspergillus flavus*. *J. Microbiol. Methods* 81, 240–246. doi: 10.1016/j.mimet.2010.03.010

Chang, P. K., Yu, J., Bhatnagar, D., and Cleveland, T. E. (2000). Characterization of the *Aspergillus parasiticus* major nitrogen regulatory gene, areA. *Biochim. Biophys. Acta* 1491, 263–266. doi: 10.1016/S0167-4781(00)00004-X

Chettri, P., and Bradshaw, R. E. (2016). LaeA negatively regulates dothistromin production in the pine needle pathogen *Dothistroma septosporum*. *Fungal Genet. Biol.* 97, 24–32. doi: 10.1016/j.fgb.2016.11.001

Chu, Y. J., Yang, H. I., Wu, H. C., Liu, J., Wang, L. Y., Lu, S. N., et al. (2017). Aflatoxin B1 exposure increases the risk of cirrhosis and hepatocellular carcinoma in chronic hepatitis B virus carriers. *Int. J. Cancer* 141, 711–720. doi: 10.1002/ijc.30782

Crespo-Sempere, A., Marin, S., Sanchis, V., and Ramos, A. J. (2013). VeA and LaeA transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. *Int. J. Food Microbiol.* 166, 479–486. doi: 10.1016/j.ijfoodmicro.2013.07.027

Delgado-Virgen, F., and Guzman-de-Pena, D. (2009). Mechanism of sterigmatocystin biosynthesis regulation by pH in *Aspergillus nidulans*. *Braz. J. Microbiol.* 40, 933–942. doi: 10.1590/S1517-83822009000400027

Ehrlich, K. C., and Cotty, P. J. (2002). Variability in nitrogen regulation of aflatoxin production by *Aspergillus flavus* strains. *Appl. Microbiol. Biotechnol.* 60, 174–178. doi: 10.1007/s00253-002-1094-5

Ehrlich, K. C., Montalbano, B. G., and Cary, J. W. (1999). Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene* 230, 249–257. doi: 10.1016/S0378-1119(99)00075-X

Ehrlich, K. C., Montalbano, B. G., and Cotty, P. J. (2005). Divergent regulation of aflatoxin production at acidic pH by two *Aspergillus* strains. *Mycopathologia* 159, 579–581. doi: 10.1007/s11046-005-1150-7

Estiarte, N., Lawrence, C. B., Sanchis, V., Ramos, A. J., and Crespo-Sempere, A. (2016). LaeA and VeA are involved in growth morphology, asexual development, and mycotoxin production in *Alternaria alternata*. *Int. J. Food Microbiol.* 238, 153–164. doi: 10.1016/j.ijfoodmicro.2016.09.003

Fountain, J. C., Scully, B. T., Chen, Z. Y., Gold, S. E., Glenn, A. E., Abbas, H. K., et al. (2015). Effects of hydrogen peroxide on different toxigenic and atoxigenic isolates of *Aspergillus flavus*. *Toxins* 7, 2985–2999. doi: 10.3390/toxins7082985

Georgianna, D. R., Fedorova, N. D., Burroughs, J. L., Dolezal, A. L., Bok, J. W., Horowitz-Brown, S., et al. (2010). Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol. Plant Pathol.* 11, 213–226. doi: 10.1111/j.1364-3703.2009.00594.x

Georgianna, D. R., and Payne, G. A. (2009). Genetic regulation of aflatoxin biosynthesis: from gene to genome. *Fungal Genet. Biol.* 46, 113–125. doi: 10.1016/j.fgb.2008.10.011

Gilbert, M. K., Mack, B. M., Wei, Q., Bland, J. M., Bhatnagar, D., and Cary, J. W. (2016). RNA sequencing of an nsdC mutant reveals global regulation of secondary metabolic gene clusters in *Aspergillus flavus*. *Microbiol. Res.* 182, 150–161. doi: 10.1016/j.micres.2015.08.007

Han, X., Qiu, M., Wang, B., Yin, W. B., Nie, X., Qin, Q., et al. (2016). Functional analysis of the nitrogen metabolite repression regulator gene nmrA in *Aspergillus flavus*. *Front. Microbiol.* 7:1794. doi: 10.3389/fmicb.2016.01794

Iqbal, S. Z., Asi, M. R., Nisar, S., Zia, K. M., Jinap, S., and Malik, N. (2016). A limited survey of aflatoxins and zearalenone in feed and feed ingredients from Pakistan. *J. Food Prot.* 79, 1798–1801. doi: 10.4315/0362-028X.JFP-16-091

Kabak, B., and Dobson, A. D. (2017). Mycotoxins in spices and herbs–An update. *Crit. Rev. Food Sci. Nutr.* 57, 18–34. doi: 10.1080/10408398.2013.772891

Kale, S. P., Milde, L., Trapp, M. K., Frisvad, J. C., Keller, N. P., and Bok, J. W. (2008). Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal Genet. Biol.* 45, 1422–1429. doi: 10.1016/j.fgb.2008.06.009

Kew, M. C. (2003). Synergistic interaction between aflatoxin B1 and hepatitis B virus in hepatocarcinogenesis. *Liver Int.* 23, 405–409. doi: 10.1111/j.1478-3231.2003.00869.x

Kiliç Altun, S., Gurbuz, S., and Ayağ, E. (2017). Aflatoxin M_1 in human breast milk in southeastern Turkey. *Mycotoxin Res.* 33, 103–107. doi: 10.1007/s12550-016-0268-4

Kim, H. K., Lee, S., Jo, S. M., Mccormick, S. P., Butchko, R. A., Proctor, R. H., et al. (2013). Functional roles of FgLaeA in controlling secondary metabolism, sexual development, and virulence in *Fusarium graminearum*. *PLoS One* 8:e68441. doi: 10.1371/journal.pone.0068441

Kosalkova, K., Garcia-Estrada, C., Ullan, R. V., Godio, R. P., Feltrer, R., Teijeira, F., et al. (2009). The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. *Biochimie* 91, 214–225. doi: 10.1016/j.biochi.2008.09.004

Kumar, D., Barad, S., Chen, Y., Luo, X., Tannous, J., Dubey, A., et al. (2017a). LaeA regulation of secondary metabolism modulates virulence in *Penicillium expansum* and is mediated by sucrose. *Mol. Plant Pathol.* 18, 1150–1163. doi: 10.1111/mpp.12469

Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., and Kang, S. G. (2017b). Aflatoxins: a global concern for food safety, human health and their management. *Front. Microbiol.* 7:2170. doi: 10.3389/fmicb.2016.02170

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 9:559. doi: 10.1186/1471-2105-9-559

Llewellyn, G. C., Jones, H. C., Gates, J. E., and Eadie, T. (1980). Aflatoxigenic potential for aspergilli on sucrose substrates. *J. Assoc. Off. Anal. Chem.* 63, 622–625.

Lohmar, J. M., Harris-Coward, P. Y., Cary, J. W., Dhingra, S., and Calvo, A. M. (2016). rtfA, a putative RNA-Pol II transcription elongation factor gene, is necessary for normal morphological and chemical development in *Aspergillus flavus*. *Appl. Microbiol. Biotechnol.* 100, 5029–5041. doi: 10.1007/s00253-016-7418-7

Minto, R. E., and Townsend, C. A. (1997). enzymology and molecular biology of aflatoxin biosynthesis. *Chem. Rev.* 97, 2537–2556. doi: 10.1021/cr960032y

Narasaiah, K. V., Sashidhar, R. B., and Subramanyam, C. (2006). Biochemical analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. *Mycopathologia* 162, 179–189. doi: 10.1007/s11046-006-0052-7

Palmer, J. M., Theisen, J. M., Duran, R. M., Grayburn, W. S., Calvo, A. M., and Keller, N. P. (2013). Secondary metabolism and development is mediated by LlmF control of VeA subcellular localization in *Aspergillus nidulans*. *PLoS Genet.* 9:e1003193. doi: 10.1371/journal.pgen.1003193

Payne, G. A., and Hagler, W. M. Jr. (1983). Effect of specific amino acids on growth and aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus* in defined media. *Appl. Environ. Microbiol.* 46, 805–812.

Pfannenstiel, B. T., Zhao, X., Wortman, J., Wiemann, P., Throckmorton, K., Spraker, J. E., et al. (2017). Revitalization of a forward genetic screen identifies three new regulators of fungal secondary metabolism in the genus *Aspergillus*. *mBio* 8:e01246-17. doi: 10.1128/mBio.01246-17

Pitt, J. I., and Miller, J. D. (2017). A concise history of mycotoxin research. *J. Agric. Food Chem.* 65, 7021–7033. doi: 10.1021/acs.jafc.6b04494

Rao, J. P., and Subramanyam, C. (2000). Calmodulin mediated activation of acetyl-CoA carboxylase during aflatoxin production by *Aspergillus parasiticus*. *Lett. Appl. Microbiol.* 30, 277–281. doi: 10.1046/j.1472-765x.2000.00717.x

Reddy, T. V., Viswanathan, L., and Venkitasubramanian, T. A. (1979). Factors affecting aflatoxin production by *Aspergillus parasiticus* in a chemically defined medium. *J. Gen. Microbiol.* 114, 409–413. doi: 10.1099/00221287-114-2-409

Roze, L. V., Hong, S. Y., and Linz, J. E. (2013). Aflatoxin biosynthesis: current frontiers. *Annu. Rev. Food Sci. Technol.* 4, 293–311. doi: 10.1146/annurev-food-083012-123702

Senyuva, H. Z., Gilbert, J., Ozturkoglu, S., Ozcan, S., and Gurel, N. (2008). Changes in free amino acid and sugar levels of dried figs during aflatoxin B1 production by *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Agric. Food Chem.* 56, 9661–9666. doi: 10.1021/jf801912m

Shimizu, K., and Keller, N. P. (2001). Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* 157, 591–600.

Shin, J. Y., Bui, D. C., Lee, Y., Nam, H., Jung, S., Fang, M., et al. (2017). Functional characterization of cytochrome P450 monooxygenases in the cereal head blight fungus *Fusarium graminearum*. *Environ. Microbiol.* 19, 2053–2067. doi: 10.1111/1462-2920.13730

Squire, R. A. (1981). Ranking animal carcinogens: a proposed regulatory approach. *Science* 214, 877–880. doi: 10.1126/science.7302565

Sun, G., Wang, S., Hu, X., Su, J., Zhang, Y., Xie, Y., et al. (2011). Co-contamination of aflatoxin B_1 and fumonisin B_1 in food and human dietary exposure in three areas of China. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 28, 461–470. doi: 10.1080/19440049.2010.544678

Tiwari, R. P., Mittal, V., Bhalla, T. C., Saini, S. S., Singh, G., and Vadehra, D. V. (1986). Effect of metal ions on aflatoxin production by *Aspergillus parasiticus*. *Folia Microbiol.* 31, 124–128. doi: 10.1007/BF02926830

Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25, 1105–1111. doi: 10.1093/bioinformatics/btp120

Tudzynski, B. (2014). Nitrogen regulation of fungal secondary metabolism in fungi. *Front. Microbiol.* 5:656. doi: 10.3389/fmicb.2014.00656

Wang, B., Han, X., Bai, Y., Lin, Z., Qiu, M., Nie, X., et al. (2017). Effects of nitrogen metabolism on growth and aflatoxin biosynthesis in *Aspergillus flavus*. *J. Hazard. Mater.* 324, 691–700. doi: 10.1016/j.jhazmat.2016.11.043

Wee, J., Hong, S. Y., Roze, L. V., Day, D. M., Chanda, A., and Linz, J. E. (2017). The fungal bZIP transcription factor AtfB controls virulence-associated processes in *Aspergillus parasiticus*. *Toxins* 9:E287. doi: 10.3390/toxins9090287

Wilkinson, J. R., Yu, J., Bland, J. M., Nierman, W. C., Bhatnagar, D., and Cleveland, T. E. (2007). Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* SRRC 143. *Appl. Microbiol. Biotechnol.* 74, 1308–1319. doi: 10.1007/s00253-006-0768-9

Wu, D., Oide, S., Zhang, N., Choi, M. Y., and Turgeon, B. G. (2012). ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen *Cochliobolus heterostrophus*. *PLoS Pathog.* 8:e1002542. doi: 10.1371/journal.ppat.1002542

Yang, K., Liu, Y., Liang, L., Li, Z., Qin, Q., Nie, X., et al. (2017). The high-affinity phosphodiesterase PdeH regulates development and aflatoxin biosynthesis in *Aspergillus flavus*. *Fungal Genet. Biol.* 101, 7–19. doi: 10.1016/j.fgb.2017.02.004

Yao, G., Zhang, F., Nie, X., Wang, X., Yuan, J., Zhuang, Z., et al. (2017). Essential APSES transcription factors for mycotoxin synthesis, fungal development, and pathogenicity in *Aspergillus flavus*. *Front. Microbiol.* 8:2277. doi: 10.3389/fmicb.2017.02277

Young, M. D., Wakefield, M. J., Smyth, G. K., and Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* 11:R14. doi: 10.1186/gb-2010-11-2-r14

Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Dominguez, Y., and Scazzocchio, C. (2004). Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* 41, 973–981. doi: 10.1016/j.fgb.2004.08.001

Zhao, S.-P., Zhang, D., Tan, L.-H., Yu, B., and Cao, W.-G. (2016). Analysis of aflatoxins in traditional Chinese medicines: classification of analytical method on the basis of matrix variations. *Sci. Rep.* 6:30822. doi: 10.1038/srep30822

Zhao, X., Spraker, J. E., Bok, J. W., Velk, T., He, Z. M., and Keller, N. P. (2017). A cellular fusion cascade regulated by LaeA is required for sclerotial development in *Aspergillus flavus*. *Front. Microbiol.* 8:1925. doi: 10.3389/fmicb.2017.01925

Zhuang, Z., Lohmar, J. M., Satterlee, T., Cary, J. W., and Calvo, A. M. (2016). The master transcription factor mtfa governs aflatoxin production, morphological development and pathogenicity in the fungus *Aspergillus flavus*. *Toxins* 8:E29. doi: 10.3390/toxins8010029

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Yao, Yue, Fu, Fang, Xu, Ma and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership