BIOLOGICAL HAZARDS IN FOOD

EDITED BY: Maria Schirone, Pierina Visciano, Rosanna Tofalo and Giovanna Suzzi
PUBLISHED IN: Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-113-5
DOI 10.3389/978-2-88945-113-5

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**

BIOLOGICAL HAZARDS IN FOOD

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

The ingestion of food containing pathogenic microorganisms (i.e. bacteria and their toxins, fungi, viruses) and parasites can cause food-borne diseases in humans. A growing number of emerging pathogens, changes of virulence of known pathogens and appearance of antibiotic resistance has recently exposed consumers to a major risk of illness. Also infected people and the environment can spread microorganisms on raw or processed food.

Outbreaks of food-borne diseases are often unrecognized, unreported, or not investigated and particularly in developing countries their agents and sources are mostly unknown. Surveillance and analytical methods aiming at their detection are to be hoped, as well as good strategies to struggle against these threats.

This E-book is subdivided in chapters regarding to pathogenic and spoiling microorganisms, chemical hazards produced by biological agents and food safety management systems.

Citation: Schirone, M., Visciano, P., Tofalo, R., Suzzi, G., eds. (2017). Biological Hazards in Food. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-113-5

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 09 January 2017
doi: 10.3389/fmicb.2016.02154

Editorial: Biological Hazards in Food

*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-borne pathogens, food safety, food spoilage, food management

Editorial on the Research Topic

Biological Hazards in Food

HISTORY AND INTRODUCTION

Food safety is one of the main objectives related to public health protection. It is expected to prevent, minimize or eliminate risks on different stages of the food chain and in the meantime maintain, provide, and distribute high-quality food to meet consumer demands. However, millions of cases of food-borne diseases occur every year worldwide (Martinović et al., 2016). Their global impact on health and food quality assurance is well-known even if the full health effects, the kinds of unsafe food, and the economic costs are often undervalued or miscalculated, as well as the outbreaks of food-borne diseases are often unrecognized, unreported, or not investigated. The globalization can lead to a widespread distribution of foods with the introduction of new pathogens strictly associated to a specific geographical area. Nowadays foods travel long distances to be consumed worldwide but only in developed countries consumers are aware of potential presence of food-borne pathogens and surveillance and analytical methods for their detection are really effective, while in developing countries the agents and sources of food-borne diseases are mostly unknown (Wang et al., 2016). In addition, the growing number of emerging pathogens, changes of virulence of known pathogens and appearance of antibiotic resistance has potentially exposed consumers to a major risk of illness and on the other hand food industry has been required to improve the strategies to struggle of these threats.

Food-borne diseases can be caused consuming food or water contaminated by pathogenic microorganisms such as bacteria and their toxins, fungi, viruses, and parasites. Food can be contaminated both at the source as raw material, and during food processing up to storage and distribution. Also people (infected persons or carriers of pathogens) and the environment (food contact surfaces and facilities) can spread microorganisms on raw or processed food.

FOOD-BORNE PATHOGENS AND FOOD SPOILAGE

Among the major food-borne pathogens there are *Listeria monocytogenes*, *Campylobacter* spp., *Escherichia coli*, *Staphylococcus aureus,* and with respect to these microorganisms the most surveillance attention from both government agencies and food industry is recommended. These pathogens are different in terms of epidemiology, physiology, host association, and virulence, but for their overall persistence a great and continue monitoring is required. Outbreaks of listeriosis in humans are reported worldwide every year, also with a moderate percentage of fatal cases (Scallan et al., 2011). The potential pathogenic profile and virulence characterization were investigated in *L. monocytogenes* strains isolated from Chinese retail ready-to-eat food (Wu et al.). The genetic variation and phenotypic characteristics of *L. monocytogenes* isolates from retail raw foods (Chen et al.), as well as the growth potential of isolates from milkshakes, prepared from naturally contaminated ice-cream scoops linked to a listeriosis outbreak (Chen et al.) were studied

OPEN ACCESS

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Masoomeh Shams-Ghahfarokhi, Tarbiat Modares University, Iran
Ioannis S. Boziaris, University of Thessaly, Greece

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

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

Received: *16 November 2016*
Accepted: *21 December 2016*
Published: *09 January 2017*

Citation:
Schirone M, Visciano P, Tofalo R and Suzzi G (2017) Editorial: Biological Hazards in Food. Front. Microbiol. 7:2154. doi: 10.3389/fmicb.2016.02154

and reported in this eBook. The methods for cultural detection, enumeration, and molecular identification of this pathogen in different foods were also exposed (Law et al.).

Human campylobacteriosis is one the most commonly reported food-borne diseases connected with the consumption of dairy and poultry products. *Campylobacter jejuni*, followed by *Campylobacter coli* and *Campylobacter lari* are the most common species associated with human infections. The high genome diversity and plasticity within the *Campylobacter* genus need of genotypic methods for outbreak investigations (Di Giannatale et al.).

Escherichia coli is a facultative anaerobic microorganism commonly found in human intestine but several strains have acquired virulence traits and can cause illness in humans. Among these strains *E. coli* O104:H4 has been associated with food-borne diseases recently occurred in some countries in the world. The development of an immune-magnetic separation method, by using of beads coated with monoclonal antibodies specific for the lipopolysaccharide of this pathogen from milk samples, was described in detail by Luciani et al.

Some original papers included in this research topic showed the prevalence of different pathogenic microorganisms such as *S. aureus* (Yang et al.) and *Vibrio parahaemolyticus* (Xie et al.) in a particular category of food (ready-to-eat) which do not need further processing before consumption and thus, the identification of microbial contamination is critical for assuring food safety. *Vibrio parahaemolyticus* has emerged as a major food-borne pathogen in China, Japan, Thailand, and other Asian countries. A total of 72 strains were isolated from clinical (i.e. patients with food poisoning) and food samples in China (Li et al.). The environmental parameters, strain sources and genotypes can affect the strain growth variability of *V. parahaemolyticus* and these researches can be helpful in predictive microbiology and microbial risk assessment (Liu et al.).

Another microorganism increasing importance as a food poisoning pathogen is *Bacillus cereus*. Some parameters (i.e., enterotoxin gene sequences, transcription, toxin secretion, and cytotoxicity) were studied in different *B. cereus* strains isolated from food and food poisoning outbreaks (Jeßberger et al.).

Cronobacter sakazakii is an opportunistic food-borne pathogen linked with life-threatening infections in infants (Song et al.). The virulent characterization (biofilm formation and flagella motility) studied by Ye et al. provided novel insides and better knowledge of the pathogenic mechanism of this microorganism.

Human noroviruses are major contributors to acute nonbacterial gastroenteritis outbreaks. A novel approach to characterize their interaction with receptors or ligands was explored by Niu et al.

If the consumption of unsafe foods can cause food-borne illness in consumers, microbial spoilage of food can be considered a greater issue than safety in terms of economic loss. It is estimated that food spoilage results in the loss of high rates of the total food supply (Pinu, 2016). Some acetic acid bacteria growing in wine can be considered spoilage bacteria because they metabolize ethanol to acetaldehyde by alcohol dehydrogenase and then produce acetic acid by acetaldehyde dehydrogenase (Longin et al.). Spoilage microorganisms such as *Lactobacillus buchneri* are able to produce acetic acid from lactic acid consumption under anaerobic conditions also in table olives, with consequent loss of their quality. In their review, Medina-Pradas and Arroyo-López reported other microbial metabolites of the product, which can also be considered toxic. Bacteria named macergens, which are responsible for plant tissue maceration by releasing pectic enzymes were described in the review of Aremu and Babalola.

OTHER MICROBIAL TOXINS AS PUBLIC HEALTH HAZARDS

Some chemical hazards are produced by biological agents and affect people with very different symptoms ranging from relatively mild discomfort to serious and life-threatening illness up to a fatal outcome. Among these hazards, marine biotoxins are produced by harmful algal blooms and can accumulate in bivalve molluscs. They are classified on the basis of their poisoning symptoms or their chemical structures. Regulatory limits and official detection methods have been established by the European legislation for these compounds (Visciano et al.).

The presence of biogenic amines in food and food products can be due to microorganisms (mainly bacteria) through the action of decarboxylases and environmental factors such as temperature, salt concentrations, and pH can influence their formation. Histamine and tyramine cause more severe acute effects in consumers. Some technological factors (i.e., addition of starter cultures, additives, effects of packaging) can be used for controlling their production (Gardini et al.).

Mycotoxin contamination in cereals and derived products represents an important problem for agriculture and food industries because it can cause substantial economic losses, mycotoxicoses in farmed animals and also toxic effects for human health. Aflatoxins are the most serious carcinogenic, hepatotoxic, teratogenic, and mutagenic secondary metabolites produced by fungi at any stage of food production from pre-harvest to storage. In recent years researchers reported new interventions to reduce or prevent the growth of fungi and subsequently mycotoxin production. The use of plant aqueous extracts was tested for their antifungal potential against aflatoxigenic isolates of *Aspergillus* spp. by Iram et al. and Iram et al.

FOOD SAFETY MANAGEMENT SYSTEMS

A food safety management system includes both control and assurance activities. Preventive measures aiming at avoiding contamination or outgrowth of microorganisms, as well as at their reduction or elimination, can be physical, chemical, and/or biological (Zwietering et al., 2016).

Equipment and food contact surfaces in food industries can provide a substrate for the development of the so-called biofilm, which represent a microbial community where bacteria can live in an extracellular matrix made of polysaccharides, extracellular DNA, and proteins. Cleaning

and disinfection chemical products, such as surfactants and alkali compounds, can be used for the prevention of biofilm formation (Campana et al., 2017). Biofilm can be formed both on abiotic and biotic surfaces (Diaz et al.). The adherence of pathogenic microorganisms to surfaces and the formation of biofilm can be associated also to the production of biosurfactants. Rossi et al. reported that the biosurfactants produced by *Salmonella* Enteritidis SE86 contributed to adherence to slices of lettuce leaf and decreased the antimicrobial action of sanitizers used to sanitize whole lettuce leaves.

Some bacterial strains able to produce antagonistic molecules used as antimicrobials and preservatives can represent bio-control agents against harmful or pathogenic microorganisms in food. *Lactobacillus plantarum* is one of the most versatile species used in food industry as microbial starter or probiotic microorganism. Different strains of this species were shown to produce antimicrobial compounds and bacteriocins (Arena et al.).

Among physical approaches for food safety management, Patrignani and Lanciotti reported the application of high and ultra-high pressure homogenization for microbial inactivation and food safety purpose, even if they are not yet implemented in food industry, because they do not guarantee the sterilization of food.

CONCLUSIONS

Food-borne diseases represent a significant problem for individuals, communities and food industries. Many factors are involved in these outbreaks such as a lacking quality of raw material, uncorrected handling of food, poor personal hygiene, and improper holding times/temperatures along the food chain. The articles included in this research topic range from food-borne pathogens and their metabolites/microbial toxins to preventive measures and management approaches to control and reduce/eliminate these hazards for the public health.

All the papers published within this eBook are reported in the references.

AUTHOR CONTRIBUTIONS

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

REFERENCES

Campana, R., Casettari, L., Fagioli, L., Cespi, M., Bonacucina, G., and Baffone, W. (2017). Activity of essential oil-based microemulsions against *Staphylococcus aureus* biofims developed on stainless steel surface in different culture media and growth conditions. *Int. J. Food Microbiol.* 241, 132–140. doi: 10.1016/j.ijfoodmicro.2016.10.021

Martinović, T., Andjelković, U., Šrajer Gajdošik, M., Rešetar, D., and Josić, D. (2016). Foodborne pathogens and their toxins. *J. Proteomics* 146, 226–235. doi: 10.1016/j.jprot.2016.04.029

Pinu, F. R. (2016). Early detection of food pathogens and food spoilage microorganisms: application of metabolomics. *Trends Food Sci. Tech.* 54, 213–215. doi: 10.1016/j.tifs.2016.05.018

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

Wang, S., Weller, D., Falardeau, J., Strawn, L. K., Mardones, F. O., Adell, A. D., et al. (2016). Food satety trends: from globalization of whole genome sequencing to application of new tools to prevent foodborne diseases. *Trends Food Sci. Technol.* 57, 188–198. doi: 10.1016/j.tifs.2016.09.016

Zwietering, M. H., Jacxsens, L., Membré, J.-M., Nauta, M., and Peterz, M. (2016). Relevance of microbial finished product testing in food safety management. *Food Control* 60, 31–43. doi: 10.1016/j.foodcont.2015.07.002

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.

frontiers in Microbiology

REVIEW
published: 12 August 2016
doi: 10.3389/fmicb.2016.01218

Technological Factors Affecting Biogenic Amine Content in Foods: A Review

Fausto Gardini[1,2], Yesim Özogul[3], Giovanna Suzzi[4], Giulia Tabanelli[1,2] and Fatih Özogul[3]*

[1] Centro Interdipartimentale di Ricerca Industriale Agroalimentare, Università degli Studi di Bologna, Cesena, Italy, [2] Dipartimento di Scienze e Tecnologie Agro-alimentari, Università degli Studi di Bologna, Cesena, Italy, [3] Department of Seafood Processing Technology, Faculty of Fisheries, Çukurova University, Adana, Turkey, [4] Faculty of BioScience and Technology for Food, Agriculture and Environment, University of Teramo, Mosciano Sant'Angelo, Italy

Biogenic amines (BAs) are molecules, which can be present in foods and, due to their toxicity, can cause adverse effects on the consumers. BAs are generally produced by microbial decarboxylation of amino acids in food products. The most significant BAs occurring in foods are histamine, tyramine, putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermine, spermidine, and agmatine. The importance of preventing the excessive accumulation of BAs in foods is related to their impact on human health and food quality. Quality criteria in connection with the presence of BAs in food and food products are necessary from a toxicological point of view. This is particularly important in fermented foods in which the massive microbial proliferation required for obtaining specific products is often relater with BAs accumulation. In this review, up-to-date information and recent discoveries about technological factors affecting BA content in foods are reviewed. Specifically, BA forming-microorganism and decarboxylation activity, genetic and metabolic organization of decarboxylases, risk associated to BAs (histamine, tyramine toxicity, and other BAs), environmental factors influencing BA formation (temperature, salt concentration, and pH). In addition, the technological factors for controlling BA production (use of starter culture, technological additives, effects of packaging, other non-thermal treatments, metabolizing BA by microorganisms, effects of pressure treatments on BA formation and antimicrobial substances) are addressed.

Keywords: biogenic amines, fermented foods, lactic acid bacteria, decarboxylase activity, a_w, pH, temperature

OPEN ACCESS

Edited by:
Andrea Gomez-Zavaglia, National Scientific and Technical Research Council (CONICET), Argentina

Reviewed by:
Giuseppe Spano, University of Foggia, Italy
Elvira Maria Hebert, National Scientific and Technical Research Council (CONICET), Argentina

****Correspondence:***
Giulia Tabanelli
giulia.tabanelli2@unibo.it

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

Received: *22 March 2016*
Accepted: *21 July 2016*
Published: *12 August 2016*

Citation:
Gardini F, Özogul Y, Suzzi G, Tabanelli G and Özogul F (2016) Technological Factors Affecting Biogenic Amine Content in Foods: A Review. Front. Microbiol. 7:1218. doi: 10.3389/fmicb.2016.01218

INTRODUCTION

Biogenic amines (BAs) are organic bases, which can be present in foods and can cause several adverse reaction in the consumers. They are produced by microorganisms (mainly bacteria) through the action of decarboxylases (carboxy-lyases EC number 4.1.1.1.), which act selectively on specific amino acids in which they remove the carboxyl group with the formation of the correspondent amine and CO_2.

In relation to their amounts and their toxicological effects, the most important BAs in foods are histamine (an heterocyclic amine deriving from histidine), tyramine and 2-phenylethylamine (deriving from the aromatic amino acids tyrosine and phenylalanine, respectively), tryptamine

(heterocyclic BA from tryptophan), putrescine (a polyamine obtained through a direct decarboxylation of ornithine or through the agmatine deiminase pathway, which follow the decarboxylation of arginine to agmatine), and cadaverine (a polyamine derived from lysine; Silla Santos, 1996; Landete et al., 2008a; Tanaka et al., 2008; Marcobal et al., 2012; Wunderlichová et al., 2014). In addition, other polyamines (spermine and spermidine) can be produced with a more complex pathway, which starts from putrescine (Bardócz, 2005; Kalač and Krausová, 2005).

There are two essential physiological reasons leading to the activation of decarboxylative pathways. From one side, decarboxylation is one of the responses of cells to acid stress and the final balance of the pathway, which consists in the loss of a carboxylic group, contributes to the intracellular (and extracellular) pH increase (Connil et al., 2002; Tanaka et al., 2008; Pereira et al., 2009; Perez et al., 2015). Furthermore, it has been demonstrated that these pathways can bring supplementary energy for the cells by energizing the protonmotive force associated to the membrane (**Figure 1**). In fact, a net positive charge is transferred outside the cell during the exchange between precursor (amino acid) and BA (Molenaar et al., 1993; Konings et al., 1997; Konings, 2006; Wolken et al., 2006; Pereira et al., 2009).

The presence of dangerous amounts of BAs is associated with a relevant growth (>7 log cfu/g) of decarboxylating microorganisms. For this reason, some authors proposed microbial quality indices based on food BA content as indirect indicators of excessive microbial proliferation (Karmas, 1981; Ruiz-Capillas and Jiménez-Colmenero, 2004; Baixas-Nogueras et al., 2005; Özogul and Özogul, 2006; Al Bulushi et al., 2009). Nevertheless, fermented foods requires (by definition) a massive growth of microorganisms which are often responsible for noteworthy BA accumulation, especially during the ripening phase (when selected starter cultures can be replaced by wild strains) or when natural (spontaneous) fermentations are adopted (Suzzi and Gardini, 2003; Ancín-Azpilicueta et al., 2008; Rabie et al., 2011a; Linares et al., 2012).

BAs can be produced both by Gram-positive and Gram-negative bacteria (Landete et al., 2008a; Marcobal et al., 2012; Wunderlichová et al., 2014). Also some fungi (yeast and molds) are involved in BA accumulation (in particular cadaverine and putrescine), but their role is debated and, for many aspects, controversial (Caruso et al., 2002; Gardini et al., 2006; Kiss et al., 2006; Tristezza et al., 2013; Qi et al., 2014).

In general, there is no legal regulation about the BA content in food. This is mainly due to the individual toxicological threshold, which can be extremely variable from few mg/kg in sensitive person to some hundred mg/kg in healthy person (Shalaby, 1996; McCabe-Sellers et al., 2006; Fogel et al., 2007; Hungerford, 2010; Knope et al., 2014). The only exception is the scombroid fish, because its richness in histidine and aptitude to support the growth of decarboxylating microorganisms, for which several national and international authorities define a limit for histamine (Anonymous, 2001; The Australian and New Zealand Food Standards Code, 2002; European Community Commission Regulation, 2005; Prester, 2011). In alcoholic beverages (wine, beer, etc.) the toxicity of BAs is enhanced by the presence of ethanol, an inhibitor of mono amino oxidases (Ancín-Azpilicueta et al., 2008). Nevertheless, the presence of high amounts of BA can be related to scarce microbiological quality of raw materials, during the improper storage of the products, as well as an uncontrolled fermentation (Suzzi and Gardini, 2003; Naila et al., 2010; Linares et al., 2012).

Recently, a qualitative risk assessment concerning BA in fermented foods was conducted in the European Union

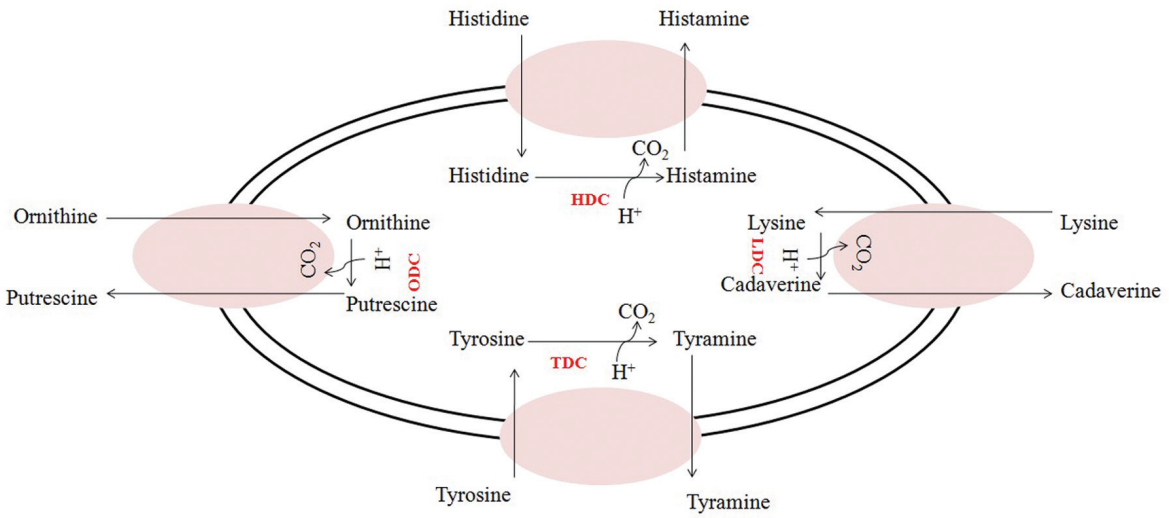


FIGURE 1 | Amino acid decarboxylation-antiporter reactions in which an amino acid is transported into the cell, where decarboxylation occurs. A proton (H^+) is consumed, and a carbon dioxide (CO_2) is removed during the reaction, and the product (biogenic amines) is exported from the cell via an antiporter. HDC, histidine decarboxylase; TDC, tyrosine decarboxylase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase.

(EFSA, 2011). According to this study, histamine was present in detectable amounts in the 85% of the dairy fermented products, 83% of fermented vegetables, 45% of fermented meats, and 73% of fish products. The higher concentrations of that were found in fish sauces, dried anchovies, and cheeses. Tyramine was detected in 75% of dairy products and 83% of fish products, but the higher concentrations were found in meat product (fermented sausages and cured meats) and cheeses. Similar results were characterized in 2-phenylethylamine formation in various food products as well. In addition, putrescine, cadaverine, and tryptamine were most frequently detected in dairy food products.

This review summarizes the current state of knowledge of the effects of the major environmental and process factors on the decarboxylase activity of microorganisms. It also highlights the strategies available to reduce the BA accumulation in food products.

BIOGENIC AMINE TOXICITY AND DECARBOXYLATING MICROORGANISMS IN FOOD

The decarboxylation process can be catalyzed through two biochemical pathways. The first is catalyzed by naturally occurring endogenous amino acid decarboxylases present in animal or vegetable cells and the second by exogenous enzymes produced by various microorganisms under favorable conditions (Halász et al., 1994).

Natural polyamines represent the main amines found in fresh food products (fish, fruits, vegetable, milk, and meat) where they have a physiological role associated with cell growth and proliferation (Bover-Cid et al., 2014). The intracellular biosynthesis of endogenous amines such as the polyamines spermine and spermidine and other amines like histamine involves the incorporation of aminopropyl groups into their precursor putrescine (Bardócz, 2005).

Exogenous BAs derive from decarboxylases secreted by microorganisms, which are present naturally in food products, introduced by contamination, or also added to foods as a starter culture. The enzymatic decarboxylation process depends on various factors such as the availability of the substrate in free form, the presence of decarboxylase-producing microorganisms and the medium conditions (pH, temperature, O_2, etc.). The free amino acid are either naturally present in the food or produced via proteolysis both by endogenous proteases in the raw products and microbial enzymes (Danquah et al., 2012). In fact, proteolysis may play an important role in the release of free amino acids from tissue proteins, which offer a substrate for decarboxylases reactions (Shalaby, 1996).

Risk Associated to Biogenic Amines in Food

The presence of BAs in food can constitute a risk to the consumer health (Gram and Dalgaard, 2002; Gram et al., 2002; Özogul et al., 2011). Ingestion of food containing high amounts of BAs is implicated in various pharmacological and toxicological reactions. In fact, BA intake can cause headaches, heart palpitations, vomiting, and diarrhea. Moreover, hypertensive crises have been reported after consuming food containing BAs, such as cheese, wine, beer, and vegetables including sauerkraut, broad bean, banana peel, and avocado (Moret et al., 2005; Maintz and Novak, 2007; Hungerford, 2010).

Once the decarboxylase enzymes are synthesized by the bacteria, BA production can continue even if the bacteria are eliminated from the food product by cooking or other technological treatment. The BAs produced are heat stable and, once formed, are not destroyed by cooking, smoking, freezing, or some other type of preservation techniques (Becker et al., 2001).

Under normal conditions in humans, exogenous amines ingested with food are rapidly detoxified (Hornero-Mendez and Garrido-Fernandez, 1997). The enzymes monoamine oxidase (MAO) and diamine oxidase play an important role in this detoxification process. However, the severity of BA toxicological effects depends on the intake with food, on individual allergy and on the consumption of MAO inhibiting drugs, alcohol, and other food amines (Silla Santos, 1996; Sathyanarayana Rao and Yeragani, 2009).

The BAs with the more severe acute effects for human health are histamine and tyramine.

Histamine causes a symptomatology known as "scombroid fish poisoning" consisting in flushing of the face, neck and upper arms, oral numbness and/or burning, metallic taste, headache, itchy rash, heart palpitations, asthma attacks, hives, gastrointestinal symptoms, and difficulties in swallowing (Lehane and Olley, 2000; Maintz and Novak, 2007; Hungerford, 2010; Knope et al., 2014). This type of food intoxication often results from consumption of scombroid fish (such as tuna, sardines, anchovies, bonito, mackerel, etc.), which are rich in histidine, because of the proliferation of histidine decarboxylative Gram-negative bacteria. However, these BAs can also be found in fermented products (wine, cheese, fish sauce, and fermented meat) where it is mainly produced by lactic acid bacteria (LAB).

Tyramine toxicity is known as "cheese reaction" because it was initially observed following the consumption of cheeses with high level of this BA (Shalaby, 1996). Tyramine has a vasoconstrictor effect and causes dietary-induced migraine, increased cardiac output, nausea, vomiting, respiratory disorders, and elevated blood glucose (Shalaby, 1996; McCabe-Sellers et al., 2006; Scheepens et al., 2010; Stadnik and Dolatowski, 2010; Marcobal et al., 2012). This increase in blood pressure due to tyramine can cause heart failure or brain hemorrhage (Naila et al., 2010). Besides to these effects, tyramine has also been determined to have an effect on the gut microbiota. The adherence of some enteropathogens, such as *Escherichia coli* O157:H7, to intestinal mucosa is increased in the presence of tyramine (Russo et al., 2012). This type of food intoxication is usually associated with the consumption of fermented foods because LAB are the most efficient producers of tyramine (Shalaby, 1996; Ladero et al., 2012).

As far as other BAs, putrescine and cadaverine have low toxicological properties on their own (Košmerl et al., 2013). However, they could potentiate the effects of histamine and

tyramine toxicity by inhibiting their metabolizing enzymes (Landete et al., 2007; Al Bulushi et al., 2009). Moreover, putrescine and cadaverine can act as a precursor to the formation of carcinogenic *N*-nitrosamines in the presence of nitrite (Rauscher-Gabernig et al., 2012; De Mey et al., 2014). High concentrations of putrescine have been linked to tumor development such as promotion of CT-26 colon tumor cell growth (Farriol et al., 2001).

Despite their cellular functions, the polyamine agmatine, spermine, and spermidine catabolism and their excess levels can lead to toxicity. It has been demonstrated that spermine and spermidine could decrease blood pressure, inhibit blood clotting and provoke respiratory symptoms and neurotoxicity resulting in renal insufficiency (Pegg, 2013).

Even if 2-phenylethylamine is present naturally in several mammalian tissues such as the brain, it is found in certain foodstuffs (chocolate, cheese and red wine) and has been known to trigger migraine attacks and increase blood pressure (Panoutsopoulos et al., 2004; Souza et al., 2005).

Main Microbial Groups Involved in BA Production in Foods

Among Gram-negative bacteria, spoilage microorganisms belonging to enterobacteria and pseudomonads are known as the major producers of histamine, cadaverine, and putrescine (Ben-Gigirey et al., 2000; de las Rivas et al., 2007; Pircher et al., 2007; Lorenzo et al., 2010). The genus *Photobacterium* is often involved in the accumulation of histamine in fish and seafood products, together with *Aeromonas hydrophila* and enterobacteria such as *Morganella morganii*, *Enterobacter aerogenes*, *Raoultella planticola*, and *Klebsiella oxytoca* (Morii and Kasama, 2004; Veciana-Nogués et al., 2004; Kanki et al., 2007; Al Bulushi et al., 2009; Fernández-No et al., 2010; Küley et al., 2013). BAs produced by enterobacteria were also found in fermented sausages, meat, and cheeses (Ruiz-Capillas and Jiménez-Colmenero, 2004; Bover-Cid et al., 2009; Linares et al., 2011).

The ability to produce BAs is widespread also among Gram-positive bacteria. The decarboxylase activity has been found in strains belonging to the genera *Staphylococcus* and *Bacillus* (Landeta et al., 2007; Chang and Chang, 2012). However, the attention has been mainly focused on LAB, which are commonly present in the ripening microbiota of several fermented foods. In fact, LAB can produce histamine, cadaverine, putrescine, but, in particular, they are the most efficient producers of tyramine (Arena and Manca de Nadra, 2001; Pereira et al., 2001; Suzzi and Gardini, 2003; Pircher et al., 2007; Buňková et al., 2009, 2011; Kuley and Özogul, 2011; Ladero et al., 2012). The tyrosine decarboxylase of LAB is often able to decarboxylate also phenylalanine (producing 2-phenylethylamine) even if with a minor efficiency compared to tyrosine (Marcobal et al., 2006a). This ability was confirmed by several authors which also underlined that phenylalanine was used as substrate by the enzyme only when tyrosine was not available (Beutling and Walter, 2002; Pessione et al., 2009; Bargossi et al., 2015b).

Oenococcus oeni, *Lactobacillus hilgardii*, *Lactobacillus fructivorans*, *Pediococcus parvulus*, *Lactobacillus brevis* are responsible for amines accumulation in wine. Decarboxylating strains of *Lactobacillus curvatus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus paracasei* were isolated from cheese, meat, and sausage with high BA content (Moreno-Arribas et al., 2003; Smit et al., 2008; Marcobal et al., 2012; Russo et al., 2012; Wunderlichová et al., 2014).

Yeast strains belonging to various species including *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Candida stellata*, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Brettanomyces bruxellensis* are also capable of aminogenesis (Romano et al., 2007). Yeast strains isolated from grapes and wines are able to yield high BA amounts (Caruso et al., 2002). In addition, the fungus *Botrytis cinerea* is considered a producer of BAs in grape must (Bäumlisberger et al., 2015).

The genetic organization of decarboxylase clusters has been reviewed for tyramine (Marcobal et al., 2012), histamine (Landete et al., 2008a), and putrescine (Wunderlichová et al., 2014).

ENVIRONMENTAL FACTORS INFLUENCING BIOGENIC AMINE FORMATION

The main environmental factors affecting microbial activities in foods are temperature, salt concentration, and pH. These factors can influence the formation of BAs in two ways. In first instance, they are responsible for the overall metabolism of the decarboxylating cells. In addition, the activity of decarboxylases depends on the same parameters. The optimal values of environmental factors for these two different aspects can be different (Bargossi et al., 2015a) and the final amount of BAs is the result of this double influence. In other words, growth of aminobiogenetic bacteria is an essential but not sufficient condition for BA production (Marcobal et al., 2006b). Moreover, some decarboxylase can maintain their activity independently of the integrity of the microbial cells in a wide range of conditions. This has been demonstrated for tyrosine decarboxylase in lactobacilli (Moreno-Arribas and Lonvaud-Funel, 2001), histidine decarboxylase in *S. thermophilus* (Tabanelli et al., 2012) and Gram-negative bacteria such *as Photobacterium phosphoreum*, *Photobacterium damselae*, *M. morganii*, and *R. planticola* (Kanki et al., 2007).

In general, the data reported indicate a great variability in the response of the cell decarboxylase systems to the environmental factors, which reflects differences among species and genus metabolic pathways, experimental conditions, type of matrix (food) considered, but also it is the results of the great heterogeneity characterizing decarboxylase activity, even within strains of the same species.

Even if the environmental factors significantly affect the rate and the entity of BA accumulation, in fermented foods their modulation is often limited by the conditions, which allow the fermentation and ripening processes (in turn linked to the

"traditional" features of the products) and by health trends, as in the case of the reduction of NaCl content.

Temperature

Temperatures close to the optimum growth values, promoting cell metabolism and proliferation, generally favor the production of BAs, which is often related to the number of cells present in the system. However, the presence of high number of decarboxylating cells is not a sufficient condition to explain the final BA amount (Marcobal et al., 2006b, 2012).

Studies carried out in a model system using a *E. faecalis* EF37, demonstrated that the increase of temperature from 16 to 44°C coincided with a faster growth and with a more rapid and intense accumulation of tyramine (Gardini et al., 2001). More recently, Bargossi et al. (2015a) studied the effect of temperature on the activity of a pure commercial tyrosine decarboxylase extracted from *E. faecalis* and found the highest decarboxylation efficiency at a temperature comprised between 30 and 37°C. By contrast, Zhang and Ni (2014) found that a tyrosine decarboxylase from *L. brevis* had its optimum temperature at 50°C, but it was rapidly inactivated at higher temperature. However, the activity of the enzyme at the optimum temperature was rapidly decreased during the permanence at 50°C for an hour.

Cells of *E. faecalis* and *E. faecium* strains suspended in buffered systems containing tyrosine and incubated at different temperatures confirmed the maximum decarboxylase activity at 37°C after 2 h of incubation. However, after 24 h the maximum tyramine content was surprisingly found in the sample incubated at the less favorable temperature (20°C; Bargossi et al., 2015a).

Using an experimental design in which several parameters were taken into consideration, Marcobal et al. (2006b) found that the optimum temperature for tyramine production under aerobic condition by *E. faecium* and *L. brevis* was 32°C. By contrast, under anaerobic conditions, the maximum tyramine concentration was obtained at 22.0–24.5°C.

The histidine decarboxylase of a cell free extract of a *S. thermophilus* strain had its maximum activity at 50°C. It decreased rapidly at higher temperature (60°C), while it maintained a detectable activity at 5°C (Tabanelli et al., 2012). By contrast, active cells of the same strain produced more rapidly histamine at 40°C, while the BA accumulation was limited or negligible at 25 and 20°C within the incubation period considered. In addition, an histamine producing strain (*S. thermophilus*) incubated at low-temperature (4°C) in milk produced less histamine than did the same strain kept at 42°C. This reduction was attributed to a lower activity of the histidine decarboxylase enzyme rather than to a reduction in gene expression or the presence of a lower cell number (Calles-Enríquez et al., 2010).

Regarding Gram-negative bacteria, Morii and Kasama (2004) studied in *P. phosphoreum* cell free extract the activity of two different histidine decarboxylases and found that the inducible enzyme had its maximum activity at 30°C while the constitutive one at 40°C. The optimum growth temperature of the strain was 25°C while the specific activity of histidine decarboxylase of cell free extracts was extremely high in the cells grown at low temperature (7°C). The histamine producing potential of the Gram-negative bacterium *Mycobacterium psychrotolerans* was studied in relation to the temperature in the range 0–20°C. Increasing temperature enhanced the rate of accumulation but also the final tyramine accumulation (Emborg and Dalgaard, 2008). The optimum temperature for histidine decarboxylase activity of bacteria belonging to different species (*M. morganii*, *R. planticola*, *P. phosphoreum*, and *P. damselae*) ranged between 30 and 40°C. It was still active at 5°C but not at 60°C (Kanki et al., 2007).

Scarce reports are available concerning the relation between other BAs (putrescine, cadaverine, and tryptamine) and temperature. Generally, the accumulation of BAs, among which cadaverine and putrescine increased with temperature (Wunderlichová et al., 2014); nevertheless, prolonged storage at low temperatures can result in accumulation of putrescine explained by the metabolism of psychrotrophic pseudomonads (Paulsen and Bauer, 1997).

Bubelová et al. (2015) studied the production of putrescine and cadaverine in relation to temperature in *Serratia marcescens*. They found that the maximum amount of these two BAs was reached at 20–30°C. If the production was compared to the biomass ("yield factor"), the decarboxylase activity of the single cells was maximum at 10°C.

In general, the ability to produce BAs is limited by the decreasing of the temperature. This implies that the control of the cold chain during storage and commercialization is a main tool to avoid the accumulation of undesired products after manufacturing, especially in not fermented foods, such as fishery products (Knope et al., 2014). Several authors stressed the crucial effect of the storage temperature on the histamine formation (and other BAs) in fish such as tuna (Veciana-Nogués et al., 2004; Emborg and Dalgaard, 2006; Kanki et al., 2007) and anchovies (Veciana-Nogués et al., 1997; Visciano et al., 2007).

Regarding fermented foods, the temperature of fermentation and during ripening has to allow the microbiological activity of the desired microbiota and the range within they can be modulated is rather strict, defined by the protocols for the production of the different fermented food typologies. The temperature applied during the first 3 days of fermentation of dry sausages influenced the BA accumulation (tyramine, 2-phenylethylamine, cadaverine, and putrescine) during all the ripening period (1 month) with increasing values in the presence of higher temperature, which was between 15 and 25°C (Gardini et al., 2008; Bover-Cid et al., 2009). Higher fermentation temperatures (and higher relative humidity) favored tyramine and 2-phenylethylamine accumulation in the Spanish fermented sausages Fuet and Llonganissa inoculated with *L. curvatus* (Latorre-Moratalla et al., 2012). By contrast, no differences were found in Turkish sausages ripened at 22 or 26°C (Gücükoğlu and Küplülu, 2010).

Ruiz-Capillas et al. (2007) found that tyramine was accumulated in higher amounts in pressurized sliced cooked ham packaged under vacuum when the storage temperature was higher. Also fermented sausages stored after production under room temperature were characterized by higher BA content than refrigerated products (Komprda et al., 2001).

A higher tyramine content was found in salted duck inoculated with *E. faecalis* stored at 20°C compared with the samples incubated at 4°C; however, no differences were found in the tyrosine decarboxylase gene expression indicating an univocal effect of temperature on enzymatic activity (Liu et al., 2014). Also in green fermented olives, the adoption of a low fermentation temperature reduced the accumulation of cadaverine and tyramine together with the formation of "zapatera" defect (García García et al., 2004).

The application of thermal treatments (when possible) to raw material such as milk before fermentation (pasteurization) can contribute to the elimination of the wild decarboxylating microbiota. The Gram-negative BA producers (enterobacteria and pseudomonads) are rapidly inactivated by temperatures higher than 60°C. LAB are more resistant and require more drastic thermal treatments. For this reason, usually cheeses from pasteurized milk are characterized by lower BA content (Schneller et al., 1997; Novella-Rodríguez et al., 2003; Novella-Rodríguez et al., 2004; Marino et al., 2008). However, Ladero et al. (2011) observed the survival in skim milk of a tyraminogenic strain of *L. curvatus* after a treatment at 78°C. Independently of the cell viability, the information concerning the thermal stability of decarboxylase is scarce. The histamine decarboxylase produced by *S. thermophilus* maintained a residual activity after treatments at 70 and 75° for 10 min (Tabanelli et al., 2012). In any case, the pasteurization of milk does not avoid the presence of a ripening microbiota, which can contribute to BA accumulation. Pinho et al. (2001) found a relevant increase in BA content of a Portuguese cheese (Azeitao) when the storage temperature increased from 4 to 25°C, related to the higher protease activity. In addition, it has been observed that the BAs content was higher in Dutch type cheese when the temperature of ripening and storage increased (Buňková et al., 2010; Pachlová et al., 2012).

Salt Concentration

In general, increasing salt concentrations contribute to the reduction of BA accumulation in foods, mainly reducing the metabolic activities of decarboxylating microorganisms. In particular, Gram-negative bacteria are more inhibited by increasing salt concentrations than Gram-positive microbiota. However, the health trend to reduce NaCl concentration is in contrast with this possible tool to reduce BA accumulation in foods.

The pure tyrosine decarboxylase tested by Bargossi et al. (2015a) demonstrated a limited loss of its relative activity in the presence of increasing amount of NaCl up to 10%. Only the addition of 15% of salt determined a more marked reduction of the decarboxylation potential, which remained, however, higher than 50% of the activity recorded in the absence of salt added.

In an *E. faecalis* strain (EF37) grown on a synthetic medium, the ability to accumulate tyramine and phenylethylamine was inversely related to the NaCl concentration, in a range comprised between 2 and 6%, while the proteolysis showed an optimum between 2 and 3% (Gardini et al., 2001). In fermented sausages inoculated with the same tyraminogenic *E. faecalis* strain, increasing amounts of salt reduced the concentration of tyramine, 2-phenylethylamine produced by enterococci, but also limited cadaverine and putrescine production by enterobacteria (Gardini et al., 2008; Bover-Cid et al., 2009).

The tyramine production of different strains of *E. faecalis* and *E. faecium* was studied in buffered systems containing tyrosine; the results indicated that *E. faecalis* partially reduced its tyraminogenic potential of cells passing from 0 to 5% of NaCl but the decarboxylation activity did not change significantly increasing NaCl concentration up to 15%. On the other hand, the same enzymatic activity in cells of *E. faecium* remained quite constant independently of the NaCl concentration (Bargossi et al., 2015a). Strains of *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* reached their maximum tyramine production in synthetic medium only in the presence of the higher salt concentration used in the trials (2%; Buňková et al., 2011); in addition the same condition allowed the maximum tyramine production rate as well as the lowest time for the production of detectable amounts of the BA.

A different role of salt on the histidine decarboxylase activity in a strain of *S. thermophilus* was highlighted in viable cells and in cell free extract. While the production of histamine was almost completely prevented in living cells by a salt concentration of 2.5% (by limiting or inhibiting the growth potential of *S. thermophilus*), the activity of the decarboxylase in cell free extract was unaffected up to 5% NaCl and then slowly decreasing, maintaining an activity, even if reduced, at 20–30% NaCl (Tabanelli et al., 2012). The presence of NaCl led to an up-regulation of histidine decarboxylase gene in the same strain grown on skim milk, suggesting a potential role of this enzyme also in osmoprotection mechanisms (Rossi et al., 2011). This confirmed that the activation of decarboxylase systems is a part of complex metabolic responses in the presence of different stress conditions (Pessione et al., 2009).

A halophilic LAB strain of *Tetragenococcus muriaticus* isolated from fish sauce produced histamine during the late exponential growth phase, reached a maximum production of this BA at 5–7% of NaCl, and was able to maintain a histidine decarboxylase activity also in the presence of 20% of salt (Kimura et al., 2001). On the other hand, the histamine decarboxylase activity of two strains of *P. phosphoreum* decreased rapidly with the increase of salt (2–5% of the optimum activity in the presence of 10% of NaCl), while more resistant (40–50% of the optimum activity) resulted the activity of the same enzyme in strains of *R. planticola*, *P. damselae*, and *M. morganii* (Kanki et al., 2007). Using a strain of *P. phosphoreum*, Morii and Kasama (2004) found that histidine decarboxylase activity in cell free extract was higher at level of 5% NaCl, while the cells were not able to multiply (and produce BA) under this salt concentration. Similar effects were described also for Bifidobacteria (Lorencová et al., 2014). The hypothesis of this possible enhancing effect on the BA production of NaCl has been found by some authors in the essential role of Na^+ ion in the sodium/proton antiport system through which H^+ ions are removed from the cell (Pereira et al., 2009; Buňková et al., 2011; Lorencová et al., 2014; Bubelová et al., 2015).

Serratia marcescens produced putrescine and cadaverine with more efficiency in the presence of 1–3% NaCl (3–5 in the yield factor was applied; Bubelová et al., 2015). Cells of *M. psychrotolerans* produced more histamine when grown in the

presence of 4% NaCl compared with lower salt concentration (Emborg and Dalgaard, 2008) even if this salt concentration slowed the growth of the strain. In other words, stressed cells seem to activate the decarboxylating pathways in the framework of more complex response systems. This make the potential of BA production by each single cell more efficient. Also *E. aerogenes* produced the maximum amounts of cadaverine, putrescine, and histamine in the presence of the 3% of NaCl (Greif et al., 2006).

In fermented sausages, BAs are accumulated during ripening. However, the rate of accumulation decreases with the decrease of a_w due to the water losses. Products packaged under modified atmosphere packaging (MAP), in which the weight losses were inhibited, continued to accumulate BAs when the packaging was carried out at high a_w (0.92 and more; González-Tenorio et al., 2013; Tabanelli et al., 2013). The Greek cheese Feta, characterized by a high salt content, with a ripening carried out in brine and with a low pH, was characterized by a noteworthy amine concentration (about 200 mg/kg of tyramine, 90 mg/kg of histamine, and 200 mg/kg of putrescine; Valsamaki et al., 2000).

pH

Since the decarboxylation is a mechanism of cells to counteract acidic stress, it is clear that several studies were focused on the study of the relationships between pH and BA accumulation. Also in this case, the effect of pH is different if the focus is directed toward the activity of the pure enzyme or the decarboxylase activity of the living cells. In any case, it has been extensively demonstrated that the transcription of genes of many decarboxylase clusters are induced by low pH and improves the fitness of cells subjected to acidic stress (Pereira et al., 2009; Pessione et al., 2009; Marcobal et al., 2012; Romano et al., 2012, 2014; Perez et al., 2015)

A commercial pure tyrosine decarboxylase had its maximum activity in buffered systems at pH between 5 and 6 (Bargossi et al., 2015a), while at pH 4 the same activity was extremely weak. The tyrosine decarboxylase obtained from a strain of *L. brevis* had its maximum relative activity at pH 5, while it maintained the higher stability at pH 7.4, e.g., 92% activity retained after 7 days of incubation (Zhang and Ni, 2014).

By contrast, a strain of *E. faecalis* under the same conditions showed the maximum tyramine accumulation at pH 4 after 2 h of incubation and pH between 4 and 5 after 24 h. In the same system, a strain of *E. faecium* did not show relevant differences in its decarboxylase activity at pH between 4 and 6. No pH differences in relation to the production of tyramine were observed in whole cells or cell free extract of *L. brevis*, with optimum activity at pH 5 (in the range 2–9); however, the cell-free extract had a higher activity compared with the whole cells (Moreno-Arribas and Lonvaud-Funel, 2001). Two tyrosine decarboxylases from *E. faecalis* and *E. faecium* (heterologously expressed in *E. coli*) had their optimum pH for the activity at 5.5 and 6, respectively (Liu et al., 2014).

The enhancing effects of lower pH on tyramine production (as responses to acidic stress) was observed also in *Enterococcus durans* (Fernández et al., 2007), *E. faecium* (Marcobal et al., 2006a; Pereira et al., 2009). Similar effects were observed for the histidine decarboxylase in *L. lactis* (Trip et al., 2012) and *L. brevis* (Marcobal et al., 2006b).

The histidine decarboxylase of *S. thermophilus* has its optimum pH at pH 4.5, measured in cell free extract, while histamine accumulation by viable cell cultures was very low at the same pH, due to the negative effect of acidity on the overall metabolism of the strain (Tabanelli et al., 2012).

Regarding Gram-negative bacteria, the pure histidine decarboxylase from *P. phosphoreum* had its higher activity at pH 7; this value decreased at 6.0 for *P. damselae* and 6.5 for *M. morganii* and *R. planticola* (Kanki et al., 2007). Morii and Kasama (2004) found that an optimal histidine decarboxylase activity was slightly lower (pH 6) in *P. phosphoreum*, while BA production by cells of *Enterobacter cloacae* and *E. aerogenes* was higher at pH 6 (Greif et al., 2006).

Cid et al. (2008) suggested that in fermented sausages, the accumulation of tyramine by LAB (*L. curvatus*) started immediately at the end of fermentation, when pH of the sausages has already reached its minimum value. Other BAs, produced by the same LAB, were produced more gradually only at a later stage of ripening.

TECHNOLOGICAL FACTORS FOR CONTROLLING BIOGENIC AMINE ACCUMULATION

Use of Starter Culture

The addition of selected starter cultures is one of the main tools able to counteract BA accumulation in fermented foods. In first instance, the microorganisms used have to be characterized by the absence of any decarboxylating activity. Then, they had to be active in inhibiting the growth performances and the aminobiogenetic potential of wild decarboxylating bacteria. In addition, aspects such as the production of antimicrobial compounds like bacteriocins, as well as the ability to degrade BAs have to be considered (Ayhan et al., 1999; Bover-Cid et al., 2000). The possibility of some microorganisms to metabolize BA will be specifically discussed in Section "Microorganisms Able to Metabolize Biogenic Amine." The use of LAB cultures producing bacteriocins can have an important potential in limiting BA accumulation, even if further researches are needed to clarify this potential. Recently it has been demonstrated that using bacteriocinogenic strains of *L. lactis* it is possible to limit BA production by *S. thermophilus* and *E. faecalis* (Tabanelli et al., 2014).

The use of starter cultures has a consolidated application in cheese and the selection of not decarboxylative cultures for dairy products is well established since long time (Cogan et al., 2007). The use of selected starter cultures aimed to limit BA accumulation in dairy products has been recently reviewed by Linares et al. (2012). The use of autochthonous starter has been tested successfully in ewe's milk cheeses by Renes et al. (2014) who found a significantly lower BA content when *L. lactis* starter cultures were used. These results confirmed the observation of Novella-Rodríguez et al. (2002a) regarding goat cheeses. Also in

Manchego cheese, the use of autochthonous starter cultures of *L. paracasei* decreased the accumulation of BA, even if compared with commercial starter cultures (Poveda et al., 2015).

European Food Safety Agency (EFSA) recommends that so-called autochthonous strains (i.e., selected strains originating from each specific fermented product) with suitable technological profiles and reduced tendencies to produce BA should be selected as starter cultures (EFSA, 2011). Recently, it was suggested to use autochthonous starter cultures for fermentation of artisanal sausages (Talon et al., 2007; Casquete et al., 2011). Latorre-Moratalla et al. (2012) have extensively reviewed many of these aspects relevant in fermented sausage production. The addition of pure or mixed selected starter cultures can decrease BAs accumulation in sausages. Mixed starters were reported to perform better than single starters to control the growth of different bacterial groups (Latorre-Moratalla et al., 2010b; Naila et al., 2010). In fermented sausages, autochthonous starter cultures determined BA reductions higher (Latorre-Moratalla et al., 2010a; Casquete et al., 2011; Renes et al., 2014) than commercial mixed starter cultures (Ayhan et al., 1999; Gücükoğlu and Küplülu, 2010). In fact, Latorre-Moratalla et al. (2012) reported that mixed starter cultures of amine negative strains of LAB and coagulase-negative staphylococci, well adapted to the meat fermentation environment, were the best choice to reduce BA content in sausages. In order to avoid formation of high level of BAs during fermentation of sausages, the use of raw materials with low microbial counts is recommended. The main level of BA production was during the first 3 days, when a sharp pH decrease and the development of LAB occurred during the fermentation process of dry sausages (Bover-Cid et al., 1999). LAB used as starter cultures can induce rapid acidification, thus inhibiting the growth of decarboxylating microorganisms, resulting in decreasing formation of BAs (Zhang et al., 2013). However, it is well known that the use of selected starter cultures alone cannot assure the reduction or inhibition of BA production (Parente et al., 2001).

The production of BA in wine is mainly associated with the activity of LAB (Lonvaud-Funel, 2001). For this reason, particular attention has been posed on the selection of starter cultures used for malolactic fermentation. Strains of *O. oeni*, the main responsible for the conversion of malic acid into lactic acid in wine, can produce histamine (Lonvaud-Funel and Joyeux, 1994; Guerrini et al., 2002) and tyramine (Gardini et al., 2005) and selected strains without this potential should be used for the conduction of this process in winemaking (Moreno-Arribas et al., 2003).

Also in the production of sauerkraut, the use of starter cultures can contribute to the reduction of BA content (Kalač et al., 2000; Rabie et al., 2011a).

Technological Additives

The use of some additives is widespread to improve the appearance and the quality of the final product such as development of the typical color of the cured meat, inhibition of mold growth and decrease of toxic compounds in the product. Thus, the effects of these additives on BA generation are important.

The scarcity of sugars, and in general poor nutritional environments, has been often associated to higher BA accumulation, being decarboxylative pathways secondary transport system providing metabolic energy (Konings, 2006). For this reason, the *in vitro* production of BAs has been tested by several authors in relation to sugar supply. Buňková et al. (2012) found the maximum tyramine accumulation by *E. durans* in the presence of the higher lactose concentration (5%). A less clear effect of this sugar was observed for *L. lactis*: increasing lactose concentration did not result in higher tyramine concentration and this was attributed to the excess of metabolic energy obtained by primary fermentation (Buňková et al., 2011). Higher amount of tyrosine were produced by *S. thermophilus* in the presence of limiting amounts of lactose (0.1%; La Gioia et al., 2011). Also Landete et al. (2008b) observed that increasing concentration of fructose and glucose progressively inhibited the histamine accumulation of enological LAB belonging to the species *L. hilgardii*, *P. parvulus*, and *O. oeni*.

In industrial formulations, especially in fermented sausages, sugars (mainly glucose, sucrose, and lactose) are added in order to improve the LAB fermentation process. González-Fernández et al. (2003) investigated the influence of three decarboxylase negative starter cultures in relation to different composition and concentration of sugars, on the presence of BAs in *chorizo*, a typical Spanish dry fermented sausage. The highest concentrations of BAs were found at the end of the ripening process in the control sausage with no starter culture irrespective of the use of different sugar concentrations. However, when a starter culture and sugar concentrations equal to 0.5% or 1% were used, the presence of BAs in the sausage decreased considerably in comparison with control and low sugar concentration sausages. The production of high amounts of putrescine (223–252 mg/kg) and tyramine (64–102 mg/kg) was observed when the concentration of sugar in the sausage was only 0.1%, even in sausages with a starter culture added. Bover-Cid et al. (2001a) also found contents of tyramine and cadaverine significantly higher in sausages without sugar in their formulation. They concluded that sugar omission is not recommended since it might increase BA accumulation during the manufacture and storage of slightly fermented sausages. The amount of sugar added can be a key factor in determining the equilibrium among the microbial communities during the fermentation step of sausages favoring the accumulation of different BAs. The modulation of sugar addition between 0 and 1.4% determined the maximum tyramine content at intermediate level, in correspondence with the maximum LAB and enterococci growth. By contrast, at the extreme levels (0 and 1.4%), the accumulation of putrescine and cadaverine was higher, associated with the best performance of enterobacteria (Bover-Cid et al., 2009). High accumulation of cadaverine and putrescine were observed also in chorizo in the presence of low sugar addition (0.1%) even in the presence of starter cultures added (González-Fernández et al., 2003).

The use of essential oils as aroma and flavor ingredients has increased recently because of the growing consumer demand for natural products as natural food preservatives and the replacement of synthetic additives in the food industry.

Wendakoon and Sakaguchi (1993) reported that antibacterial activity of essential oil such as eugenol present in clove might delay the BA formation of *E. aerogenes* in mackerel muscle extract. No amines were observed in blue fish burgers treated with thymol, lemon extract, and grapefruit seed extract combination with MAP (Del Nobile et al., 2009). Özogul et al. (2015) investigated the impact of carvacrol at different level (0.1, 0.5 and 1 ml/100 ml) on BAs production by foodborne pathogens including, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, *E. faecalis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *A. hydrophila*, and *Salmonella* Paratyphi A in histidine decarboxylase broth. The results of this study showed that all bacteria tested were able to decarboxylate more than one amino acid and that carvacrol was able to reduce BAs formation depending on its concentration and bacterial species. Cai et al. (2015) reported low BAs content, especially histamine, putrescine, cadaverine in red drum (*Sciaenops ocellatus*) filets treated with 4 ml/l clove, cumin, and spearmint oils. In addition, Bozkurt (2006) reported that the levels of histamine, putrescine, and tyramine in *sucuk* (Turkish dry-fermented sausage) were lower in sausages with green tea extract than control. In Gouda cheese, the addition to curd of *Zataria multiflora* essential oil, whose major constituent was carvacrol (71.1%) determined a significant reduction of tyramine and histamine in the final product (Gorji et al., 2014).

In this perspective, spices can affect BA contents in food. Spices are often defined as aromatic, dried plant substances used in foods for flavoring and coloring. Komprda et al. (2004) reported that high content of red pepper, together with starter cultures, contributed to the lower BA content in dry fermented sausages. Red pepper contains capsaicin, known to prevent the growth of some bacteria (Hirasa and Takemasa, 1998). The effects of a variety of spices including ginger, garlic, green onion, red pepper, clove, and cinnamon were investigated to reduce BA contents in *myeolchi-jeot*, Korean salted and fermented anchovy (Mah et al., 2009). The greatest inhibitory effect on BA production was found in the culture treated by garlic extract while the other spice extracts showed minor effect in reducing BA contents. In particular, ginger extract reduced putrescine contents while red pepper extract decreased cadaverine accumulation. Since garlic contains allicin, antimicrobial component, its efficacy is probably related to the presence of this molecule. Other studies (Shakila et al., 1996) reported that clove and cinnamon reduced histamine production of *M. morganii* by 95%.

Effects of Packaging on Biogenic Amine Formation

There is evidence that oxygen can affect the BA production. For example, it has been demonstrated that *E. durans* (Buňková et al., 2012) and *L. lactis* (Buňková et al., 2011) tyramine accumulation is favored by anaerobic conditions. A similar trend was reported also by Cid et al. (2008) using a strain of *L. curvatus*. However, the main technologies for food preservation based on atmosphere modification are focused on oxygen exclusion. Nevertheless, in such strategy, the principal aim, in relation to BA presence, is not the inactivation of decarboxylase activity but the inhibition of microbial population with decarboxylating properties.

In this perspective, the atmosphere used for packaging can affect the qualitative and quantitative formation of BAs. MAP and vacuum packaging (VP) play an important role in the selection of spoilage microorganisms and, particularly, on decarboxylating bacteria (Curiel et al., 2011).

CO_2 is the main gas used as bacteriostatic agent. Different concentrations of this gas in MAP have been applied to prolong shelf-life of foods by inhibiting microbial growth of Enterobacteria and H_2S-producers bacteria (Lopez-Caballero et al., 2002), histamine forming bacteria (Özogul and Özogul, 2006), *Pseudomonas* spp. (Li et al., 2014), *Lactobacillus sakei* (Devlieghere et al., 1998) and psychrophilic microorganisms (Arashisar et al., 2004). The CO_2 concentration (>60%) in the MAP caused significant reductions in the contents of total BAs in barramundi filets (Yassoralipour et al., 2012) and sardine (Özogul and Özogul, 2006). Rodrigues et al. (2016) also indicated that lower production of putrescine and cadaverine was observed in MAP (80% CO_2/20% N_2) and VP samples of rainbow trout. Yew et al. (2014) investigated the major BA profile in Indian mackerel packed in different carbon dioxide compositions (30, 60, 80, and 100% CO_2) with content 5% O_2 and corresponding N_2 level. Each amine responded differently to different CO_2 levels. Histamine concentration was reduced by 6.4, 8.5, 70.3, 78.8, and 90.2% in fish packed under VP, 30, 60, 80, and 100% CO_2, respectively. In particular, histamine and tyramine increased rapidly in fish packed under VP and 30% CO_2. This was attributed to the presence of histidine and tyrosine decarboxylase bacteria that cannot grow in the presence of increasing CO_2 concentration (Barakat et al., 2000).

LAB, due to their capacity to grow under high concentrations of CO_2, constitute a substantial part of the natural microbiota of MAP meats (Chouliara et al., 2007). Curiel et al. (2011) demonstrated that under vacuum conditions or MAP (20% CO_2 and 80% N_2) the enterobacteria producing putrescine, cadaverine, and agmatine were inhibited. In addition, the packaging under vacuum did not reduce significantly the tyraminogenic potential of strains of *Carnobacterium divergens*.

Zhang et al. (2015) evaluated the effect of air and MAP on the shelf life of chilled chicken. The putrescine and cadaverine content of gas mixture-packaged samples was significantly lower than that of the air-packaged samples during the storage period. The production of these BAs was slowed as the CO_2 content increased, indicating that the increasing concentration of CO_2 inhibited the growth of putrescine and cadaverine producing bacteria. The results are in agreement with those reported by Rodriguez et al. (2015) who investigated the effect of CO_2 concentration on the formation of BAs in shredded cooked chicken breast filet packed in modified atmosphere. They reported that the putrescine and cadaverine are affected by storage time and CO_2 concentration. Putrescine-producing bacteria are more CO_2-resistant. The correlation between BA formation and bacterial growth showed that putrescine and cadaverine concentrations could be used as quality indicators as their formation is related to total viable count.

Nowadays the most widely used active packaging technology for food is oxygen scavengers which eliminate oxygen in the packaging and in the product or permeating through the packaging material during storage (Alvarez, 2000). Mohan et al. (2009) investigated the effect of O_2 scavenger on the formation of BAs during chilled storage of seer fish (*Scomberomorus commerson*) and indicated that the use of O_2 scavenger with the proper maintenance of chilled storage temperature helped in reducing the formation of BAs and also reduced the risk of *Clostridium botulinum* toxin. Similar results using commercial O_2 scavengers were reported by Mohan et al. (2008) for catfish (*Pangasius sutchi*) steaks and Goncalves et al. (2004) for gilthead sea bream (*Sparus aurata*).

Naila et al. (2010) indicated that active packaging, VP and MAP inhibit formation of BAs more effectively than air packaging, through inhibition of BA forming bacteria or enzyme activity.

Other Non-thermal Treatments

Food irradiation can be used to increase the safety and shelf life of foods by reducing microbial growth. Gamma-irradiation has been reported to reduce BAs contents in food due to the delay in the initial growth of adventitious microorganisms (Kim et al., 2003, 2004). Low levels of BAs by gamma irradiation in pepperoni sausage during storage were observed (Kim et al., 2005a). Gamma irradiation at 5, 10, or 15 kGy reduced putrescine, cadaverine, agmatine, histamine, tryptamine, spermine, and spermidine during fermentation of low-salt fermented soy paste (Kim et al., 2005b). Rabie et al. (2010) investigated the effects of different doses (2, 4, and 6 kGy) of gamma irradiation on BA formation in Egyptian fermented sausages. Histamine was detected in irradiated samples, immediately after irradiation, but not afterward and low levels of all BAs were observed during storage period especially in products treated at 6 kGy. Similar results were obtained for Blue cheese (Rabie et al., 2011b). Chub mackerel (*Scomber japonicus*) in chilled storage, after irradiation followed by vacuum packing slowed down the formation of BAs (Mbarki et al., 2009). Özogul and Ozden (2013) reported that radiation levels of (2.5 and 5 kGy) had similar effects on reducing the BA content except for agmatine and tryptamine. Although radiation caused an increase in spermine, agmatine, and tryptamine content in sea bream muscle, the putrescine and cadaverine contents in this product significantly decreased following the radiation process.

Pulsed electric fields (PEF) is a non-thermal method of food preservation that uses short pulses of electricity for microbial inactivation and causes minimal detrimental effect on food quality attributes (Quass, 1997). Previous studies have proven that PEF treatment can be useful in the sterilization of fruit juice under various conditions (Gurtler et al., 2010; Guo et al., 2014). They observed a clear microbial inactivation, depending on the juice, microorganisms, treatment conditions, and equipment. In addition, increasing the electric field strength (>600 kV/m) improved the inhibition of microorganism growth in tilapia during storage (Ko et al., 2016).

Microorganisms Able to Metabolize Biogenic Amine

Many microorganisms can produce amino oxidases, which are the enzyme responsible for BA detoxification. These enzymes metabolize BAs firstly by deamination, with the production of NH_3 and H_2O_2 in the presence of oxygen. The aldehydes formed is further reduced to the corresponding acids which can be then transferred to the central metabolism of the cells (Cooper, 1997). This metabolic pathway can be used as a source of NH_3 in nitrogen poor media. These enzymatic activities have been evidenced *in vitro* in several microorganisms. Leuschner et al. (1998) found these abilities widespread among *Kocuria* (former *Micrococcus*) *varians*. In addition, they isolated amino oxidase positive *Brevibacterium linens* strains, which were used for the production of a surface ripened Munster cheese, causing the reduction of BA (Leuschner and Hammes, 1998).

Amino oxidase activity was found also in *Bacillus amyloliquefaciens* and *Bacillus subtilis* (Zaman et al., 2010, 2011). Among LAB, many strains of *Lactobacillus*, *Pediococcus*, and *Oenococcus* showed these ability in culture media (García-Ruiz et al., 2011). Similar activities were observed *in vitro* also in *Lactobacillus casei*, *Lactobacillus plantarum* (Fadda et al., 2001; Herrero-Fresno et al., 2012), and in *L. sakei* in ensiled fish slurry (Dapkevicius et al., 2000). Herrero-Fresno et al. (2012) demonstrated also that the use of two strains of *L. casei* with high degradation rates for histamine and tyramine could reduce the accumulation of these BAs in Cabrales-like mini-cheeses.

Also staphylococci were able to deaminate tyramine and histamine *in vitro* (Martuscelli et al., 2000; Zaman et al., 2011). However, the activity of selected staphylococci in real food systems was limited by the presence of other nitrogen sources easily available for these bacteria, such in the case of dry fermented sausages (Gardini et al., 2002). In addition, in real complex food systems, the BA reduction by an amino oxidase positive *L. casei* strain cannot be clearly ascribed to an effective BA deamination or to a specific antagonism of the strains toward decarboxylating microorganism (Nishino et al., 2007). More interesting results were obtained in a nitrogen poor matrix such as wine, in which the use of strains of *L. casei* and *Pediococcus* spp. (García-Ruiz et al., 2011), as well as *L. plantarum* (Capozzi et al., 2012), determined a BA deamination.

Finally, some authors suggested the possibility to use purified amino oxidase in foods with negligible results (Hobson and Anderson, 1985; Dapkevicius et al., 2000). More interesting reductions were observed in wine using fungal amino oxidases (Cueva et al., 2012).

Effects of Pressure Treatments on BA Formation

Among the alternative processes to thermal treatment for food preservation, the field of high pressure processing, better known as high hydrostatic pressure (HHP) or high pressure homogenization (HPH), is one of the most scientifically explored. Given the antimicrobial effects of HHP, this technology can modify the microbiota of treated foods both quantitatively and qualitatively, affecting also the food matrix characteristics

(Georget et al., 2015). Novella-Rodríguez et al. (2002b) studied the differences in BA formation in goat cheeses by using pasteurized and pressurized milk and found no significant effects between the two trials on tyramine, histamine, and putrescine accumulations. The treatment at 400 MPa or 600 MPa of 21- and 35-day ripened cheeses determined a significant increase of both aminopeptidase activity and free amino acid concentration. By contrast, the total BA concentration was higher in the not treated cheeses, especially in samples treated at 600 MPa (50% and more of BA reduction; Calzada et al., 2013). In other words, HHP treatment reduced the population of potentially decarboxylating microorganisms, limiting BA accumulation also in the presence of a higher concentration of precursors. Novella-Rodríguez et al. (2002a) used HHP to accelerate cheese ripening, founding no differences in tyramine content between treated and not-treated goat cheese samples when 400 MPa were applied; however, in the same work, a prolonged treatment at 50 MPa increased the tyramine content.

Ruiz-Capillas et al. (2007) studied the BA formation in sliced dry-cured "chorizo" sausage, HHP treated at 350 MPa for 15 min, during chilled storage at 2°C and found a significant reduction of tyramine, putrescine, and cadaverine concentrations. The reason for this decrease was found in the higher susceptibility of decarboxylating microorganisms. However, during the storage, BAs continued to increase, probably due to the residual decarboxylases, whose activity was independent on the cell viability. Ruiz-Capillas et al. (2007) described a positive effect of HHP treatment of 400 MPa for 10 min on tyramine accumulation in vacuum packaged cooked sliced ham, with an increase of the shelf-life by at least 35 days. Similar results were observed by treating at 500 MPa/10 min Hungarian dry fermented sausages cut in 5 cm long pieces under vacuum (Simon-Sarkadi et al., 2012). Under these conditions, BA content was reduced during the storage.

In addition to HHP, also HPH can be a strategy to control the presence of decarboxylating microorganisms in raw materials such as the milk used for cheese making. Lanciotti et al. (2007) demonstrated that a HPH milk treatment at 100 MPa can significantly reduce the BAs accumulation compared with the thermal treated milk, due to a deep modification of the microbiota during ripening.

Antimicrobial Substances

The use of substance with antimicrobial properties can modify the BA profile of foods interfering with the equilibrium among microbial population rather than affecting directly the decarboxylase efficiency. Nitrate and nitrite salts are commonly used in fermented sausages for different purposes. In fact, they influence the color, the flavor and the oxidation of cured meat. In addition, they are used for controlling hazardous bacteria, such as clostridia. Nevertheless, their action can also interfere with BA accumulation. The addition of increasing concentration of nitrite (up to 150 mg/kg) reduced tyramine and cadaverine accumulation in Sucuk, a Turkish fermented sausage (Gençcelep et al., 2008). Opposite results were obtained in a Spanish not fermented cured meat (Lacon) in which the addition of nitrate and nitrite significantly increased the BA content (Lorenzo et al., 2007). This trend was explained by the fact that the addition of nitrite favored the selection of a superficial microbiota mainly constituted by LAB with relevant decarboxylative activity. The addition of nitrite reduced also the BA content in fresh beef, pork, and poultry meats stored at 4°C (Jastrzębska et al., 2016). These latter authors tested, under the same condition, also a weak acid (sorbic acid) whose effectiveness was rather limited. The use of two weak acids (sorbate and benzoate), in combination with clove, inhibited the decarboxylative action of an aminobiogenetic *E. aerogenes* strain isolated from fish (Wendakoon and Sakaguchi, 1993). Benzoate and sorbate were also used to limit BA production during the storage of two different type of fishery products (cod roe and pearl mullet filets, respectively; Lapa-Guimarães et al., 2011; Gençcelep et al., 2014).

Also sulfur containing antimicrobials were used to control BA accumulation in foods. Bover-Cid et al. (2001b) added sodium sulfide (maximum concentration 1000 mg/kg) to ripened sausages and observed a contradictory effect on BA accumulation. In fact, while the cadaverine content was inhibited by the addition of sulfide, tyramine, and putrescine accumulation was strongly enhanced. Jastrzębska et al. (2016) observed a contribution of sodium metabisulphite to the reduction of BA content in fresh meats. However, the most relevant matrices in which sulfur compounds play a key role in the reduction of BAs are the alcoholic beverages. In particular, in wine it is known the potential aminobiogenetic potential of the LAB responsible for malolactic fermentation (Lonvaud-Funel, 2001; Landete et al., 2005). Some authors demonstrated that the addition of SO_2 when malate was completely converted into lactate prevented amine formation in subsequent stages (Vidal-Carou et al., 1990; Marcobal et al., 2006c). A similar effect of SO_2 on tyramine accumulation was evidenced in a model system inoculated with an *O. oeni* strain (Gardini et al., 2005). The control of BA accumulation due to SO_2 is related to the inhibiting effect on cell metabolism rather than to the repression of the decarboxylases activity (Landete et al., 2008b). Interesting results were obtained in wine using lysozyme instead of SO_2. In fact, using this enzyme in Rioja wines subjected to malolactic fermentation, a drastic decrease of histamine content was found (López et al., 2009).

CONCLUSION

Even if the presence of BAs in food (and the risks associated with them) is known since a long period (Gale, 1946), systematic studies regarding their presence have been carried out only in relatively recent times. The reviews of Shalaby (1996) and Silla Santos (1996) had the merit to collect the fragmented information about this issue and were the starting point for a drastic multiplication of scientific publications regarding the presence of BA in food products and the elucidation of the metabolic and genetic drivers of their production by microorganisms. Combining the words "biogenic amine" and "food" the number of publication selected by the Web of Science passed from about 500 in the year 2000 to more than 4500 in 2015.

This increasing scientific effort allowed obtaining a deeper knowledge about the genetic and biochemical mechanisms

responsible for BA production by foodborne microorganisms, but also furnished important information about the possibility to reduce their accumulation in food and the risks associated with their presence.

The possible ways reviewed here to achieve this goal in food are mainly based on two strategies, which always are strictly interacting each other: the modulation of process and environmental factors including storage and distribution conditions and the control of the microbiota associated with fermented foods.

While the studies regarding the genetic bases of microorganism decarboxylating activity have brought to relevant steps forward and new insights on this topic, the role of environmental and technological factors on the overall activity of aminobiogenic microorganisms and on their decarboxylases requires deeper researches aimed to improve the possibility of intervention on the food processes in the perspective of the reduction of the risks associated to the BA presence.

For the fermented foods, from a strictly microbiological point of view, more information is available regarding the possible role of selected starter cultures aimed to overcome and inhibit decarboxylating microbiota. Further work is required to evaluate the real potential and optimize the use of microbial cultures able to degrade BAs and detoxifying them through the action of amino oxidases. In this perspective, also the use of bioprotective cultures producing bacteriocins or other antimicrobial substances needs greater attention due to their not fully explored potential in this field.

AUTHOR CONTRIBUTIONS

FG and GS wrote the introduction and conclusion sections; YO and FO wrote chapters 2, 3, 4, and 5; GT coordinated contributions and provided the final draft of the manuscript.

REFERENCES

Al Bulushi, I., Poole, S., Deeth, H. C., and Dykes, G. A. (2009). Biogenic amines in fish: roles in intoxication, spoilage, and nitrosamine formation, A review. *Crit. Rev. Food Sci. Nutrit.* 49, 369–377. doi: 10.1080/10408390802067514

Alvarez, M. F. (2000). Review: active food packaging. *Food Sci. Technol. Int.* 6, 97–108. doi: 10.1177/108201320000600203

Ancín-Azpilicueta, C., González-Marco, A., and Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Crit. Rev. Food Sci. Nutr.* 48, 257–275. doi: 10.1080/10408390701289441

Anonymous (2001). *Fish and Fisheries Products Hazards and Controls Guidance, June 2001*, 3rd Edn, Chap. 7. Silver Spring, MD: U.S. Food and Drug Administration. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Seafood/ucm2018426.htm

Arashisar, S., Hisar, O., Kaya, M., and Yanik, T. (2004). Effect of modified atmosphere and vacuum packaging on microbiological and chemical properties of rainbow trout (*Oncorynchus mykiss*) fillets. *Int. J. Food Microbiol.* 97, 209–214. doi: 10.1016/j.ijfoodmicro.2004.05.024

Arena, M. E., and Manca de Nadra, M. C. (2001). Biogenic amine production by *Lactobacillus*. *J. Appl. Microbiol.* 90, 158–162. doi: 10.1046/j.1365-2672.2001.01223.x

Ayhan, K., Kolsarici, N., and Ozkan, G. A. (1999). The effect of a starter culture on the formation of biogenic amines in Turkish soudjoucks. *Meat Sci.* 53, 183–188. doi: 10.1016/S0309-1740(99)00046-7

Baixas-Nogueras, S., Bover-Cid, S., Veciana-Nogués, M. T., Mariné-Font, A., and Vidal-Carou, M. C. (2005). Biogenic amine index for freshness evaluation in iced Mediterranean hake (*Merluccius merluccius*). *J. Food Prot.* 68, 2433–2438.

Barakat, R. K., Griffiths, M. W., and Harris, L. J. (2000). Isolation and characterization of *Carnobacterium*, *Lactococcus*, and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat. *Int. J. Food Microbiol.* 62, 83–94. doi: 10.1016/S0168-1605(00)00381-0

Bardócz, S. (2005). Polyamines in food and their consequences for food quality and human health. *Trends Food Sci. Technol.* 6, 341–346. doi: 10.1016/S0924-2244(00)89169-4

Bargossi, E., Gardini, F., Gatto, V., Montanari, C., Torriani, S., and Tabanelli, G. (2015a). The capability of tyramine production and correlation between phenotypic and genetic characteristics of *Enterococcus faecium* and *Enterococcus faecalis* strains. *Front. Microbiol.* 6:1371. doi: 10.3389/fmicb.2015.01371

Bargossi, E., Tabanelli, G., Montanari, C., Lanciotti, R., Gatto, V., Gardini, F., et al. (2015b). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and tyrDC gene expression. *Front. Microbiol.* 6:259. doi: 10.3389/fmicb.2015.00259

Bäumlisberger, M., Moellecken, U., König, H., and Claus, H. (2015). The potential of the yeast *Debaryomyces hansenii* H525 to degrade biogenic amines in food. *Microorganisms* 3, 839–850. doi: 10.3390/microorganisms3040839

Becker, K., Southwick, K., Reardon, J., Berg, R., and MacCormack, J. N. (2001). Histamine poisoning associated with eating tuna burgers. *JAMA* 285, 1327–1330. doi: 10.1001/jama.285.10.1327

Ben-Gigirey, B., de Sousa, J. M. V. B., Villac, T. G., and Barros-Velazquez, J. (2000). Characterization of biogenic amine-producing *Stenotrophomonas maltophilia* strains isolated from white muscle of fresh and frozen albacore tuna. *Int. J. Food Microbiol.* 57, 19–31. doi: 10.1016/S0168-1605(00)00240-3

Beutling, D. M., and Walter, D. (2002). 2-Phenylethylamine formation by enterococci in vitro. *Eur. Food Res. Technol.* 215, 240–242. doi: 10.1007/s00217-002-0525-y

Bover-Cid, S., Hugas, M., Izquierdo-Pulido, M., and Vidal-Carou, M. C. (2000). Reduction of biogenic amine formation using a negative amino acid-decarboxylase starter culture for fermentation of fuet sausages. *J. Food Prot.* 63, 237–243.

Bover-Cid, S., Izquierdo-Pulido, M., and Vidal-Carou, M. C. (1999). Effect of proteolytic starter cultures of *Staphylococcus* spp. on biogenic amine formation during the ripening of dry fermented sausages. *Int. J. Food Microbiol.* 46, 95–104. doi: 10.1016/S0168-1605(98)00170-6

Bover-Cid, S., Izquierdo-Pulido, M., and Vidal-Carou, M. C. (2001a). Changes in biogenic amine and polyamine contents in slightly fermented sausages manufactured with and without sugar. *Meat Sci.* 57, 215–221. doi: 10.1016/S0309-1740(00)00096-6

Bover-Cid, S., Miguel-Arrizádo, M. J., and Vidal-Carou, M. C. (2001b). Biogenic amine accumulation in ripened sausages affected by the addition of sodium sulphite. *Meat Sci.* 59, 391–396. doi: 10.1016/S0309-1740(01)00091-2

Bover-Cid, S., Latorre-Moratalla, M. L., Veciana-Nogués, M. T., and Vidal-Carou, M. C. (2014). "Biogenic amines" in *encyclopedia of food safety*, eds Y. Motarjemi, G. Moy, and E. Todd (San Diego, CA: Academic Press), 381–391.

Bover-Cid, S., Torriani, S., Gatto, V., Tofalo, R., Suzzi, G., Belletti, N., et al. (2009). Relationships between microbial population dynamics and putrescine and cadaverine accumulation during dry fermented sausage ripening. *J. Appl. Microbiol.* 106, 1397–1407. doi: 10.1111/j.1365-2672.2008.04108.x

Bozkurt, H. (2006). Utilization of natural antioxidants: green tea extract and *Thymbra spicata* oil in Turkish dry-fermented sausage. *Meat Sci.* 73, 442–450. doi: 10.1016/j.meatsci.2006.01.005

Bubelová, Z., Buňka, F., Taťáková, M., Štajnochová, K., Purevdorj, K., and Buňková, L. (2015). Effects of temperature, pH and NaCl content on in vitro putrescine and cadaverine production through the growth of *Serratia*

marcescens CCM 303. *J. Environ. Sci. Health Part B-Pestic. Contam. Agric. Wastes* 50, 797–808. doi: 10.1080/03601234.2015.1058097

Buňková, L., Buňka, F., Dráb, V., Kráčmar, S., and Kubáň, V. (2012). Effects of NaCl, lactose and availability of oxygen on tyramine production by the *Enterococcus durans* CCDM 53. *Eur. Food Res. Technol.* 234, 973–979. doi: 10.1007/s00217-012-1714-y

Buňková, L., Buňka, F., Hlobilová, M., Vakátková, Z., Nováková, D., and Dráb, V. (2009). Tyramine production of technological important strains of *Lactobacillus*, *Lactococcus* and *Streptococcus*. *Eur. Food Res. Technol.* 229, 533–538. doi: 10.1007/s00217-009-1075-3

Buňková, L., Buňka, F., Mantlová, G., Cablová, A., Sedlácec, I., Svec, P., et al. (2010). The effect of ripening and storage conditions on the distribution of tyramine, putrescine and cadaverine in Edam-cheese. *Food Microbiol.* 27, 880–888. doi: 10.1016/j.fm.2010.04.014

Buňková, L., Buňka, F., Pollaková, E., Podešvová, T., and Dráb, V. (2011). The effect of lactose, NaCl and an aero/anaerobic environment on the tyrosine decarboxylase activity of *Lactococcus lactis* subsp. cremoris and *Lactococcus lactis* subsp. lactis. *Int. J. Food Microbiol.* 147, 112–119. doi: 10.1016/j.ijfoodmicro.2011.03.017

Cai, L., Cao, A., Li, Y., Song, Z., Leng, L., and Li, J. (2015). The effects of essential oil treatment on the biogenic amines inhibition and quality preservation of red drum (*Sciaenops ocellatus*) fillets. *Food Control* 56, 1–8. doi: 10.1016/j.foodcont.2015.03.009

Calles-Enríquez, M., Eriksen, B. H., Andersen, P. S., Rattray, F. P., Johansen, A. H., Fernández, M., et al. (2010). Sequencing and transcriptional analysis of the *Streptococcus thermophilus* histamine biosynthesis gene cluster: factors that affect differential hdcA expression. *Appl. Environ. Microbiol.* 76, 6231–6238. doi: 10.1128/AEM.00827-1

Calzada, J., del Olmo, A., Picón, A., Gaya, P., and Nuñez, M. (2013). Reducing biogenic-amine producing bacteria, decarboxylase activity, and biogenic amines in raw milk cheese by high-pressure treatments. *Appl. Environ. Microbiol.* 79, 1277–1283. doi: 10.1128/AEM.03368-12

Capozzi, V., Russo, P., Ladero, V., Fernández, M., Fiocco, D., Alvarez, M. A., et al. (2012). Biogenic amines degradation by *Lactobacillus* plantarum: toward a potential application in wine. *Front. Microbiol.* 3:122. doi: 10.3389/fmicb.2012.00122

Caruso, M., Fiore, C., Contursi, M., Salzano, G., Paparella, A., and Romano, P. (2002). Formation of biogenic amines as criteria for the selection of wine yeasts. *World J. Microbiol. Biotechnol.* 18, 159–163. doi: 10.1023/A:1014451728868

Casquete, R., Benito, M. J., Martín, A., Ruiz-Moyano, S., Hernández, A., and Córdoba, M. G. (2011). Effect of autochthonous starter cultures in the production of "salchichón", a traditional Iberian dry-fermented sausage, with different ripening processes. *Food Sci. Technol.* 44, 1562–1571. doi: 10.1016/j.lwt.2011.01.028

Chang, M., and Chang, H. C. (2012). Development of a screening method for biogenic amine producing *Bacillus* spp. *Int. J. Food Microbiol.* 153, 269–274. doi: 10.1016/j.ijfoodmicro.2011.11.00

Chouliara, E., Karatapanis, A., Savvaidis, I. N., and Kontominas, M. G. (2007). Combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, stored at 4°C. *Food Microbiol.* 24, 607–617. doi: 10.1016/j.fm.2006.12.005

Cid, S. B., Miguélez-Arrizado, M. J., Beckerc, B., Holzapfel, W. H., and Vidal-Carou, M. C. (2008). Amino acid decarboxylation by *Lactobacillus* curvatus CTC273 affected by the pH and glucose availability. *Food Microbiol.* 25, 269–277. doi: 10.1016/j.fm.2007.10.013

Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P., and Ustunol, Z. (2007). Advances in starter cultures and cultured foods. *J. Dairy Sci.* 90, 4005–4021. doi: 10.3168/jds.2006-765

Connil, N., Le Breton, Y., Dousset, X., Auffray, Y., Rincé, A., and Prévost, H. (2002). Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. *Appl. Environ. Microbiol.* 68, 3537–3544. doi: 10.1128/AEM.68.7.3537-3544.2002

Cooper, R. A. (1997). On the amine oxidases of *Klebsiella aerogenes* strain W70. *FEMS Microbiol. Lett.* 146, 85–89. doi: 10.1016/s0378-1097(96)00458-2

Cueva, C., García-Ruiz, A., González-Rompinelli, E., Bartolome, B., Martín-Alvarez, P. J., Salazar, O., et al. (2012). Degradation of biogenic amines by vineyard ecosystem fungi. Potential use in winemaking. *J. Appl. Microbiol.* 112, 672–682. doi: 10.1111/j.1365-2672.2012.05243.x

Curiel, J. A., Ruiz-Capillas, C., de las Rivas, B., Carrascosa, A. V., Jiménez-Colmenero, F., and Muñoz, R. (2011). Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration. *Meat Sci.* 88, 368–373. doi: 10.1016/j.meatsci.2011.01.011

Danquah, A. O., Benjakul, S., and Simpson, B. K. (2012). "Biogenic amines in foods," in *Food Biochemistry and Food Processing*, ed. B. K. Simpson (Ames, IA: John Wiley & Sons), 820–832.

Dapkevicius, M. L. N. E., Nout, M. J. R., Rombouts, F. M., Houben, J. H., and Wymenga, W. (2000). Biogenic amine formation and degradation by potential fish silage starter microorganisms. *Int. J. Food Microbiol.* 57, 107–114. doi: 10.1016/S0168-1605(00)00238-5

de las Rivas, B., Marcobal, A., and Munõz, R. (2007). Gene organization of the ornithine decarboxylase-encoding region in *Morganella morganii*. *J. Appl. Microbiol.* 102, 1551–1560. doi: 10.1111/j.1365-2672.2006.03188.x

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M.-C., et al. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Sci.* 96, 821–828. doi: 10.1016/j.meatsci.2013.09.010

Del Nobile, M. A., Corbo, M. R., Speranza, B., Sinigaglia, M., Conte, A., and Caroprese, M. (2009). Combined effect of MAP and active compounds on fresh blue fish burger. *Int. J. Food Microbiol.* 135, 281–287. doi: 10.1016/j.ijfoodmicro.2009.07.024

Devlieghere, F., Debevere, J., and Van Impe, J. (1998). Effect of dissolved carbon dioxide and temperature on the growth of *Lactobacillus* sake in modified atmospheres. *Int. J. Food Microbiol.* 41, 231–238. doi: 10.1016/S0168-1605(98)00052-X

EFSA (2011). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA J.* 9, 2393–2486. doi: 10.2903/j.efsa.2011.2393

Emborg, J., and Dalgaard, P. (2006). Formation of histamine and biogenic amines in cold-smoked tuna: an investigation of psychrotolerant bacteria from samples implicated in cases of histamine fish poisoning. *J. Food Prot.* 69, 897–906.

Emborg, J., and Dalgaard, P. (2008). Modelling the effect of temperature, carbon dioxide, water activity and pH on growth and histamine formation by *Morganella psychrotolerans*. *Int. J. Food Microbiol.* 128, 226–233. doi: 10.1016/j.ijfoodmicro.2008.08.016

European Community Commission Regulation (2005). Europen Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Union L* 338, 1–26.

Fadda, S., Vignolo, G., and Oliver, G. (2001). Tyramine degradation and tyramine/histamine production by lactic acid bacteria and *Kocuria* strains. *Biotechnol. Lett.* 23, 2015–2019. doi: 10.1023/A:1013783030276

Farriol, M., Segovia-Silvestre, T., Castellanos, J. M., Venereo, Y., and Orta, X. (2001). Role of putrescine in cell proliferation in a colon carcinoma cell line. *Nutrition* 17, 934–938. doi: 10.1016/S0899-9007(01)00670-0

Fernández, M., Linares, D. M., Rodríguez, A., and Alvarez, M. A. (2007). Factors affecting tyramine production in *Enterococcus durans* IPLA 655. *Appl. Microbiol. Biotechnol.* 73, 1400–1406. doi: 10.1007/s00253-006-0596-y

Fernández-No, I. C., Böhme, K., Gallardo, J. M., Barros-Velázquez, J., Cañas, B., and Calo-Mata, P. (2010). Differential characterization of biogenic amine-producing bacteria involved in food poisoning using MALDI-TOF mass fingerprinting. *Electrophoresis* 31, 1116–1127. doi: 10.1002/elps.200900591

Fogel, W. A., Lewinski, A., and Jochem, J. (2007). Histamine in food: is there anything to worry about? *Biochem. Soc. Trans.* 35, 349–352. doi: 10.1042/BST0350349

Gale, E. F. (1946). The bacterial amino acid decarboxylases. *Adv. Enzymol.* 6, 1–32.

García García, P., Romero Barranco, C., Durán Quintana, M. C., and Garrido Fernández, A. (2004). Biogenic amine formation and "Zapatera" spoilage of fermented green olives: effect of storage temperature and debittering process. *J. Food Prot.* 67, 117–123.

García-Ruiz, A., González-Rompinelli, E. M., Bartolomé, B., and Moreno-Arribas, M. V. (2011). Potential of wine-associated lactic acid bacteria to degrade biogenic amines. *Int. J. Food Microbiol.* 148, 115–120. doi: 10.1016/j.ijfoodmicro.2011.05.009

Gardini, F., Bover-Cid, S., Tofalo, R., Belletti, N., Gatto, V., Suzzi, G., et al. (2008). Modeling the aminogenic potential of *Enterococcus faecalis* EF37 in dry fermented sausages through chemical and molecular approaches. *Appl. Environ. Microbiol.* 74, 2740–2750. doi: 10.1128/AEM.02267-07

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., et al. (2001). Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *Int. J. Food Microbiol.* 64, 105–117. doi: 10.1016/S0168-1605(00)00445-1

Gardini, F., Martuscelli, M., Crudele, M. A., Paparella, A., and Suzzi, G. (2002). Use of *Staphylococcus xylosus* as a starter culture in dried sausages: effect on the biogenic amine content. *Meat Sci.* 61, 275–283. doi: 10.1016/S0309-1740(01)00193-0

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., et al. (2006). Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiol.* 23, 641–648. doi: 10.1016/j.fm.2005.12.005

Gardini, F., Zaccarelli, A., Belletti, N., Faustini, F., Cavazza, A., Martuscelli, M., et al. (2005). Factors influencing biogenic amine production by a strain of *Oenococcus oeni* in a model system. *Food Control* 16, 609–616. doi: 10.1016/j.foodcont.2004.06.023

Gençcelep, H., Andiç, S., and Köse, Ş. (2014). Effects of potassium sorbate application on shelf life and biogenic amines of pearl mullet (*Chalcalburnus tarichi* pallas, 1811) fillets packaged with vacuum. *J. Aquatic Food Product. Technol.* 23, 347–357. doi: 10.1080/10498850.2012.719588

Gençcelep, H., Kaban, G., Aksu, M. I., Öz, F., and Kaya, M. (2008). Determination of biogenic amine in sucuk. *Food Control* 19, 868–872. doi: 10.1016/j.foodcont.2007.08.013

Georget, E., Sevenich, R., Reineke, K., Mathys, A., Heinz, V., Callanan, M., et al. (2015). Inactivation of microorganisms by high isostatic pressure processing in complex matrices: a review. *Innov. Food Sci. Emerg. Technol.* 27, 1–14. doi: 10.1016/j.ifset.2014.10.015

Goncalves, A., Mendes, R., and Nunes, M. L. (2004). Effect of oxygen absorber on the shelf life of gilthead sea bream (*Spratus aurata*). *J. Aquat Food Prod. Technol.* 13, 49–59. doi: 10.1300/J030v13n03_05

González-Fernández, C., Santos, E., Jaime, I., and Rovira, J. (2003). Influence of starter cultures and sugar concentrations of biogenic amine contents in chorizo dry sausage. *Food Microbiol.* 20, 275–284. doi: 10.1016/S0740-0020(02)00157-0

González-Tenorio, R., Fonseca, B., Caro, I., Fernández-Diez, A., Kuri, V., Soto, S., et al. (2013). Changes in biogenic amine levels during storage of Mexican-style soft and Spanish-style dry-ripened sausages with different aw values under modified atmosphere. *Meat Sci.* 94, 369–375. doi: 10.1016/j.meatsci.2013.03.017

Gorji, M. E., Noori, N., Nodehi, R. N., Khaniki, G. J., Rastkari, N., and Alimohammadi, M. (2014). The evaluation of *Zataria multiflora* Boiss. essential oil effect on biogenic amines formation and microbiological profile in Gouda cheese. *Lett. Appl. Microbiol.* 59, 621–630. doi: 10.1111/lam.12319

Gram, L., and Dalgaard, P. (2002). Fish spoilage bacteria-problems and solutions. *Curr. Opin. Biotechnol.* 13, 262–266. doi: 10.1016/S0958-1669(02)00309-9

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., and Givskov, M. (2002). Food spoilage-interactions between food spoilage bacteria. *Int. J. Food Microbiol.* 78, 79–97. doi: 10.1016/S0168-1605(02)00233-7

Greif, G., Greifova, M., and Karovicova, J. (2006). Effects of NaCl concentration and initial pH value on biogenic amine formation dynamics by *Enterobacter* spp. bacteria in model conditions. *J. Food Nutr. Res.* 45, 21–29.

Gücükoğlu, A., and Küplülu, O. (2010). The effect of different starter cultures and ripening temperatures on formation of biogenic amine in Turkish fermented sausages. *Eur. Food Res. Technol.* 230, 875–884. doi: 10.1007/s00217-010-1220-z

Guerrini, S., Mangani, S., Granchi, L., and Vincenzini, M. (2002). Biogenic amine production by *Oenococcus oeni*. *Curr. Microbiol.* 44, 374–378. doi: 10.1007/s00284-001-0021-9

Guo, M., Jin, T., Geveke, D., Fan, X., Sites, J., and Wang, L. (2014). Evaluation of microbial stability, bioactive compounds, physicochemical properties, and consumer acceptance of pomegranate juice processed in a commercial scale pulsed electric field system. *Food Bioprocess. Technol.* 7, 2112–2120. doi: 10.1007/s11947-013-1185-6

Gurtler, J. B., Rivera, R. B., Zhang, H. Q., and Geveke, D. J. (2010). Selection of surrogate bacteria in place of *E. coli* O157:H7 and *Salmonella typhimurium* for pulsed electric field treatment of orange juice. *Int. J. Food Microbiol.* 139, 1–8. doi: 10.1016/j.ijfoodmicro.2010.02.023

Halász, A., Baráth, A., Simon-Sarkadi, L., and Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* 5, 42–49. doi: 10.1016/0924-2244(94)90070-1

Herrero-Fresno, A., Martínez, N., Sánchez-Llana, E., Díaz, M., Fernández, M., Martin, M. C., et al. (2012). *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental mode. *Int. J. Food Microbiol.* 157, 297–304. doi: 10.1016/j.ijfoodmicro.2012.06.002

Hirasa, K., and Takemasa, M. (1998). "Antimicrobial and antioxidant properties of spices," in *Spice Science and Technology*, eds K. Hirasa and M. Takemasa (New York, NY: Marcel Dekker Inc.), 163–200.

Hobson, J. C., and Anderson, D. A. G. (1985). Amine Removal. EP0132674.

Hornero-Mendez, D., and Garrido-Fernandez, A. (1997). Rapid high-performance liquid chromatography analysis of biogenic amines in fermented vegetable brines. *J. Food Prot.* 60, 414–419.

Hungerford, J. M. (2010). Scombroid poisoning: a review. *Toxicon* 56, 231–243. doi: 10.1016/j.toxicon.2010.02.006

Jastrzębska, A., Kowalska, S., and Szłyk, E. (2016). Studies of levels of biogenic amines in meat samples in relation to the content of additives. *Food Addit. Contam. Part A Chem.* 33, 27–40. doi: 10.1080/19440049.2015.1111525

Kalač, P., and Krausová, P. (2005). A review of dietary polyamines: formation, implications for growth and health and occurrence in foods. *Food Chem.* 90, 219–230. doi: 10.1016/j.foodchem.2004.03.044

Kalač, P., Špička, J., Křížek, M., and Pelikánová, T. (2000). The effects of lactic acid bacteria inoculants on biogenic amines formation in sauerkraut. *Food Chem.* 70, 355–359. doi: 10.1016/S0308-8146(00)00103-5

Kanki, M., Yoda, T., Tsukamoto, T., and Baba, E. (2007). Histidine decarboxylases and their role in accumulation of histamine in tuna and dried saury. *Appl. Environ. Microbiol.* 73, 1467–1473. doi: 10.1128/AEM.01907-06

Karmas, E. (1981). Biogenic amines as indicators of sea food freshness. *Lebensm. Wiss. Technol.* 14, 273–275.

Kim, J. H., Ahn, H. J., Jo, C., Park, H. J., Chung, Y. J., and Byun, M. W. (2004). Radiolysis of biogenic amines in model system by gamma irradiation. *Food Control* 15, 405–408. doi: 10.1016/S0956-7135(03)00102-6

Kim, J. H., Ahn, H. J., Kim, D. H., Jo, C., Yook, H. S., and Byun, M. W. (2003). Irradiation effects on biogenic amines in Korean fermented soybean paste during fermentation. *J. Food Sci.* 68, 80–84. doi: 10.1111/j.1365-2621.2003.tb14118.x

Kim, J. H., Ahn, H. J., Lee, J. W., Park, H. J., Ryu, G. H., Kang, I. J., et al. (2005a). Effects of gamma irradiation on biogenic amines in pepperoni with different packaging conditions. *J. Agric. Food Chem.* 89, 199–205. doi: 10.1016/j.foodchem.2004.02.026

Kim, J. H., Kim, D. H., Ahn, H. J., Park, H. J., and Byun, M. W. (2005b). Reduction of the biogenic amine contents in low salt-fermented soy bean paste by gamma irradiation. *Food Control* 16, 43–49. doi: 10.1016/j.foodcont.2003.11.004

Kimura, B., Konagaya, Y., and Fujii, T. (2001). Histamine formation by *Tetragenococcus muriaticus*, a halophilic lactic acid bacterium isolated from fish sauce. *Int. J Food Microbiol.* 70, 71–77. doi: 10.1016/S0168-1605(01)00514-1

Kiss, J., Korbász, M., and Sass-Kiss, A. (2006). Study of amine composition of botrytized grape berries. *J. Agric. Food Chem.* 54, 8909–8918. doi: 10.1021/jf061578g

Knope, K. E., Sloan-Gardner, T. S., and Stafford, R. J. (2014). Histamine fish poisoning in Australia, 2001 to 2013. *Commun. Dis. Intell. Q. Rep.* 38, 285–293.

Ko, W. C., Yang, S. Y., Chang, C. K., and Hsieh, C. W. (2016). Effects of adjustable parallel high voltage electrostatic field on the freshness of tilapia (*Orechromis niloticus*) during refrigeration. *LWT - Food Sci. Technol.* 66, 151–157. doi: 10.1016/j.lwt.2015.10.019

Komprda, T., Neznalova, J., Standara, S., and Bover-Cid, S. (2001). Effect of starter culture and storage temperature on the content of biogenic amines in dry fermented sausage poličan. *Meat Sci.* 59, 267–276. doi: 10.1016/S0309-1740(01)00079-1

Komprda, T., Smělá, D., Pechová, P., Kalhotka, L., Štencl, J., and Klejdus, B. (2004). Effect of starter culture, spice mix and storage time and temperature on biogenic amine content of dry fermented sausages. *Meat Sci.* 67, 607–616. doi: 10.1016/j.meatsci.2004.01.003

Konings, W. N. (2006). Microbial transport: adaptations to natural environments. *Antonie Van Leeuwenhoek* 90, 325–342. doi: 10.1007/s10482-006-9098-3

Konings, W. N., Lolkema, J. S., Bolhuis, H., van Veen, H. W., Poolman, B., and Driessen, A. J. M. (1997). The role of transport processes in survival of lactic acid bacteria, energy transduction and multidrug resistance. *Antonie Van Leeuwenhoek* 71, 117–128. doi: 10.1023/A:1000143525601

Košmerl, T., Šućur, S., and Prosen, H. (2013). Biogenic amines in red wine: the impact of technological processing of grape and wine. *Acta Agric. Slov.* 101, 249–261. doi: 10.2478/acas-2013-0021

Kuley, E., and Özogul, F. (2011). Synergistic and antagonistic effect of lactic acid bacteria on tyramine production by food-borne pathogenic bacteria in tyrosine decarboxylase broth. *Food Chem.* 127, 1163–1168. doi: 10.1016/j.foodchem.2011.01.118

Küley, E., Özogul, F., Balikçi, E., Durmus, M., and Ayas, D. (2013). The influences of fish infusion broth on the biogenic amines formation by lactic acid bacteria. *Braz. J. Microbiol.* 44, 407–415. doi: 10.1590/S1517-83822013000200010

La Gioia, F., Rizzotti, L., Rossi, F., Gardini, F., Tabanelli, G., and Torriani, S. (2011). Identification of a tyrosine decarboxylase (tdcA) gene in *Streptococcus thermophilus* 1TT45: analysis of its expression and tyramine production in milk. *Appl. Environ. Microbiol.* 77, 1140–1144. doi: 10.1128/AEM.01928-10

Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martín, M. C., et al. (2012). Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiol.* 30, 132–138. doi: 10.1016/j.fm.2011.12.016

Ladero, V., Sánchez-Llana, E., Fernández, M., and Alvarez, M. A. (2011). Survival of biogenic amine-producing dairy LAB strains at pasteurisation conditions. *Int. J. Food Sci. Technol.* 46, 516–521. doi: 10.1111/j.1365-2621.2010.02508.x

Lanciotti, R., Patrignani, F., Iucci, L., Guerzoni, M. E., Suzzi, G., Belletti, N., et al. (2007). Effects of milk high pressure homogenization on biogenic amine accumulation during ripening of ovine and bovine Italian cheeses. *Food Chem.* 104, 693–701. doi: 10.1016/j.foodchem.2006.12.017

Landeta, G., de las Rivas, B., Carrascosa, A. V., and Munõz, R. (2007). Screening of biogenic amine production by coagulase-negative staphylococci isolated during industrial Spanish dry-cured ham processes. *Meat Sci.* 77, 556–661. doi: 10.1016/j.meatsci.2007.05.004

Landete, J. M., de las Rivas, B., Marcobal, A., and Muñoz, R. (2007). Molecular methods for the detection of biogenic amine-producing bacteria on foods. *Int. J. Food Microbiol.* 117, 258–269. doi: 10.1016/j.ijfoodmicro.2007.05.001

Landete, J. M., de las Rivas, B., Marcobal, A., and Muñoz, R. (2008a). Updated molecular knowledge about histamine biosynthesis by bacteria. *Crit. Rev. Food Sci. Nutr.* 48, 697–714. doi: 10.1080/10408390701639041

Landete, J. M., Pardo, I., and Ferrer, S. (2008b). Regulation of hdc expression and HDC activity by enological factors in lactic acid bacteria. *J. Appl. Microbiol.* 105, 1544–1551. doi: 10.1111/j.1365-2672.2008.03865.x

Landete, J. M., Ferrer, S., and Pardo, I. (2005). Which lactic acid bacteria are responsible for histamine production in wine? *J. Appl. Microbiol.* 99, 580–586. doi: 10.1111/j.1365-2672.2005.02633.x

Lapa-Guimarães, L., Trattner, S., and Pickova, J. (2011). Effect of processing on amine formation and the of cod (*Gadus morhua*) roe. *Food Chem.* 129, 716–723. doi: 10.1016/j.foodchem.2011.05.010

Latorre-Moratalla, M. L., Bover-Cid, S., Bosch-Fusté, J., and Vidal-Carou, M. C. (2012). Influence of technological conditions of sausage fermentation on the aminogenic activity of L. curvatus CTC273. *Food Microbiol.* 29, 43–48. doi: 10.1016/j.fm.2011.08.004

Latorre-Moratalla, M. L., Bover-Cid, S., Talon, R., Garriga, M., Aymerich, T., Zanardi, E., et al. (2010a). Distribution of aminogenic activity among potential autochthonous starter cultures. *J. Food Prot* 73, 524–525.

Latorre-Moratalla, M. L., Bover-Cid, S., Talon, R., Garriga, M., Aymerich Zanardi, E., Ianieri, A., et al. (2010b). Strategies to reduce biogenic amine accumulation in traditional sausage manufacturing. *Food Sci. Technol.* 43, 20–25. doi: 10.1016/j.lwt.2009.06.018

Lehane, L., and Olley, J. (2000). Histamine fish poisoning revisited. *Int. J. Food Microbiol.* 58, 1–37. doi: 10.1016/S0168-1605(00)00296-8

Leuschner, R. G., and Hammes, W. P. (1998). Degradation of histamine and tyramine by *Brevibacterium* linens during surface ripening of Munster cheese. *J. Food Prot.* 61, 874–878.

Leuschner, R. G., Heidel, M., and Hammes, W. P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. *Int. J. Food Microbiol.* 39, 1–10. doi: 10.1016/S0168-1605(97)00109-8

Li, M., Tian, L., Zhao, G., Zhang, Q., Gao, X., Huang, X., et al. (2014). Formation of biogenic amines and growth of spoilage-related microorganisms in pork stored under different packaging conditions. *Meat Sci.* 96, 843–848. doi: 10.1016/j.meatsci.2013.09.023

Linares, D. M., del Río, B., Ladero, V., Martínez, N., Fernández, M., Martín, M. C., et al. (2012). Factors influencing biogenic amines accumulation in dairy products. *Front. Microbiol.* 3:180. doi: 10.3389/fmicb.2012.00180

Linares, D. M., Martín, M. C., Ladero, V., Alvarez, M. A., and Fernández, M. (2011). Biogenic amines in dairy products. *Crit. Rev. Food Sci. Nutr.* 51, 691–703. doi: 10.1080/10408398.2011.582813

Liu, F., Xu, W., Du, L., Wang, D., Zhu, Y., Geng, Z., et al. (2014). Heterologous expression and characterization of tyrosine decarboxylase from *Enterococcus faecalis* R612Z1 and *Enterococcus faecium* R615Z1. *J. Food Prot.* 77, 592–598. doi: 10.4315/0362-028X.JFP-13-326

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiol. Lett.* 199, 9–13. doi: 10.1111/j.1574-6968.2001.tb10643.x

Lonvaud-Funel, A., and Joyeux, A. (1994). Histamine production by wine lactic acid bacteria. Isolation of a histamine-producing strain of *Leuconostoc oenos*. *J. Appl. Bacteriol.* 77, 401–407. doi: 10.1111/j.1365-2672.1994.tb03441.x

López, I., Santamaría, P., Tenorio, C., Garijo, P., Gutiérrez, A. R., and López, R. (2009). Evaluation of lysozyme to control vinification process and histamine production in Rioja wines. *J. Microbiol. Biotechnol.* 19, 1005–1012. doi: 10.4014/jmb.0811.602

Lopez-Caballero, M. E., Goncalves, A., and Nunes, M. L. (2002). Effect of CO_2/O_2-containing modified atmospheres on packed deepwater pink shrimp (*Parapenaus longirostris*). *Eur. Food Res. Technol.* 214, 192–197. doi: 10.1007/s00217-001-0472-z

Lorencová, E., Buňková, L., Pleva, P., Dráb, V., Kubán, V., and Buňka, F. (2014). Selected factors influencing the ability of *Bifidobacterium* to form biogenic amines. *Int. J. Food Sci. Technol.* 49, 1302–1307. doi: 10.1111/ijfs.12427

Lorenzo, J. M., Cachaldora, A., Fonseca, S., Gómez, M., Franco, I., and Carballo, J. (2010). Production of biogenic amines "in vitro" in relation to the growth phase by *Enterobacteriaceae* species isolated from traditional sausages. *Meat Sci.* 86, 684–691. doi: 10.1016/j.meatsci.2010.06.005

Lorenzo, J. M., Martínez, S., Franco, I., and Carballo, J. (2007). Biogenic amine content during the manufacture of dry-cured lacón, a Spanish traditional meat product: effect of some additives. *Meat Sci.* 77, 287–293. doi: 10.1016/j.meatsci.2007.03.020

Mah, J.-H., Kim, Y. J., and Hwang, H.-J. (2009). Inhibitory effects of garlic and other spices on biogenic amine production in Myeolchi-jeot, Korean salted and fermented anchovy product. *Food Control* 20, 449–454. doi: 10.1016/j.foodcont.2008.07.006

Maintz, L., and Novak, N. (2007). Histamine and histamine intolerance. *Am. J. Clin. Nutr.* 85, 1185–1196.

Marcobal, A., De Las Rivas, B., Landete, J. M., Tabera, L., and Muñoz, R. (2012). Tyramine and phenylethylamine biosynthesis by food bacteria. *Crit. Rev. Food Sci. Nutr.* 52, 448–467. doi: 10.1080/10408398.2010.500545

Marcobal, A., de las Rivas, B., and Muñoz, R. (2006a). First genetic characterization of a bacterial β-phenylethylamine biosynthetic enzyme in *Enterococcus faecium* RM58. *FEMS Microbiol. Lett.* 258, 144–149. doi: 10.1111/j.1574-6968.2006.00206.x

Marcobal, A., Martín-Álvarez, P. J., Moreno-Arribas, M. V., and Muñoz, R. (2006b). A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. *Res. Microbiol.* 157, 417–424. doi: 10.1016/j.resmic.2005.11.006

Marcobal, A., Martín-Alvarez, P. J., Polo, M. C., Muñoz, R., and Moreno-Arribas, M. V. (2006c). Formation of biogenic amines throughout the industrial manufacture of red wine. *J. Food Prot.* 69, 391–396.

Marino, M., Maifreni, M., Bartolomeol, I., and Rondinini, G. (2008). Evaluation of amino acid-decarboxylative microbiota throughout the ripening of an Italian PDO cheese produced using different manufacturing practices. *J. Appl. Microbiol.* 105, 540–549. doi: 10.1111/j.1365-2672.2008.03793.x

Martuscelli, M., Crudele, M. A., Gardini, F., and Suzzi, G. (2000). Biogenic amine formation and oxidation by *Staphylococcus xylosus* strains from artisanal fermented sausages. *Lett. Appl. Microbiol.* 31, 228–232. doi: 10.1046/j.1365-2672.2000.00796.x

Mbarki, R., Ben Miloud, N., Selmi, S., Dhib, S., and Sadok, S. (2009). Effect of vacuum packaging and low-dose irradiation on the microbial, chemical and sensory characteristics of chub mackerel (*Scomber japonicus*). *Food Microbiol.* 26, 821–826. doi: 10.1016/j.fm.2009.05.008

McCabe-Sellers, B., Staggs, C. G., and Bogle, M. L. (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. *J. Food Compost. Anal.* 19, S58–S65. doi: 10.1016/j.jfca.2005.12.008

Mohan, C. O., Ravishankar, C. N., and Gopal, T. K. S. (2008). Effect of O_2 scavenger on the shelf-life of catfish (*Pangasius sutchi*) steaks during chilled storage. *J. Sci. Food Agric.* 88, 442–448. doi: 10.1002/jsfa.3105

Mohan, C. O., Ravishankar, C. N., Gopal, T. K. S., Kumar, K. A., and Lalitha, K. V. (2009). Biogenic amines formation in seer fish (*Scomberomorus commerson*) steaks packed with O_2 scavenger during chilled storage. *Food Res. Int.* 42, 411–416. doi: 10.1016/j.foodres.2009.01.015

Molenaar, D., Bosscher, J. S., Ten Brink, B., Driessen, A. J. M., and Konings, W. N. (1993). Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *J. Bacteriol.* 175, 2864–2870.

Moreno-Arribas, M. V., Polo, M. C., Jorganes, F., and Muñoz, R. (2003). Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* 84, 117–123. doi: 10.1016/S0168-1605(02)00391-4

Moreno-Arribas, V., and Lonvaud-Funel, A. (2001). Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine. *FEMS Microbiol. Lett.* 195, 103–107. doi: 10.1111/j.1574-6968.2001.tb10505.x

Moret, S., Smela, D., Populin, T., and Conte, L. S. (2005). A survey on free biogenic amine content of fresh and preserved vegetables. *Food Chem.* 89, 355–361. doi: 10.1016/j.foodchem.2004.02.050

Morii, H., and Kasama, K. (2004). Activity of two histidine decarboxylases from *Photobacterium phosphoreum* at different temperatures, pHs, and NaCl concentrations. *J. Food Prot.* 67, 1736–1742.

Naila, A., Flint, S., Fletcher, G., Bremer, P., and Meerdink, G. (2010). Control of biogenic amines in food - Existing and emerging approaches. *J. Food Sci.* 75, R139–R150. doi: 10.1111/j.1750-3841.2010.01774.x

Nishino, N., Hattori, H., Wada, H., and Touno, E. (2007). Biogenic amine production in grass, maize and total mixed ration silages inoculated with *Lactobacillus* casei or *Lactobacillus buchneri*. *J. Appl. Microbiol.* 103, 325–332. doi: 10.1111/j.1365-2672.2006.03244.x

Novella-Rodríguez, S., Veciana-Nogués, M. T., Izquierdo-Pulido, M., and Vidal-Carou, M. C. (2003). Distribution of biogenic amines and polyamines in cheese. *J. Food Sci.* 68, 750–755. doi: 10.1111/j.1365-2621.2003.tb08236.x

Novella-Rodríguez, S., Veciana-Nogués, M. T., Roig-Sagués, A. X., Trujillo-Mesa, A. J., and Vidal-Carou, M. C. (2004). Evaluation of biogenic amines and microbial counts throughout the ripening of goat cheeses from pasteurized and raw milk. *J. Dairy Res.* 71, 245–252. doi: 10.1017/S0022029904000147

Novella-Rodríguez, S., Veciana-Nogués, M. T., Saldo, J., and Vidal-Carou, M. C. (2002a). Effects of high hydrostatic pressure treatments on biogenic amine contents in goat cheeses during ripening. *J. Agric. Food Chem.* 50, 7288–7292. doi: 10.1021/jf025665u

Novella-Rodríguez, S., Veciana-Nogués, M. T., Trujillo-Mesa, A. J., and Vidal-Carou, M. C. (2002b). Profile of biogenic amines in goat cheese made from pasteurized and pressurized milks. *J. Food Sci.* 67, 2940–2944. doi: 10.1111/j.1365-2621.2002.tb08842.x

Özogul, F., Kacar, Ç, and Hamed, I. (2015). Inhibition effects of carvacrol on biogenic amine formation by common food-borne pathogens in histidine decarboxylase broth. *LWT - Food Sci. Technol.* 64, 50–55. doi: 10.1016/j.lwt.2015.05.027

Özogul, F., Kuley, E., and Kenar, M. (2011). Effects of rosemary and sage tea extract on biogenic amines formation of sardine (*Sardina pilchardus*) fillets. *Int. J. Food Sci. Technol.* 46, 761–766. doi: 10.1111/j.1365-2621.2011.02560.x

Özogul, F., and Ozden, O. (2013). The effects of gamma irradiation on the biogenic amine formation in sea bream (*Sparus aurata*) stored in ice. *Food Bioprocess. Technol.* 6, 1343–1349. doi: 10.1007/s11947-011-0593-8

Özogul, F., and Özogul, Y. (2006). Biogenic amine content and biogenic amine quality indices of sardines (*Sardina pilchardus*) stored in modified atmosphere packaging and vacuum packaging. *Food Chem.* 99, 574–578. doi: 10.1016/j.foodchem.2005.08.029

Pachlová, V., Buňka, F., Flasarová, R., Valková, P., and Buňková, L. (2012). The effect of elevated temperature on ripening of Dutch type cheese. *Food Chem.* 132, 1846–1854. doi: 10.1016/j.foodchem.2011.12.017

Panoutsopoulos, G. I., Kouretas, D., Gounaris, E. G., and Beedham, C. (2004). Metabolism of 2-phenylethylamine and phenylacetaldehyde by precision-cut guinea pig fresh liver slices. *Eur. J. Drug Metab. Pharmacokinet.* 29, 111–118. doi: 10.1007/BF03190585

Parente, E., Martuscelli, M., Gardini, F., Grieco, S., Crudele, M. A., and Suzzi, G. (2001). Evolution of microbial populations and biogenic amine production in dry sausages produced in Southern Italy. *J. Appl. Microbiol.* 90, 882–891. doi: 10.1046/j.1365-2672.2001.01322.x

Paulsen, P., and Bauer, F. (1997). Biogenic amines in fermented sausages: 2. Factors influencing the formation of biogenic amines in fermented sausages. *Fleisch. Int.* 77, 32–34.

Pegg, A. E. (2013). Toxicity of polyamines and their metabolic products. *Chem. Res. Toxicol.* 26, 1782–1800. doi: 10.1021/tx400316s

Pereira, C. I., Barreto Crespo, M. T., and San Romão, M. V. (2001). Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and L. homohiochii. *Int. J. Food Microbiol.* 68, 211–216. doi: 10.1016/S0168-1605(01)00534-7

Pereira, C. I., Matos, D., Romão, M. V. S., and Barreto Crespo, M. T. (2009). Dual role for the tyrosine decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge and generation of a proton motive force. *Appl. Environ. Microbiol.* 75, 345–352. doi: 10.1128/AEM.01958-08

Perez, M., Calles-Enríquez, M., Nes, I., Martin, M. C., Fernandez, M., Ladero, V., et al. (2015). Tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of *Enterococcus faecalis* in acidic environments. *Appl. Microbiol. Biotechnol.* 99, 3547–3558. doi: 10.1007/s00253-014-6301-7

Pessione, E., Pessione, A., Lamberti, C., Coïsson, D. J., Riedel, K., Mazzoli, R., et al. (2009). First evidence of a membrane-bound, tyramine and β-phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a two-dimensional electrophoresis proteomic study. *Proteomics* 9, 2695–2710. doi: 10.1002/pmic.200800780

Pinho, O., Ferreira, I. M. P. L. V. O., Mendes, E., Oliveira, B. M., and Ferreira, M. (2001). Effect of temperature on the evolution of free amino acid and biogenic amine contents during storage of Azeitão cheese. *Food Chem.* 75, 287–291. doi: 10.1016/S0308-8146(01)00109-1

Pircher, A., Bauer, F., and Paulsen, P. (2007). Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. *Eur. Food Res. Technol.* 226, 225–231. doi: 10.1007/s00217-006-0530-7

Poveda, J. M., Chicon, R., and Cabezas, L. (2015). Biogenic amine content and proteolysis in Manchego cheese manufactured with *Lactobacillus paracasei* subsp. paracasei as adjunct and other autochthonous strains as starters. *Int Dairy J* 47, 94–101. doi: 10.1016/j.idairyj.2015.03.004

Prester, L. (2011). Biogenic amines in fish, fish products and shellfish: a review. *Food Addit. Contam. Part A-Chem.* 28, 1547–1560. doi: 10.1080/19440049.2011.600728

Qi, W., Hou, L. H., Guo, H. L., Wang, C. L., Fan, Z. C., Liu, J. F., et al. (2014). Effect of salt-tolerant yeast of *Candida versatilis* and *Zygosaccharomyces rouxii* on the production of biogenic amines during soy sauce fermentation. *J. Sci. Food Agric.* 94, 1537–1542. doi: 10.1002/jsfa.6454

Quass, D. W. (1997). *Pulsed Electric Field Processing in the Food Industry. A Status Report on Pulsed Electric Field*. Palo Alto, CA: Electric Power Research Institute, 23–35.

Rabie, M. A., Siliha, H., el-Saidy, S., el-Badawy, A.-A., and Malcata, F. X. (2010). Effects of γ-irradiation upon biogenic amine formation in Egyptian ripened sausages during storage. *Innov. Food Sci. Emerg. Technol.* 11, 661–665. doi: 10.1016/j.ifset.2010.08.007

Rabie, M. A., Siliha, H., el-Saidy, S., el-Badawy, A. A., and Malcata, F. X. (2011a). Reduced biogenic amine contents in sauerkraut via addition of selected lactic acid bacteria. *Food Chem.* 129, 1778–1782. doi: 10.1016/j.foodchem.2011.05.106

Rabie, M. A., Siliha, H. I., El-Saidy, S. M., El-Badawya, A. A., and Malcata, F. X. (2011b). Effect of γ-irradiation upon biogenic amine formation in blue cheese during storage. *Int. Dairy J.* 21, 373–376. doi: 10.1016/j.idairyj.2010.11.009

Rauscher-Gabernig, E., Gabernig, R., Brueller, W., Grossgut, R., Bauer, F., and Paulsen, P. (2012). Dietary exposure assessment of putrescine and cadaverine and derivation of tolerable levels in selected foods consumed in Austria. *Eur. Food Res. Technol.* 235, 209–220. doi: 10.1007/s00217-012-1748-1

Renes, E., Diezhandino, I., Fernandez, D., Ferrazza, R. E., Tornadijo, M. E., and Fresno, J. M. (2014). Effect of autochthonous starter cultures on the biogenic

amine content of ewe's milk cheese throughout ripening. *Food Microbiol.* 44, 271–277. doi: 10.1016/j.fm.2014.06.001

Rodrigues, B. L., da Silveira Alvaresb, T., Sampaio, G. S. L., Cabral, C. C., Araujo, J. V. A., Franco, R. M., et al. (2016). Influence of vacuum and modified atmosphere packaging in combination with UV-C radiation on the shelf life of rainbow trout (*Oncorhynchus mykiss*) fillets. *Food Control* 60, 596–605. doi: 10.1016/j.foodcont.2015.09.004

Rodriguez, M. B. R., Conte-Junior, C. A., Carneiro, C. S., Lázaro, C. A., and Mano, S. B. (2015). Biogenic amines as a quality index in shredded cooked chicken breast fillet stored under refrigeration and modified atmosphere. *J. Food Process. Preserv.* 39, 2043–2048. doi: 10.1111/jfpp.12447

Romano, A., Ladero, V., Alvarez, M. A., and Lucas, P. M. (2014). Putrescine production via the ornithine decarboxylation pathway improves the acid stress survival of *Lactobacillus brevis* and is part of a horizontally transferred acid resistance locus. *Int. J. Food Microbiol.* 175, 14–19. doi: 10.1016/j.ijfoodmicro.2014.01.009

Romano, A., Trip, H., Lonvaud-Funel, A., Lolkema, J. S., and Lucas, P. M. (2012). Evidence of two functionally distinct ornithine decarboxylation systems in lactic acid bacteria. *Appl. Environ. Microbiol.* 78, 1953–1961. doi: 10.1128/AEM.07161-11

Romano, P., Capece, A., and Poeta, C. (2007). Biogenic amine formation in alcoholic fermentation. *Bull. OIV* 80, 251–262.

Rossi, F., Gardini, F., Rizzotti, L., La Gioia, F., Tabanelli, G., and Torriani, S. (2011). Quantitative analysis of histidine decarboxylase gene (hdcA) transcription and histamine production by *Streptococcus thermophilus* PRI60 under conditions relevant to cheese making. *Appl. Environ. Microbiol.* 77, 2817–2822. doi: 10.1128/AEM.02531-10

Ruiz-Capillas, C., Carballo, J., and Jiménez Colmenero, F. (2007). Biogenic amines in pressurized vacuum-packaged cooked sliced ham under different chilled storage conditions. *Meat Sci.* 75, 397–405. doi: 10.1016/j.meatsci.2006.08.004

Ruiz-Capillas, C., and Jiménez-Colmenero, F. (2004). Biogenic amines in meat and meat products. *Crit. Rev. Food Sci. Nutr.* 44, 489–499. doi: 10.1080/10408690490489341

Russo, P., de Palencia, P. F., Romano, A., Fernández, M., Lucas, P., Spano, G., et al. (2012). Biogenic amine production by the wine *Lactobacillus brevis* IOEB 9809 in systems that partially mimic the gastrointestinal tract stress. *BMC Microbiol.* 12:247. doi: 10.1186/1471-2180-12-247

Sathyanarayana Rao, T. S., and Yeragani, V. K. (2009). Hypertensive crisis and cheese. *Indian J. Psychiatry* 51, 65–66. doi: 10.4103/0019-5545.44910

Scheepens, A., Tan, K., and Paxton, J. W. (2010). Improving the oral bioavailability of beneficial polyphenols through designed synergies. *Genes Nutr.* 5, 75–87. doi: 10.1007/s12263-009-0148-z

Schneller, R., Good, P., and Jenny, M. (1997). Influence of pasteurised milk, raw milk and different ripening cultures on biogenic amine concentrations in semi-soft cheeses during ripening. *Z. Lebensm Unters. Forsch. A* 204, 265–272. doi: 10.1007/s002170050075

Shakila, R. J., Vasundhara, T. S., and Rao, D. V. (1996). Inhibitory effect of spices on in vitro histamine production and histidine decarboxylase activity of *Morganella morganii* and on the biogenic amine formation in mackerel stored at 30 oC. *Z. Lebensm. Unters. Forsch.* 203, 71–76. doi: 10.1007/BF012 67773

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Res. Int.* 29, 675–690. doi: 10.1016/S0963-9969(96)00066-X

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* 29, 213–231. doi: 10.1016/0168-1605(95)00032-1

Simon-Sarkadi, L., Pásztor-Huszár, K., Dalmadi, I., and Kiskó, G. (2012). Effect of high hydrostatic pressure processing on biogenic amine content of sausage during storage. *Food Res. Int.* 47, 380–384. doi: 10.1016/j.foodres.2011.10.029

Smit, A. Y., Du Toit, W. J., and Du Toit, M. (2008). Biogenic amines in wine: understanding the headache. *S. Afr. J. Enol. Viticol.* 29, 109–127.

Souza, S. C., Theodoro, K. H., Souza, E. R., Motta, S. D., and Glória, M. B. A. (2005). Bioactive amines in Brazilian wines: types, levels and correlation with physico-chemical parameters. *Braz. Arch. Biol. Technol.* 48, 53–62. doi: 10.1590/s1516-89132005000100009

Stadnik, J., and Dolatowski, Z. J. (2010). Biogenic amines in meat and fermented meat products. *Acta Sci. Pol. Technol. Aliment.* 9, 251–263.

Suzzi, G., and Gardini, F. (2003). Biogenic amines in dry fermented sausages: a review. *Int. J. Food Microbiol.* 88, 41–54. doi: 10.1016/S0168-1605(03)00080-1

Tabanelli, G., Montanari, C., Bargossi, E., Lanciotti, R., Gatto, V., Felis, G., et al. (2014). Control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin forming lactococci. *Int. J. Food Microbiol.* 190, 14–23. doi: 10.1016/j.ijfoodmicro.2014.08.023

Tabanelli, G., Montanari, C., Grazia, L., Lanciotti, R., and Gardini, F. (2013). Effect of aw at packaging time and atmosphere composition on aroma profile, biogenic amine content and microbiological features of dry fermented sausage. *Meat Sci.* 94, 177–186. doi: 10.1016/j.meatsci.2013.01.018

Tabanelli, G., Torriani, S., Rossi, F., Rizzotti, L., and Gardini, F. (2012). Effect of chemico-physical parameters on the histidine decarboxylase (HdcA) enzymatic activity in *Streptococcus thermophilus* PRI60. *J. Food Sci.* 77, M231–M237. doi: 10.1111/j.1750-3841.2012.02628.x

Talon, R., Leroy, S., and Lebert, I. (2007). Microbial ecosystems of traditional fermented meat products: the importance of indigenous starters. *Meat Sci.* 77, 55–62. doi: 10.1016/j.meatsci.2007.04.023

Tanaka, Y., Kimura, B., Takahashi, H., Watanabe, T., Obata, H., Kai, A., et al. (2008). Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of transcription and role in acid resistance. *J. Appl. Microbiol.* 104, 1283–1293. doi: 10.1111/j.1365-2672.2007.03652.x

The Australian and New Zealand Food Standards Code (2002). *Food Standards Code 2.2.3*, Vol. 2, Ann Arbor, MI: The Australian and New Zealand Food Standards Code. 22301.

Trip, H., Mulder, N. L., and Lolkema, J. S. (2012). Improved acid stress survival of *Lactococcus lactis* expressing the histidine decarboxylation pathway of *Streptococcus thermophilus* CHCC1524. *J. Biol. Chem.* 287, 11195–11204. doi: 10.1074/jbc.M111.330704

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., et al. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region. *Food Microbiol* 36, 335–342. doi: 10.1016/j.fm.2013.07.001

Valsamaki, K., Michaelidou, A., and Polychroniadou, A. (2000). Biogenic amine production in Feta cheese. *Food Chem.* 71, 259–266. doi: 10.1016/S0308-8146(00)00168-0

Veciana-Nogués, M. T., Bover-Cid, S., Mariné-Font, A., and Vidal-Carou, M. C. (2004). Biogenic amine production by *Morganella morganii* and *Klebsiella oxytoca* in tuna. *Eur. Food Res. Technol.* 218, 284–288. doi: 10.1007/s00217-003-0860-7

Veciana-Nogués, M. T., Mariné-Font, A., and Vidal-Carou, M. C. (1997). Changes in biogenic amines during the storage of Mediterranean anchovies immersed in oil. *J. Agric. Food Chem.* 45, 1385–1389. doi: 10.1021/jf9605714

Vidal-Carou, M. C., Ambatle-Espunyes, A., Ulla-Ulla, M. C., and Mariné-Font, A. (1990). Histamine and tyramine in Spanish wines: their formation during the winemaking process. *Am. J. Enol. Vitic.* 41, 160–167.

Visciano, P., Campana, G., Annunziata, L., Vergara, A., and Ianieri, A. (2007). Effect of storage temperature on histamine formation in *Sardina pilchardus* and *Engraulis encrasicolus* after catch. *J. Food Biochem.* 31, 577–588. doi: 10.1111/j.1745-4514.2007.00131.x

Wendakoon, C. N., and Sakaguchi, M. (1993). Combined effects of cloves with potassium sorbate and sodium benzoate on the growth and biogenic amine production of *Enterobacter aerogenes*. *Biosci. Biotechnol. Biochem.* 57, 678–679. doi: 10.1271/bbb.57.678

Wolken, W. A. M., Lucas, P., Lonvaud-Funel, A., and Lolkema, J. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis*. *J. Bacteriol.* 188, 2198–2206. doi: 10.1128/JB.188.6.2198-2206.2006

Wunderlichová, L., Buňková, L., Koutný, M., Jančová, P., and Buňka, F. (2014). Formation, degradation, and detoxification of putrescine by foodborne bacteria: a review. *Compr. Rev. Food Sci. Food Saf.* 13, 1012–1033. doi: 10.1111/1541-4337.12099

Yassoralipour, A., Bakar, J., Rahman, R. A., and Bakar, F. A. (2012). Biogenic amines formation in barramundi (*Lates calcarifer*) fillets at 8°C kept in modified atmosphere packaging with varied CO_2 concentration. *LWT - Food Sci. Technol.* 48, 142–146. doi: 10.1016/j.lwt.2012.01.033

Yew, C., Bakar, F. A., Rahman, R. A., Bakar, J., Zaman, M. Z., Velu, S., et al. (2014). Effects of modified atmosphere packaging with various carbon dioxide composition on biogenic amines formation in Indian Mackerel (*Rastrelliger kanagurta*) stored at 5 ± 1°C. *Packag. Technol. Sci.* 27, 249–254. doi: 10.1002/pts.2020

Zaman, M. Z., Bakar, F. A., Jinap, S., and Bakar, J. (2011). Novel starter cultures to inhibit biogenic amines accumulation during fish sauce fermentation. *Int. J. Food Microbiol.* 145, 84–91. doi: 10.1016/j.ijfoodmicro.2010.11.031

Zaman, M. Z., Bakar, F. A., Selamat, J., and Bakar, J. (2010). Occurrence of biogenic amines and amines degrading bacteria in fish sauce. *Czech. J. Food Sci.* 28, 440–449.

Zhang, K., and Ni, Y. (2014). Tyrosine decarboxylase from *Lactobacillus brevis*: soluble expression and characterization. *Protein Expr. Purif.* 94, 33–39. doi: 10.1016/j.pep.2013.10.018

Zhang, Q., Lin, S., and Nie, X. (2013). Reduction of biogenic amine accumulation in silver carp sausage by an amine-negative *Lactobacillus plantarum*. *Food Control* 32, 496–500. doi: 10.1016/j.foodcont.2013.01.029

Zhang, X., Wang, H., Li, N., Li, M., and Xu, X. (2015). High CO_2-modified atmosphere packaging for extension of shelf-life of chilled yellow-feather broiler meat: a special breed in Asia. *LWT - Food Sci. Technol.* 64, 1123–1129. doi: 10.1016/j.lwt.2015.07.039

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.

frontiers in Microbiology

REVIEW
published: 03 August 2016
doi: 10.3389/fmicb.2016.01132

Applications of High and Ultra High Pressure Homogenization for Food Safety

*Francesca Patrignani and Rosalba Lanciotti**

Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy

Traditionally, the shelf-life and safety of foods have been achieved by thermal processing. Low temperature long time and high temperature short time treatments are the most commonly used hurdles for the pasteurization of fluid foods and raw materials. However, the thermal treatments can reduce the product quality and freshness. Consequently, some non-thermal pasteurization process have been proposed during the last decades, including high hydrostatic pressure, pulsed electric field, ultrasound (US), and high pressure homogenization (HPH). This last technique has been demonstrated to have a great potential to provide "fresh-like" products with prolonged shelf-life. Moreover, the recent developments in high-pressure-homogenization technology and the design of new homogenization valves able to withstand pressures up to 350–400 MPa have opened new opportunities to homogenization processing in the food industries and, consequently, permitted the development of new products differentiated from traditional ones by sensory and structural characteristics or functional properties. For this, this review deals with the principal mechanisms of action of HPH against microorganisms of food concern in relation to the adopted homogenizer and process parameters. In addition, the effects of homogenization on foodborne pathogenic species inactivation in relation to the food matrix and food chemico-physical and process variables will be reviewed. Also the combined use of this alternative technology with other non-thermal technologies will be considered.

Keywords: high pressure homogenization, ultra high pressure homogenization, pathogenic species, inactivation, food matrices, non-thermal technologies, spores, enzymes

OPEN ACCESS

Edited by:
Maria Schirone,
University of Teramo, Italy

Reviewed by:
Luca Settanni,
University of Palermo, Italy
Lucilla Iacumin,
University of Udine, Italy

****Correspondence:***
Rosalba Lanciotti
rosalba.lanciotti@unibo.it

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

Received: *23 March 2016*
Accepted: *07 July 2016*
Published: *03 August 2016*

Citation:
Patrignani F and Lanciotti R (2016) Applications of High and Ultra High Pressure Homogenization for Food Safety. Front. Microbiol. 7:1132. doi: 10.3389/fmicb.2016.01132

INTRODUCTION

For several decades, the food research has been focused on finding new solutions to old problems to ensure food safety without losing nutrients and more recently, in response to market demand, to preserve the sensory and the freshness of the products as well as to produce innovative foods, using safe, quick, economical, and environmental friendly processes (Zamora and Guamis, 2015). In a globalized market, the solution to these goals is fundamental for the survival of food and equipment enterprises, especially the small and medium enterprises (SMEs). The enterprise competition makes crucial the innovation in processes and products and user-friendly processes have been designed, focusing the interest on the application of new processing technologies including high pressure homogenization (HPH) and ultra high pressure homogenization (UHPH; Dumay et al., 2013).

The word "homogenization" is referred to the ability to produce a homogeneous size distribution of particles suspended in a liquid, by forcing the liquid under the effect of pressure through a specifically designed homogenization valve. Homogenizer able to process fluid matrices at pressure ranging between 20–100 MPa are nowadays employed in the dairy beverage, pharmaceutical, and cosmetic industries mainly to reduce particle size and consequently increase stability of emulsions in order to avoid creaming and coalescence phenomena (**Figure 1**). However, HPH was firstly employed as an useful method for cell disruption and recovery of intracellular bio-products (Keshavarz Moore et al., 1990; Shirgaonkar et al., 1998). The successful results obtained on cell disruption of dense microbial cultures stimulated researches on the application of HPH for food safety and shelf-life improvement. In fact, in the food industry, there is a growing interest in mild non-thermal processes, which combine an efficient microbial reduction with a maximal retention of physic-chemical product properties, as well as nutritional and sensory characteristics of the raw materials and ingredients used. Among the non-thermal treatments studied and proposed, HPH is regarded by a wide literature as one of the most encouraging alternatives to traditional heat treatments for food preservation and diversification dairy products, emulsions, egg based foods and fruit juices. Its effectiveness in the deactivation of spoilage microorganisms in model and real systems is well documented since 1994 (Lanciotti et al., 1994, 1996; Guerzoni et al., 1999; Kheadr et al., 2002; Wuytack et al., 2002; Vannini et al., 2004; Diels and Michiels, 2006; Bevilacqua et al., 2009; Patrignani et al., 2009a, 2010, 2013a,b; Zhao et al., 2014; Ferragut et al., 2015). However, HPH effectiveness for microbial inactivation is affected by several parameters such as process and microbial-physiological factors and aspects related to the characteristics of the treated fluid.

The HPH has been proposed also for the bacterial spore inactivation, generally in combination with other physic-chemical hurdles for spore surviving and/or germination due to the ability of bacterial spores to survive to the most prohibitive conditions (Wuytack et al., 2002; Bevilacqua et al., 2007, 2012; Chaves-López et al., 2009; Chen et al., 2013; Roig-Sagues et al., 2015). In addition to the effects on microbial cells, the HPH treatment is reported to act on food constituents, especially proteins and enzymes, modifying their functional properties and activities (Kheadr et al., 2002; Hayes and Kelly, 2003; Vannini et al., 2004; Iucci et al., 2008; Navarro et al., 2014). In fact, it is reported to improve food microstructure, rheology and availability of food bioactive compounds (Guerzoni et al., 1997, 2002; Lanciotti et al., 2004a,b; Sandra and Dalgleish, 2005; Betoret et al., 2015). Moreover, it has been proposed as a useful tool to enhance the cheese yield and reduce the cheese ripening times due to the enhancement of proteolysis and lipolysis of milk naturally occurring or microbial enzymes (Guerzoni et al., 1999; Lanciotti et al., 2004b, 2006, 2007a; Burns et al., 2008; Vannini et al., 2008; Burns et al., 2015). Moreover, the effects of HPH, applied at sub-lethal level (50 MPa), was studied on several strains of *Lactobacillus* spp., and particularly non-starter LAB (NSLAB), inoculated in milk as adjunct and involved in dairy product ripening (Lanciotti et al., 2007b). The literature data demonstrated that this cold technology is able to affect the strain metabolic activity and enzymes leading to a modification of the cheese ripening patterns, especially linked to the breakdown of proteins. The modification of enzyme and protein activity and functionality can greatly affect not only the food quality and ripening patterns but also the food safety feature due to the increased activities of egg and milk naturally occurring antimicrobials such as lysozyme, lactoperoxidase systems, and lactoferrin (Vannini et al., 2004; Iucci et al., 2007; Patrignani et al., 2013b) or reducing the biogenic amine content in cheeses obtained from HPH treated milk (Lanciotti et al., 2007a). In addition, HPH has been proposed with several roles in the functional food sector, for the production of probiotic dairy products with improved sensory or functional properties, such as probiotic strain viability over refrigerated storage and accelerated fermentation kinetics with less environmental impact with respect to the traditional heat treatment (Burns et al., 2008, 2015; Patrignani et al., 2009b, 2015a). In fact, sub-lethal pressure levels (50 MPa) applied directly to microbial cells, increased some functional properties (i.e., hydrophobicity, resistance to simulated gastric conditions, and stomach-duodenum passage) in some probiotic strains associated to an increased maintenance of viability during refrigerated storage (Tabanelli et al., 2012, 2013, 2014). Also, Muramalla and Aryana (2011) reported low homogenization pressures (up to 13–80 MPa for five passes) to improve certain probiotic characteristics of yogurt bacteria and *Lactobacillus acidophilus* LA-K (i.e., strain acid and bile tolerance) without effects on protease activity and strain growth potential. Additionally, these low homogenization pressure treatments, applied directly to probiotic strains, modified their interaction

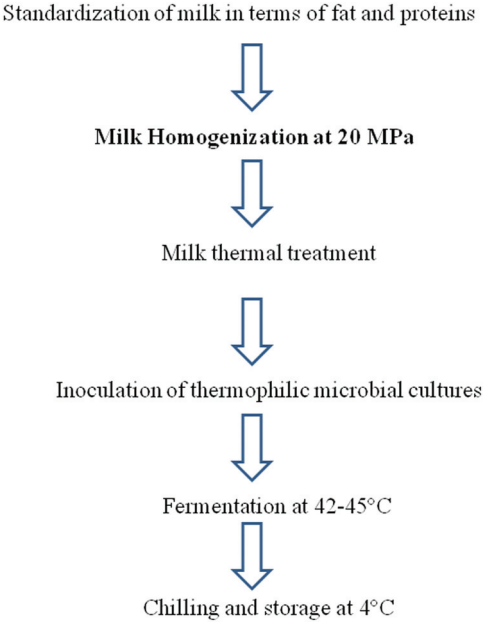


FIGURE 1 | State of art of conventional homogenization in yoghurt preparation.

with the small intestines of BALB mice and induced a higher IgA response compared with untreated mice in a strain- and feeding period-dependent way (Tabanelli et al., 2012). Burns et al. (2015) studied the effects of a sub-lethal HPH on a probiotic strains used as adjunct for producing Caciotta cheese demonstrating that the HPH-treated probiotic strain maintained high viability for 14 days whilst the physico-chemical analyses on Caciotta cheese containing HPH treated cells showed a faster ripening, compared to cheeses containing not HPH treated cells. In addition, these Authors demonstrated that the 50 MPa treatment increased the *L. paracasei* gastric resistance in Caciotta, maintaining high strain viability without any significant effects on IgA production in mice.

The HPH technology has shown a great impulse both at industrial level and research field during the last decades also with the support of some important EU projects. The most relevant EU projects relative to the effects of HPH on food safety can be envisaged in the EU project HighQ RTE FP6-FOOD-023140 "Innovative non-thermal processing technologies to improve the quality and safety of ready-to-eat (RTE) meals-HighQ RTE," EU Craft project "UHPH 512626, Development and Optimisation of a Continuous Ultra High Pressure Homogenizer for Application on Milks and Vegetable Milks" and the EU project FUNENTECH 232603 "study of functionality, nutritional and safety aspects of liquid foods, liquid food preparations, and cosmetics processed by ultrahigh-pressure homogenization". The last had the aim to reinforce transfer the HPH processing to SMEs of fluid or pumpable ingredients and foods as well as cosmetic sectors.

The research performed within national and international projects stimulated the improvement of the HPH equipments and devices and their adaptation to food processing lines. Several HPH equipments are nowadays available and some equipment producers, such as Microfluidics (USA), Bee International (USA), Avestin (Canada), Gea Niro Soavi (Italy), and APV (UK), have proposed pilot devices able to exert a pressure of 100–200 MPa, with average flow of 160 L/h at 200 MPa (Zamora and Guamis, 2015). However, Stansted Fluid Power Ltd (United Kingdom) have developed from the year 2006 pilot devices able to work up to 400 MPa giving origin to the UHPH technology applied to food sector. Also Gea Niro Soavi (Italy) developed within the project made in Italy ATENA a pilot homogenizer able to work at 400 MPa, having an average flow of 5 L/h (patent no. US-2015-0314254-A1, 11/05/2015). This prototype was used to obtained apple and tomato based formulations having a shelf-life of 30 days when stored at environmental temperature and treated at 300–400 MPa. At the expire date the levels of *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Salmonella* spp. were under the detection limits even in the samples inoculated before the HPH treatments at levels ranging between 3 and 4 log CFU/ml.

Since the safety features are prerequisite both for traditional and innovative products, this review is focused on the effects of HPH on foodborne pathogen inactivation in relation to the food matrix and food physico-chemical and process variables. Moreover, the review takes into consideration also the opportunity to inactivate the spores from pathogenic bacteria by using dynamic pressure. Also the potential of HPH to increase the activity of antimicrobial enzymes to inactivate pathogenic microorganism will be taken into consideration. Finally, the combined use of this alternative technology with other non-thermal technologies will be considered within this review.

MECHANISM OF ACTION OF HIGH PRESSURE HOMOGENIZATION

From a technological point of view, an homogenizer consists principally of a pump and a homogenizing valve (**Figure 2**). The pump is used to force the fluid into the valve where the homogenization happens (Diels and Michiels, 2006). In the homogenizing valve the fluid is forced under pressure through a small orifice between the valve and the valve seat. The operating pressure is controlled by adjusting the distance between the valve and the seat. Among the process parameters, the level of pressure applied and the temperature reached in the food matrix during the process are the variables able to affect the food components and the eventually occurring microbial cells. The microbial inactivation caused by the application of HPH, although affected by several factors and mainly by the physic chemical features of the food matrix and the sensitiveness of different microorganisms, increases with the pressure level (Diels and Michiels, 2006). The effects induced by the temperature have to be necessarily taken into account in HPH, since, during homogenization, a rise of the temperature (about 2.5°C per 10MPa), related to the fluid food employed, is observed in the fluid downstream of the valve. This is generally attributed by the literature to the viscous stresses caused by the high velocity of the fluid flow, which is then impinging on the ceramic valve of the homogenizer, leading to the dissipation of a significant fraction of the mechanical energy as heat in the fluid (Floury et al., 2000; Zamora and Guamis, 2015). However, such temperature increase did not result in the appearance of heat indicators in HPH treated food samples probably due the flash time of treatment of the food matrices (lower than 1 s; Floury et al., 2004; Pinho et al., 2011). For example HPH treated milk samples suffered less Maillard reaction, less whey protein denaturation and did not present lactose isomerisation compared to a commercial pasteurized milk with a concomitant preservation of essential amino acids and as a consequence with a better nutritional value desired by consumers (Pereda et al., 2009).

The significant improvement of HPH equipment functionality and flexibility (capacity to work in a food processing line), the design of new valves able to work at 300–400 MPa and the significant research efforts make HPH technology ready for the scaling up at industrial level for the development of new products differentiated from traditional ones by sensory and structural characteristics or functional properties. Also patents regarding continuous systems and procedure of sterilization and physical stabilization of pumpable fluids by means of HPH are available (i.e., EP 2409583 A1).

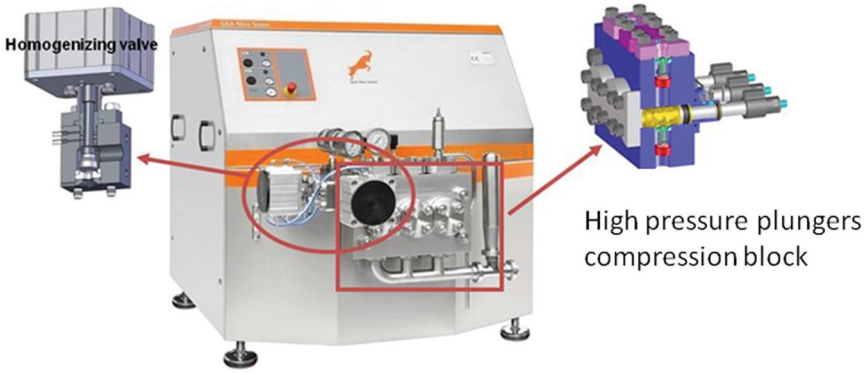


FIGURE 2 | State of the art of homogenizer able to reach 200 MPa (GEA Niro Soavi, Italy).

POTENTIAL AND APPLICATIONS OF HIGH AND ULTRA HIGH PRESSURE HOMOGENIZATION FOR PATHOGENIC SPECIES INACTIVATION *IN VITRO* AND FOOD SYSTEMS

The first application of homogenization for the stabilization of food and dairy emulsion was presented at the Paris World's Fair in 1900 where Auguste Gaulin presented an invention for "intimately mixing milk" using pressures up to 30 MPa (Gaulin, 1899). Since then conventional homogenization extended the pressure range until 50 MPa. HPH, also known as dynamic HPH, has been frequently highlighted for its potential for food cold pasteurization (Diels and Michiels, 2006; Donsì et al., 2009a,b,c; Patrignani et al., 2009a, 2010, 2013a,b; Pereda et al., 2009; Panozzo et al., 2014). Modern high pressure homogenizer enables pressures 10–15 times higher than traditional ones and covers pressure ranges between 300 and 400 MPa. These last ranges have been referred to as UHPH. The progression toward UHPH has also opened the view to new sterilization opportunities, including also the inactivation of spores by HPH. The inlet temperature of pumpable products and the level of pressure, which both determine the temperature reached during the UHPH treatment, have been considered as the main factors of the microbial inactivation (Zamora and Guamis, 2015). UHPH warrants the destruction of microorganisms reaching sterilization of liquid food products, and it has a positive influence on food stability, with few effects on nutritional value and sensory characteristics of the processed fluids. UHPH technology allows more efficient particle reduction than the classical homogenization, and its effect is the results of several mechanisms such as sudden pressure drop, torsion and shear stresses, turbulence, impingement, cavitation phenomena, shock waves, and temperature increase, with a concomitant reduction of microbial load and interesting consequences in emulsion properties (**Figure 3**). Since its first appearance, HPH and, later, UHPH have been tested on several matrixes and *in vitro* systems by using different microbial targets to demonstrate their effectiveness. The microbial targets deal with pathogenic and spoilage microorganisms but in this review only the researches regarding the pathogenic species will be reviewed.

In **Table 1**, the studies regarding the microbial inactivation in model systems by HPH or UHPH, in relation to the microbial targets, inoculation level and conditions adopted, are reported.

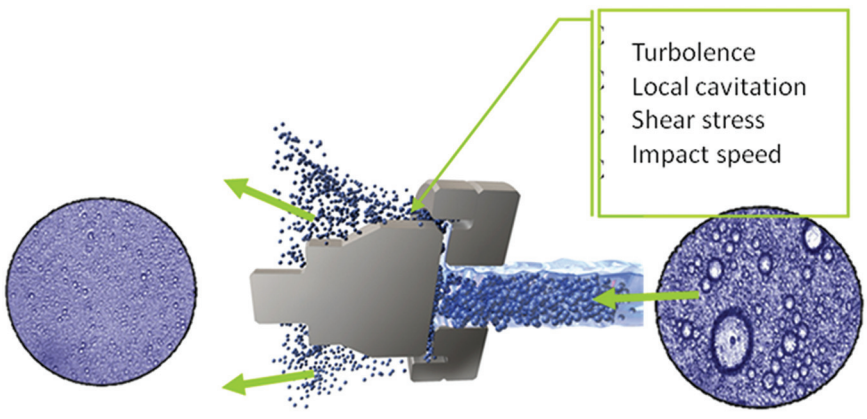


FIGURE 3 | Most probable mechanisms of action of HPH (GEA Niro Soavi, Italy).

TABLE 1 | High pressure homogenization (HPH) microbial inactivation in relation to the model system, species and process conditions adopted.

Matrix	Microorganisms	Reduction	Conditions	Homogenizer/type of valve	Reference
Water 50%, oil 25%, egg yolk 25%	*Listeria monocytogenes*, *Yersinia enterocolitica*	7 log for all	Pressure ranges: 150–200 MPa	PA′NS valve	Lanciotti et al., 1994
Water 25%, oil 53%, egg yolk 22%	*Listeria monocytogenes*, *Yersinia enterocolitica*,	7 log for all	Pressure ranges: 150–200 MPa	PA′NS valve	Lanciotti et al., 1994
Brain Heart Infusion modified in pH and water activity	*Listeria monocytogenes, Staphylococcus. aureus, Escherichia coli*	7 log for all	Pressure ranges: 150–200 MPa	′PA′NS valve	Lanciotti et al., 1996
Model system simulating dairy product	*Listeria monocytogenes,*	No growth with respect to the inoculum	Pressure: 25 MPa	′PA′NS valve	Guerzoni et al., 1997
Brain Heart Infusion modified in pH and NaCl	*Salmonella enteritidis*	2.6 log	Pressure ranges: 0.1–140 MPa	PA′NS valve	Guerzoni et al., 2002
PBS buffer (pH 7)	*Staphylococcus aureus*	3.0 log	Tin = 50 *P* = 300 Tout = 18	Counterjet dispergator	Diels et al., 2003
PBS buffer (pH 7)	*Staphylococcus aureus*	6.0 log	Tin = 25 *P* = 300 Tout = 42	Counterjet dispergator	Wuytack et al., 2002
PBS buffer (pH 7)	*Yersinia enterocolitica*	6.0 log	Tin = 50 *P* = 250 Tout = 18	Counterjet dispergator	Diels et al., 2003
PBS buffer (pH 7)	*Salmonella enteritidis* ATCC 13047	8.0 log (after five passes)	Tin = 25 *P* = 200 Tout = NR, flow 1.5 L/h	Counterjet dispergator	Vachon et al., 2002
PBS buffer (pH 7)	*Enterococcus faecalis*	3.0 log	Tin = 25 *P* = 250 Tout = 42	Counterjet dispergator	Wuytack et al., 2002
PBS buffer (pH 7)	*Escherichia coli* K12	6.0 log	Tin = 25 *P* = 300 Tout = NR	Axial-flown through orifice valve	Diels et al., 2005
PBS buffer (pH 7)	*Escherichia coli* MG1655	7.0 log	Tin = 50 *P* = 250 Tout = 18	Counterjet dispergator	Diels et al., 2004
Saline solution and nisin	*Escherichia coli* K12	7.0 log	Tin = 5 *P* = 300 Tout = 70	Axial-flown through orifice valve	Taylor et al., 2007
PBS buffer (pH 7)	*Escherichia coli* O157:H7 ATCC 35150	8.0 log (after three passes)	Tin = 25 *P* = 200 Tout = NR flow 1.5 L/h	Counterjet dispergator	Vachon et al., 2002
LB nutrient	*Escherichia coli*	7.0 log	300 MPa	Stansted high-pressure homogenizer (model FPG11300:350	Donsì et al., 2009b

Lanciotti et al. (1994) studied the effectiveness of pressures ranging from 15 to 200 MPa, applied with a continuous homogenizer (Niro Soavi, Parma, Italy), on the cell viability of spoilage and pathogenic microorganisms in two model systems made of water 50%, oil 25%, egg yolk 25%, the first, and water 25%, oil 53%, egg yolk 22%, the second. Moreover, the effects of microstructural modifications of food systems associated with the treatments on cell viability and on its subsequent evolution were investigated. Modulation of the homogenization pressure allowed strong instantaneous reductions of the initial cell loads of *L. monocytogenes* and *Yersinia enterocolitica*. The combined effects of the initial pressure treatment and space reduction, resulting from the microstructural modifications of food systems, increased the safety and the shelf-life both of water in oil and oil in water emulsions. Moreover, Lanciotti et al. (1996) investigated also the effects of chemico-physical growth conditions such as pH, temperature and water activity *(a)* on lethal high homogenization pressure effects on *L. monocytogenes, S. aureus,* and *E. coli*. The results, based on standard medium such as Brain Heart Infusion (BHI), highlighted the importance of food system composition and its thermal history on the high pressure tolerance of the microbial population. In previous work, Lanciotti et al. (1994) showed that the relationship between survival cells and instantaneous pressure applied in medium appeared to be log-linear in a range between 40 and 200 MPa. According to the author findings, *S. aureus* was the most resistant pathogenic species while *L. monocytogenes* was resistant to pressure only when grown at highest *aw*. Also Guerzoni et al. (1997) studied the growth of *L. monocytogenes* in a model system, simulating a dairy product, when treated at 25 MPa, in relation to the lipid content NaCl and pH values, modulated according to a Central Composite Design (CCD). Polynomial equations describing the effects of such variables on the *aw*, microstructural features, and *L. monocytogenes* growth, were obtained. The three variables and their interactions, in addition to a direct effect on microbial growth, played an indirect role

due to their influence on microstructural features, such as diameter of water droplets and total water phase availability. In particular, the pH value affected the a(w) and the total space available for microbial growth, while the NaCl content had a prevalently indirect effect on space availability and on the diameter of the water droplets. The results suggested that the microstructural changes induced by HPH affected the growth of *L. monocytogenes* which was dependent on the total water phase space availability. Guerzoni et al. (2002) studied the survival of *Salmonella enteritidis* after pressure treatments, ranging between 0.1 and 140 MPa, in relation to compositive variables (NaCl content, pH). The study was performed both in model (BHI) and real systems consisting of an egg-based mayonnaise type product. Moreover, the fate of the surviving cells of *S. enteritidis* was monitored during storage at 10°C and the growth or death parameters were calculated and modeled in relation to pH, NaCl concentration of the medium and the level of pressure treatment applied. From this study, it was evident that the salt content and pH displayed a synergistic effect with pressure, whose extent was higher in the mayonnaise based products than in BHI. In fact, while in the model systems the cell recovery and growth during the subsequent incubation at 10°C was allowed in many combinations of the CCD, in the real systems no recovery or growth of *S. enteritidis* were observed. According to the Authors, this viability loss, which was maximum at pH 4.00 or 2% NaCl, is not be attributed merely to the interactions of such variables, but it probably involved the naturally occurring antimicrobial enzymes of the raw material, whose activity can be enhanced by the pressure treatment. Reviewing the literature, one of the most studied *in vitro* system is represented by PBS buffer. Different Authors have investigated in this medium the potentialities of HPH treatment in pathogenic species inactivation. For example, Vachon et al. (2002) studied the effect of an homogenization treatment performed at 200 MPa, repeated for five cycles, on the inactivation of *S. enteritidis* ATCC 13047, *E. coli* O157:H7 ATCC35150 and *L. monocytogenes* LSD 105-1 when inoculated in PBS buffer at pH 7. The results showed that *E. coli* and *L. monocytogenes* reached 8 log reduction cycles after the HPH treatment after three passes while five passes were necessary to reach the same inactivation level for *S. enteritidis*. Also Wuytack et al. (2002) found that a treatment of 300 MPa was able to inactivate *S. aureus, Enterococcus faecalis* and *L. innocua* of 6, 3, and 5 log reduction cycles in PBS buffer pH 7, using a treatment of 300 MPa and a fluid inlet temperature of 25°C. Diels et al. (2003) conduced a detailed study of the inactivation of *S. aureus* and *Y. enterocolitica* in PBS buffer by HPH at, respectively, 25 and 35 different combinations of process temperature and process pressure covering a range of 5–50°C and 100–300 MPa. In the entire studied, it was clear that *S. aureus* was more resistant to HPH than *Y. enterocolitica*, as already demonstrated by Lanciotti et al. (1996). Also, temperature between 5 and 40°C did not affect inactivation of *S. aureus* by high-pressure homogenisation, while *Y. enterocolitica* inactivation was affected by temperature over a much wider range. Later, Diels et al. (2005) investigated the resistance of *E. coli* K12 when treated in PBS buffer at 300 MPa by using a axial-flow through orifice valve, outlining reduction of 6 log cycles. Donsì et al. (2009c) studied the inactivation of *E. coli* by HPH in model systems, under a wide range of operating conditions (temperature, pressure, number of homogenization passes, cell concentration) in a lab-scale and a pilot-scale unit (Stansted Fluid Power) utilizing single or multiple passes. Results highlighted that the inactivation kinetics did not depend linearly on pressure, due to the distribution of individual cell resistance in the sample. The efficacy of the treatment at higher pressures or upon multiple passes was reduced. Moreover, the scale of the apparatus, which in this case differed of several order of magnitude (from 0.7 to 120 l/h), and consequently of the width of the gap of the valve did not affect the extent of microbial inactivation at a given pressure. Taylor et al. (2007) studied the inactivation of *E. coli* K-12 cells, grown statically or in chemostat, when exposed to HPH processing pressures of 50 to 350 MPa in the absence or presence of the antimicrobial nisin. These Authors found that pressure and temperature exhibited a quadratic relationship. Significant HPH-induced inactivations of the Gram-negative microorganism was observed in the range of 100 to 250 MPa. Above 300 MPa, heat was the main factor promoting microbial inactivation, regardless of whether cells were grown in chemostat or statically. Chemostat-grown cells were significantly more resistant to HPH processing than were statically grown cells. Moreover, the data indicated a potential synergistic effects of nisin and HPH on the inactivation of bacterial contaminants, although this antimicrobial is generally active against Gram-positive bacteria.

However, although a huge amount of data collected from model systems were available and helpful to understand the mechanisms of action of HPH against pathogenic species, many researchers tested potentialities of this technology against pathogenic species inoculated in food matrixes because the composition and viscosity of the treated food also have an indirect effect on the microbial inactivation (**Table 2**). The available literature reports about the use of HPH for the reduction of foodborne pathogens in several food matrices, such as milk (Lanciotti et al., 1994; Diels et al., 2005; Hayes et al., 2005; Brinez et al., 2006a,b, 2007; López-Pedemonte et al., 2006; Roig-Sagues et al., 2009), egg-based products (Velazquez-Estrada et al., 2008; Patrignani et al., 2013b), orange juice (Brinez et al., 2006b; Kumar et al., 2009; Pathanibul et al., 2009; Maresca et al., 2011), mayonnaise type products (Guerzoni et al., 2002). Lanciotti et al. (1994) found that the relationship between surviving cells and pressure applied was log-linear in milk inoculated with *Y. enterocolitica* and *L. monocytogenes* and processed at different pressures (40–150 MPa) at 25°C. In order to establish the fate of the surviving cells, the growth of *Y. enterocolitica,* and *L. monocytogenes*, in the samples stored at 3–4°C, was followed over 220 h. The data were analyzed according to the Gompertz equation. For *Y. enterocolitica* and *L. monocytogenes*, the treatment apparently did not induce irreversible damage to the surviving cells; in fact, although the lag phase was prolonged when pressures higher than 400 bar were used, the μmax increased with the applied pressure and the maximal cell numbers attained (A + K) were independent on the level of the applied pressure. Diels et al. (2005) studied the inactivation of *E. coli* MG1655 in skim, soy and strawberry-raspberry milk

TABLE 2 | High pressure homogenization microbial inactivation in relation to the food matrix, species and process conditions adopted.

Matrix	Microorganisms	Reduction	Conditions	Homogenizer/type of valve	Reference
Milk	*Yersinia enterocolitica, Listeria monocytoges*	*Yersinia enterocolitica* 5 log at 150 MPa *Listeria monocytogenes* : the same	*P* range = 40–150 MPa Tout max = 65	PS valve, Gea Homogenizer	Lanciotti et al., 1994
Egg yolk 10%, yoghurt 13%, sunflower oil 60%, water in relation to pH and NaCl	*Salmonella enteritidis*	Reduction was obtained at 50 MPa with pH 4 and 2% NaCl. No re-growth at 10°C	*P* range = 0.1–50 MPa	PS valve, Gea Homogenizer	Guerzoni et al., 2002
Skim, soy, and strawberry-raspberry milk	*Escherichia coli* MG1655	Skim 3.5 log Soy 3.0 log Straw/rasp 3.0 log	Tin = 25, *P* = 300 MPa, Tout = 18	Counterjet dispergator	Diels et al., 2005
Bovine milk	*Pseudomonas fluorescens* AFT 36	6 log	Tin = 45 *P* = 250 Tout = 76.8	Axial-flown through orifice valve	Hayes et al., 2005
Milk	*Staphylococcus aureus* CECT 976	7 log	Tin = 20 *P* = 330 Tout = NR flow 16 L/h	Axial-flown through orifice valve	López-Pedemonte et al., 2006
Orange juice	*Escherichia coli* O58:H21 ATCC 10536, *Escherichia coli* O157:H7 CCUG 44857	3.9 log (O58:H21) 3.7 log (O157:H7)	Tin = 20 *P* = 300 Tout = NR, flow 18 L/h	Axial-flown through orifice valve	Brinez et al., 2006b
Milk	*Listeria innocua* ATCC 33090	2.7 log	Tin = 20, *P* = 300; Tout = NR, flow = 18 L/h	Axial-flown through orifice valve	Brinez et al., 2006a
Milk and orange juice	*Staphylococcus aureus* ATCC 13565	Milk 3.6 log Orange juice 4.2 log	Tin = 20 *P* = 300 Tout = 18 flow 18 L/h	Axial-flown through orifice valve	Brinez et al., 2007
Liquid whole egg	*Salmonella enterica* serovar *senftenberg* 775W	3.2 log at 250 MPa	100,150, 200, and 250 MPa	Not found	Velazquez-Estrada et al., 2008
Milk (0.3, 3.6, 10, and 15% fat contents)	*Listeria monocytogenes* CCUG 15526	7.95 log at 400 MPa and 15% fat	200, 300, and 400 MPa	Benchtop high-pressure homogenizer (model/DRG FPG7400H:350, Stansted Fluid	Roig-Sagues et al., 2009
Apple juice and apple cider	*Escherichia coli* K12	7 log	Tin = 25 *P* = 250 Tout = 70	Axial-flown through orifice valve	Kumar et al., 2009
Apple and carrot juice	*Listeria innocua*, *Escherichia coli*	*Escherichia coli*: > 5 log reduction (>250 MPa). *Listeria innocua*: 5 log at 350 MPa	*P* range = 0–350 MPa		Pathanibul et al., 2009
Orange, red orange, and pineapple	*Escherichia coli*	100 MPa 7 log after four passes 150 MPa 8 log after three passes	*P* range = 50–250 MPa Passes = 1–5 Tin = 2–20°C	nm-GEN 7400 series by Stansted Power Fluids, UK.	Maresca et al., 2011
Liquid whole egg	*Salmonella enteritidis*	3 log	100 MPa for five cycles	PS valve, Gea Homogenizer	Patrignani et al., 2013b
Egg white	*Salmonella enterica* SDMZ 9898	5 log after eight passes at 150 MPa	*P* = 20, 50, 100, 150 MPa *P* = 150 MPa via multiple passes up to 17	Cylindrical tungsten carbide homogenising valves	Panozzo et al., 2014

subjected to 300 MPa founding that the highest inactivation was reached in skim milk with a Tin and Tout temperature of 25 and 18°C, respectively, by using a counterjet dispergator valve. Hayes et al. (2005), utilizing an axial-flown through orifice valve, found in milk treated at 250 MPa, with a Tin of 45°C and a Tout of 76.8, a 6 log reduction of *Pseudomonas fluorescens* AFT 36, resulting from the application of pressure and temperature increase. Lowest reduction (2.7 log) was found by Brinez et al. (2006b) for *L. innocua* ATCC33090 in bovine milk or *S. aureus* ATCC 13565 (3.6 log reduction) and *S. aureus* CECT 4491 in

milk (Brinez et al., 2007). López-Pedemonte et al. (2006) found high inactivation for *S. aureus* CECT 976 inoculated in milk treated at 330 MPa. Roig-Sagues et al. (2009) reported about the inactivation of *L. monocytogenes* CCUG 15526 when inoculated at 7.0log CFU/ml in milk samples having 0.3, 3.6, 10, and 15% of fat contents. The samples were subjected to a single cycle of UHPH treatment at 200, 300, and 400 MPa. Microbiological analyses were performed 2 h after the UHPH treatments and after 5, 8, and 15 days of storage at 4°C. Maximum lethality values were observed in samples treated at 400 MPa with 15 and 10% fat (7.95 and 7.46 log CFU/ml), respectively, while in skimmed and 3.6% fat milk samples, complete inactivation was not achieved and, during the subsequent 15 days of storage at 4°C, *L. monocytogenes* was able to recover. In milk samples with 10 and 15% fat, *L. monocytogenes* recovered to the level of initial counts only in the milk samples treated at 200 MPa but not in the milk samples treated at 300 and 400 MPa. According to these data, fat content increase enhanced the maximum temperature reached during UHPH treatment and this could have contributed to the lethal effect achieved. In addition the HPH treatments of milk is reported to enhance the release of free fatty acids (due to the rupture of fatty globule membranes and the activation of lipases), and mainly short and medium chain ones, having an antimicrobial effects (Lanciotti et al., 2006; Vannini et al., 2008). On the contrary, some Authors attributed to the fat content in milk a protective role against microbial species during the high pressure treatment performed at 100 MPa for several cycles. Although several Authors have tested the same matrixes, and in some case the same microbial species, different inactivation results was achieved, particularly using the multi-pass approach. For example, Patrignani et al. (2013b) found that *Salmonella* inactivation in eggs, resulting from the application of HPH at 100 MPa, seems to be linearly correlated to the number of passes. This result, although using a different substrate and microorganism, is in agreement with the findings of Patrignani et al. (2010) who demonstrated for the spoiling *Zygosaccharomyces bailii*, inoculated in apricot and carrot juice, that the effect of each pass is additive and, therefore, each homogenization pass causes almost the same reduction of the microbial load. Several other authors have also found first order inactivation kinetics as a function of the number of passes (Wuytack et al., 2002; Diels and Michiels, 2006; Tahiri et al., 2006), although the literature data concerning the inactivation kinetics by HPH are still conflicting. In fact, Donsì et al. (2009c) observed that HPH inactivation of *E. coli*, produced a non-additive trend for multiple pass processes at a given pressure level. These Authors attributed this behavior mainly to the physiological diversity within a microbial population and to the existence of resistant cells able to survive after repeated passes at the pressure applied. On the other hand, it is important to take into consideration the additional effect of HPH on the antimicrobial activity of naturally occurring enzymes, such as lysozyme, lactoperoxidase system and so on. Velazquez-Estrada et al. (2008) have published data regarding the use of HPH for *Salmonella* inactivation in liquid whole egg (LWE). In particular, these Authors proposed the single-pass treatment of inoculated LWE with ultra HPH at 100, 150, 200, and 250 MPa, demonstrating that the level of pressure applied can influence the *S. enterica* lethality attained. Moreover, Panozzo et al. (2014) outlined the potentialities of HPH as a promising alternative to thermal pasteurization of eggwhite. These Authors showed that a HPH at 150 MPa for multiple passes was able to decontaminate egg white inoculated with *S. enterica* SDMZ 9898.

Many applications of HPH or UHPH for inactivation of spoiling and pathogenic species were tested in juices and vegetable drinks, where HPH and UHPH find a great application potential. For example, Brinez et al. (2006a) tested the inactivation of *E. coli* 058:H21 ATCC 10536 and *E. coli* 0157:H7 CCUG 44857 inoculated in orange juice and treated at 300 MPa founding a log reduction of 3.9 and 3.7, respectively. Kumar et al. (2009) investigated on the inactivation of *E. coli* K12 in apple juice and apple cider obtaining an inactivation of 7 log applying a pressure of 250 MPa with a Tin of 25°C and Tout of 70°C, thus avoiding the use of a thermal exchanger. Also Pathanibul et al. (2009) inoculated *L. innocua* ATCC51742 and *E. coli*, as surrogate for foodborne pathogens, in apple and carrot juice, containing or not nisin (0-10 IU), and treated from 0.1 to 350 MPa. At 50 MPa homogenization pressure intervals, juice samples were collected, immediately cooled to 10°C, and then serially diluted and plated on non-selective recovery media. As processing pressure increased, inactivation of *E. coli* increased, and a 5 log reduction of cells was achieved following exposure to pressures in excess 250 MPa. In contrast, little inactivation was observed for *L. innocua* with pressures ranging between 250 and 350 MPa. However, several authors have been demonstrated the major efficacy of HPH against Gram-negative bacteria. The addition of 10 IU nisin, together with HPH, did not exhibit significant additional *E. coli* inactivation, but interactions were observed with *L. innocua.* On the other hand nisin is reported to be active against Gram-positive bacteria (Siroli et al., 2015).

POTENTIAL AND APPLICATION OF HIGH PRESSURE HOMOGENIZATION FOR SPORE INACTIVATION *IN VITRO* AND FOOD SYSTEMS

Bacterial spores represent one of the major hazard in food safety due to their high resistance to most hurdles. In particular, they are resistant to elevated temperatures (80°C), which distinguishes them from vegetative cells. Thermal sterilization is the method to eliminate spores in most food applications, as it provides the highest guarantee of sterility. From a safety point of view, spores of *Bacillus* spp. and *Clostridium* spp. are greatly resistant to several treatment such as heat, desiccation, lack of nutrients, exposure to UV and gamma radiation, organic chemicals, and oxidizing agents. In general, the heat resistance of spores depends on conditions such as elevated sporulation temperature, the presence of minerals and dipicolinic acid (DPA), and core dehydration. The endospores are composed of a central core, which is surrounded by several protective layers. The outermost

layer, the exosporium, is not present in spores of all species, and is the primary site of contact with the environment. Between the outer and inner membrane, there is the cortex. Since the spore structure plays a major role in spore resistance, the spore inactivation in foods requires high levels of heat treatments, which can in turn have negative effects on the sensory and nutritional profile (Schubert and Beaudet, 2011; Reineke et al., 2013; Georget et al., 2014a,b; Dong et al., 2015). For this reason, alternative and convenient methods have been studied in recent years (Chaves-López et al., 2009). In order to increase antimicrobial effectiveness and reduce side effects on food quality, the application of combined hurdles has also received great attention. Extensive literature indicates that the effects of combined stresses on microbial growth and survival may be additive or synergistic, when the outcome is usually significantly greater than the additive response (Tapia de Daza et al., 1996; Leistner, 2000; Ross et al., 2003), because of the disturbing action on microbial homeostasis in several respects (Leistner, 2000).

Because of its great potential for microbial inactivation, several authors have studied the effects of HPH or UHPH, when applied individually or in combination with other mild physical or chemical stresses (heat and H_2O_2), on the inactivation of *Bacillus* and *Clostridium* spores, whose genera, from a safety point of view, the most important species belong. The analysis of the literature shows that the major application for HPH and UHPH regards the inactivation of spores of spoiling bacteria while the reports dealing with the inactivation of spores from pathogenic species are sporadic. Focusing on pathogenic species, Chaves-López et al. (2009) evaluated the influence of HPH treatment, applied individually (one, two, or three cycles) or in combination with other mild physical or chemical stresses (mild heat treatment, H_2O_2, and low pH), on the capability of *B. cereus* and *B. subtilis* spore, suspended in sterilized double distilled water, to form colonies. These Author determined also the effects of the treatments applied on the release of DPA from the spores, since spore resistance to stresses such as temperature and pressure has been correlated to their ability to retain DPA, present in the core region of the dormant spores (Setlow, 2000; Cortezzo et al., 2004). The Authors outlined how the application of specific stress sequences can significantly inactivate *B. cereus* spores. The remarkable efficacy of repeated cycles at 150 MPa suggested that dynamic high pressure, particularly applied in combination with other sub-lethal stresses, could be a useful and innovative tool for *B. cereus* control in fluid foods. In particular, the Authors showed that, although plate count only slightly decreased in all the strains when one cycle of HPH at 150 MPa was applied alone, the spores released significant levels of DPA (up to 28%) that could indicate a possible disruption of spore layers. Three consecutive cycles of HPH determined high reduction of colony count (about 5 log CFU/ml) and high DPA release (52%). Among the stress conditions applied, it was observed that only the thermal shock after one HPH cycle reduced the colony count of 2.3 log CFU/ml and induced a DPA release up to 57%. Pinho et al. (2011) evaluated the inactivation of *Clostridium sporogenes* PA 3679 spores (considered as harmless twin of *C. botulinum*) by HPH in model system such as skim milk, showing that that pressures up to 300 MPa were not able to cause any reduction on spore counts or promote changes on their thermal resistance. The application of heat shock (100°C/15 min) before HPH treatment and the homogenization process realized at mild inlet temperature (45°C), which results in homogenization temperature of around 84°C at 300 MPa, also did not cause reduction on viable spores counts. A few spores reduction (0.67 logarithmic cycles) were only observed when the milk samples were subjected to homogenization treatment for 16 cycles (multiple passes) at 300 MPa. Therefore, although HPH be recognized as an effective method for milk pasteurization, in this specific case, HPH process is not able to guarantee the commercial sterility of milk, being necessary the association of the homogenization with another preservative method, as refrigeration.

Also Amador Espejo et al. (2014) tested the ability of Ultra High-Pressure Homogenization treatments at 300 MPa with inlet temperatures of 55, 65, 75, and 85°C to inactivate *B. cereus* spores inoculated into commercial ultra high temperature treated whole milk in order to evaluate the inactivation level achieved. These Authors provided important evidence of the suitability of UHPH technology for the inactivation of spores in high numbers, leading to the possibility of obtaining commercially sterile milk. In fact, UHPH conditions at 300 MPa with a inlet temperature of 75 and 85°C were capable of a spore inactivation of 5 log CFU/ml. Furthermore, under these processing conditions, commercial sterility (evaluated as the complete inactivation of the inoculated spores) was obtained in milk treated at 300 MPa with inlet temperature of 75°C.

POTENTIAL OF HIGH PRESSURE HOMOGENIZATION TO INCREASE THE ANTIMICROBIAL ACTIVITY OF ENZYME OF FOOD INTEREST

Because of the great interest within the food industry in aldehydes, ketones, esters as natural antimicrobial compounds (Burt, 2004; Patrignani et al., 2015b) or enzymes such as lysozyme, lactoperoxidase system and lactoferrin, many Authors have tried to find strategies to enhance their efficacy in foods. In general, the approaches used are aimed to destabilize the microbial outer membranes of Gram-negative bacteria or to modify the chemical structure of the enzyme. For example, Ibrahim et al. (1993) and Bernkop-Schnurch et al. (1998) showed that the antimicrobial spectrum of lysozyme can be extended, including also Gram-negative bacteria, by slight chemical modification of the protein with hydrophobic ligands throughtout thermal treatment. These modifications generate an amphitropic protein able to spread the cytoplasmic membrane (Vannini et al., 2004; Iucci et al., 2007). Peptic digestion or heat treatment are reported to augment the antimicrobial activity of lactoferrin. In this perspective, several authors tried to modify the enzyme structure, and

consequently its activity, by using HPH in order to increase food safety. On the other hand the effect of HPH on naturally occurring food enzymes involved also in shelf-life, ripening and functionality of several matrices has been demonstrated (Kheadr et al., 2002; Lanciotti et al., 2004b, 2006; Vannini et al., 2008).

From a safety point of view, Vannini et al. (2004) evaluated the effect of HPH on the activity of antimicrobial enzymes such as lysozyme and lactoperoxidase against a selected group of Gram-positive and Gram-negative species inoculated in skim milk, formulating the hypothesis that the interaction of HPH and enzymes is associated to conformational modifications of the two proteins with a consequent enhancement of their activity. *L. monocytogenes* was the most pressure resistant species while *S. typhimurium*, *S. aureus*, and *S. enteritidis* were found to be very sensitive to the hyperbaric treatment. The enzyme addition enhanced the immediate pressure efficacy on almost all the considered species as indicated by their instantaneous viability loss following the treatment. Moreover, the combination of the enzyme and HPH significantly affected the recovery and growth dynamics of the considered species. Although *L. monocytogenes* was slightly sensitive to pressure, the combination of the two stress factors induced a significant viability loss within 3 h and an extension of lag phases in skim milk during incubation at 37°C. Iucci et al. (2007) investigated the effects of HPH treatment at 100 MPa, in comparison to different heat treatments, 70°C for 30 s, 70°C for 5 min or 100°C for 5 min, on the activity of lysozyme and lactoferrin. Their antimicrobial activities were tested on *L. monocytogenes* inoculated in milk or cultural medium. The results indicated that antimicrobial activities of lactoferrin and lysozyme were enhanced and/or accelerated by HPH treatment. Particularly, the highest immediate inactivation values were recorded when *L. monocytogenes* cells were added to HPH-treated lactoferrin, processed simultaneously or separately with the target microorganism. Although to a lesser extent than HPH treatment the heat treatments applied also were able to increase the antimicrobial activity of lysozyme. The Author suggested that the large supramolecular structure is disrupted under pressure, allowing the components to move freely and become independent of the original structure. Interactions can reform when the pressure instantaneously decreases but the original structure is not reformed because of the independent movements of the components.

Patrignani et al. (2013b) found for *S. enteritidis*, inoculated in LWE, HPH inactivation linearly correlated to the number of passes at 100 MPa underling as for LWE, it is important to take into consideration the additional effect of HPH on the antimicrobial activity of naturally occurring enzymes, such as lysozyme. In fact, dynamic pressure is reported to enhance antimicrobial activity of lysozyme and lactoferrin, probably due to the change of the exposure of hydrophobic regions (Iucci et al., 2007). Guerzoni et al. (2002) hypothesized that the inactivation of *S. enteritidis* in egg based formulation treated at different HPH pressure and its inability to re grow in the system at 10°C was due to the increase of activity of HPH treated lysozyme from eggs. Also Velazquez-Estrada et al. (2008) attributed higher inactivation of *S. enterica* serovar *Senftenberg* 775Win HPH LWE to effects of pressure on cells and naturally occurring antimicrobial enzymes. Diels et al. (2005) observed that, above 150 MPa, *E. coli* became more sensitive to lysozyme when this compound was added before HPH treatment compared with adding the enzyme after the treatments, indicating that HPH treatment increases effectiveness and action spectrum of lysozyme.

CONCLUSION

The critical evaluation of the available literature data has showed the great potential of HPH for microbial inactivation and food safety purpose. Since 1990 several Authors have been tested its potentialities *in vitro* and *real* systems, demonstrating its different ability for pathogenic species inactivation in relation to the strains considered, the food matrix and technological procedures adopted. However, until the introduction of new valve design and ultra high pressure homogenizers, able to reach pressures of 400 MPa, this technology was implemented in food industry only for fat globule reduction, for juice treatment and emulsion creation. The introduction of these new variables have opened new field in food sector, also for food decontamination and permitting to replace or minimize the traditional thermal treatments generally applied for the safety purposes. The new main applications regard the treatment of milk (for consumption or dairy product manufacture), fruit and vegetable juice, vegetable milks, and food component (such as enzymes) obtaining more stable and safer products without detrimental effects on quality properties. In fact, in the most of the cases, the literature data have underlined an improvement of the sensory and nutritional properties and stability of the HPH and UHPH treated products. The replacement of traditional thermal treatment can represent an advantage for the industry since this HPH is a cold technology with a lower impact on the environment, more sustainable, saving energy, time and additional costs. Moreover the literature data have demonstrated that HPH, when applied to the milk for cheesemaking, can increase the cheese yield, reduce the cheese hydrolytic patterns, reducing the costs for ripening.

Moreover, another important improvement of the state of the art was done for the inactivation of resistant endospores, which still represent a great challenge for food industry. The literature data have pointed out that the combination of UHPH and several chemico-physical hurdles can be regarded as tool for spore inactivation especially for milk based products. Further improve of food safety and functional properties could be achieved exploiting the well recognized dynamic pressure potential to obtain nanoparticles of antimicrobial molecules or functional ingredients. However, also in this case the reduction of thermal treatment can represent an advantage for food industry and contribute to the maintenance of the quality and nutritional properties of foods.

However, although the achieved aims, UHPH has not been yet implemented in food industry since conventional and ultra

homogenizer do not guarantee by themselves the sterilization and, subsequently, packaging of foods in aseptic conditions is needed. This results in a great disadvantage for food industry because it limits the implementation of this technology in food process as full alternative to thermal treatment.

AUTHOR CONTRIBUTIONS

FP and RL put in writing this review together consulting the available literature, patents on the topic and trying to investigate several aspects on high pressure homogenization and safety.

REFERENCES

Amador Espejo, G. G., Hernandez-Herrero, M. M., Juan, B., and Trujillo, A. J. (2014). Inactivation of Bacillus spores inoculated in milk by Ultra High Pressure Homogenization. *Food Microbiol* 44, 204–210.

Bernkop-Schnurch, A., Krist, S., Vehabovic, M., and Valenta, C. (1998). Synthesis and evaluation of lysozyme derivates exhibiting an enhanced antimicrobial action. *Euro. J. Pharm. Sci.* 6, 301–306. doi: 10.1016/S0928-0987(97)10026-4

Betoret, E., Betoret, N., Rocculi, P., and Dalla Rosa, M. (2015). Strategies to improve food functionality: structure-property relationships on high pressures homogenization, vacuum impregnation and drying technologies. *Trends Food Sci. Technol.* 46, 1–12. doi: 10.1016/j.tifs.2015.07.006

Bevilacqua, A., Cibelli, F., Corbo, M. R., and Sinigaglia, M. (2007). Effects of high-pressure homogenization on the survival of *Alicyclobacillus acidoterrestris* in a laboratory medium. *Lett. App. Microbiol.* 45, 382–386. doi: 10.1111/j.1472-765X.2007.02219.x

Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2012). High-pressure homogenisation and benzoate to control *Alicyclobacillus acidoterrestris*: a possible way? *Int. J. Food Sci. Technol.* 47, 879–883. doi: 10.1111/j.1365-2621.2011.02913.x

Bevilacqua, A., Costa, C., Corbo, M. R., and Sinigaglia, M. (2009). Effects of the high pressure of homogenization on some spoiling microorganisms, representative of fruit juice microflora, inoculated in saline solution. *Lett. Appl. Microbiol.* 48, 261–267. doi: 10.1111/j.1472-765X.2008.02527.x

Brinez, W. J., Roig-Sagues, A. X., Herrero, M. H., and Lopez, B. G. (2006a). Inactivation by ultra high-pressure homogenization of *Escherichia coli* strains inoculated into orange juice. *J. Food Prot.* 69, 984–989.

Brinez, W. J., Roig-Sagues, A. X., Herrero, M. H., and Lopez, B. G. (2006b). Inactivation of Listeria innocua in milk and orange juice by ultrahigh-pressure homogenization. *J. Food Protect.* 69, 86–92.

Brinez, W. J., Roig-Sagues, A. X., Herrero, M. H., and Lopez, B. G. (2007). Inactivation of *Staphylococcus* spp. strains in whole milk and orange juice using ultra high pressure homogenisation at inlet temperatures of 6 and 20 degrees °C. *Food Control* 10, 1282–1288. doi: 10.1016/j.foodcont.2006.09.002

Burns, B., Patrignani, F., Serrazanetti, D., Vinderola, G. C., Reinheimer, J. A., Lanciotti, R., et al. (2008). Probiotic crescenza cheese containing *Lactobacillus casei* and *Lactobacillus acidophilus* manufactured with high pressure-homogenized milk. *J. Dairy Sci.* 91, 500–512. doi: 10.3168/jds.2007-0516

Burns, P., Patrignani, F., Tabanelli, G., Vinderola, G. C., Siroli, L., Reinheimer, J. A., et al. (2015). Potential of high pressure homogenisation on probiotic Caciotta cheese quality and functionality. *J. Funct. Foods* 13, 126–136. doi: 10.1016/j.jff.2014.12.037

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods. *Rev. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Chaves-López, C., Lanciotti, R., Serio, A., Paparella, A., Guerzoni, M. E., and Suzzi, G. (2009). Effect of high pressure homogenization applied individually or in combination with other mild physical or chemical stresses on Bacillus cereus and *Bacillus subtilis* spore viability. *Food Control* 20, 691–695. doi: 10.1016/j.foodcont.2008.09.001

Chen, W., Harte, F. M., Davidson, P. M., and Golden, D. A. (2013). Inactivation of *Alicyclobacillus acidoterrestris* using high pressure homogenization and dimethyl dicarbonate. *J. Food Protect.* 76, 1041–1045. doi: 10.4315/0362-028X.JFP-12-443

Cortezzo, D. E., Setlow, B., and Setlow, P. (2004). Analysis of the action of compounds that inhibit the germination of spores of *Bacillus* species. *J. Appl. Microbiol.* 96, 725–741. doi: 10.1111/j.1365-2672.2004.02196.x

Diels, A. M., and Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of microorganisms. *Crit. Rev. Microbiol.* 32, 201–216. doi: 10.1080/10408410601023516

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., and Michiels, C. W. (2004). Moderate temperatures affect *Escherichia coli* inactivation by high-pressure homogenization only through fluid viscosity. *Biotechnol. Prog.* 20, 1512–1517. doi: 10.1021/bp0499092

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., and Michiels, C. W. (2005). Inactivation of *Escherichia coli* by high-pressure homogenisation is influenced by fluid viscosity but not by water activity and product composition. *Int. J. Food Microbiol.* 101, 281–291. doi: 10.1016/j.ijfoodmicro.2004.11.011

Diels, A. M. J., Wuytack, E. Y., and Michiels, C. W. (2003). Modelling inactivation of *Staphylococcus aureus* and *Yersinia enterocolitica* by high-pressure homogenisation at different temperatures. *Int. J. Food Microbiol.* 87, 55–62. doi: 10.1016/S0168-1605(03)00050-3

Dong, P., Georget, E. S., Aganovic, K., Heinz, V., and Mathys, A. (2015). Ultra high pressure homogenization (UHPH) inactivation of *Bacillus amyloliquefaciens* spores in phosphate buffered saline (PBS) and milk. *Front. Microbiol.* 6:712. doi: 10.3389/fmicb.2015.00712

Donsì, F., Ferrari, G., and Maresca, P. (2009a). "High-pressure homogenisation for food sanitisation," in *Global Issues in Food Science and Technology,* eds G. Barbosa-Cànovas, A. Mortimer, D. Lineback, W. Spiess, K. Buckle, and P. Colonna (Cambridge, MA: Academic Press), 309–352.

Donsì, F., Ferrari, G., and Maresca, P. (2009b). Main factors regulating microbial inactivation by high-pressure homogenization: operating parameters and scale of operation. *Chem. Eng. Sci.* 64, 520–532. doi: 10.1016/j.ces.2008.10.002

Donsì, F., Esposito, L., Lenza, E., Senatore, B., and Ferrari, G. (2009c). Production of shelf-stable annurca apple juice with pulp by high pressure homogenization. *Int. J. Food Eng.* 5:12. doi: 10.2202/1556-3758.1602

Dumay, E., Chevalier-Lucia, D., Picart-Palmade, L., Benzaria, A., Gràcia-Julià, A., and Blayo, C. (2013). Technological aspects and potential applications of (ultra) high-pressure homogenisation. *Trends Food Sci. Technol.* 31, 13–26. doi: 10.1016/j.tifs.2012.03.005

Ferragut, M., Hernández-Herrero, M., Veciana-Nogués, T., Borras-Suarez, M., González-Linares, J., Vidal-Caroub, M. C., et al. (2015). Ultra-high-pressure homogenization UHPH system for producing high-quality vegetable-based beverages: physicochemical, microbiological, nutritional and toxicological characteristics. *J. Sci. Food Agric.* 95, 953–961. doi: 10.1002/jsfa.6769

Floury, J., Bellettre, J., Legrand, J., and Desrumaux, A. (2004). Analysis of a new type of homogenizer. A study of the flown pattern. *Chem. Eng. Sci.* 59, 843–853. doi: 10.1016/j.ces.2003.11.017

Floury, J., Desrumaux, A., and Lardières, J. (2000). Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. *Innov. Food Sci. Emerg* 1, 127–134. doi: 10.1016/S1466-8564(00)00012-6

Gaulin A. (1899). *System for Intimately Mixing Milk*. US 756953.

Georget, E., Miller, B., Aganovic, K., Callanan, M., Heinz, V., and Mathys, A. (2014a). Bacterial spore inactivation by ultra-high pressure homogenization. *Innov. Food Sci. Emerg. Technol.* 26, 116–123. doi: 10.1016/j.ifset.2014.08.004

Georget, E., Miller, B., Callanan, M., Heinz, V., and Mathys, A. (2014b). (Ultra) high pressure homogenization for continuous high pressure sterilization of pumpable foods-a review. *Front. Nutr.* 1:15. doi: 10.3389/fnut.2014.00015

Guerzoni, M. E., Lanciotti, R., Westall, F., and Pittia, P. (1997). Interrelationship between chemico-physical variables, microstructure and growth of *Listeria monocytogenes* and *Yarrowia lipolytica* in food model systems. *Sci. Aliment.* 17, 497–512.

Guerzoni, M. E., Vannini, L., Chaves Lopez, C., Lanciotti, R., Suzzi, G., and Gianotti, A. (1999). Effect of high pressure homogenization on microbial and

chemico-physical characteristics of goat cheeses. *J. Dairy Sci.* 82, 851–862. doi: 10.3168/jds.S0022-0302(99)75303-8

Guerzoni, M. E., Vannini, L., Lanciotti, R., and Gardini, F. (2002). Optimisation of the formulation and of the technological process of egg-based products for the prevention of *Salmonella enteritidis* survival and growth. *Int. J. Food Microbiol.* 73, 367–374. doi: 10.1016/S0168-1605(01)00673-0

Hayes, M. G., Fox, P. F., and Kelly, A. L. (2005). Potential applications of high pressure homogenisation in processing of liquid milk. *J. Dairy Res.* 72, 25–33. doi: 10.1017/S0022029904000524

Hayes, M. G., and Kelly, A. L. (2003). High pressure homogenisation of raw whole bovine milk (a) effects on fat globules size and other properties. *J. Dairy Res.* 70, 297–305. doi: 10.1017/S0022029903006320

Ibrahim, H. R., Kato, A., and Kobayashi, K. (1993). Length of hydrocarbon chain and antimicrobial action to gram-negative of fatty acylated lysozyme. *J. Agric. Food Chem.* 41, 1164–1188. doi: 10.1021/jf00031a029

Iucci, L., Lanciotti, R., Huppertz, T., and Kelly, A. (2008). Plasmin activity in high-pressure-homogenised bovine milk. *Milchwissenschaft* 63, 68–70

Iucci, L., Patrignani, F., Vallicelli, M., Guerzoni, M. E., and Lanciotti, R. (2007). Effects of high pressure homogenization on the activity of lysozyme and lactoferrin against *Listeria monocytogenes*. *Food Control* 18, 558–565. doi: 10.1016/j.foodcont.2006.01.005

Keshavarz Moore, E., Hoare, M., and Dunnill, P. (1990). Disruption of baker's yeast in a high–pressure homogenizer: new evidence on mechanism. *Enzyme Microb. Technol.* 12, 764–770. doi: 10.1016/0141-0229(90)90149-K

Kheadr, E. E., Vachon, J. F., Paquin, P., and Fliss, I. (2002). Effect of dynamic pressure on microbiological, rheological and microstructural quality of Cheddar cheese. *Int. Dairy J.* 12, 435–446. doi: 10.1016/S0958-6946(01)00104-2

Kumar, S., Thippareddi, H., Subbiah, J., Zivanovic, S., Davidson, P. M., and Harte, F. (2009). Inactivation of *Escherichia coli* K-12 in apple juice using combination of high-pressure homogenization and chitosan. *J. Food Sci.* 74, M8–M14. doi: 10.1111/j.1750-3841.2008.00974.x

Lanciotti, R., Chaves-Lopez, C., Patrignani, F., Paparella, A., Guerzoni, M. E., Serio, A., et al. (2004b). Effects of milk treatment with dynamic high pressure on microbial population as well as on the lipolytic and proteolytic profiles of Crescenza cheese. *Int. J. Dairy Technol.* 57, 19–25. doi: 10.1111/j.1471-0307.2004.00121.x

Lanciotti, R., Gardini, F., Sinigaglia, M., and Guerzoni, M. E. (1996). Effects of growth conditions on the resistance of some pathogenic and spoilage species to high pressure homogenization. *Lett. Appl. Microbiol.* 22, 165–168. doi: 10.1111/j.1472-765X.1996.tb01134.x

Lanciotti, R., Patrignani, F., Iucci, L., Guerzoni, M. E., Suzzi, G., Belletti, N., et al. (2007a). Effects of milk high pressure homogenization on biogenic amine accumulation during ripening of ovine and bovine Italian cheeses. *Food Chem.* 104, 693–701. doi: 10.1016/j.foodchem.2006.12.017

Lanciotti, R., Patrignani, F., Iucci, L., Saracino, P., and Guerzoni, M. E. (2007b). Potential of high pressure homogenization in the control and enhancement of proteolytic and fermentative activities of some Lactobacillus species. *Food Chem.* 102, 542–550. doi: 10.1016/j.foodchem.2006.06.043

Lanciotti, R., Sinigaglia, M., Angelini, P., and Guerzoni, M. E. (1994). Effects of homogenization pressure on the survival and growth of some food spoilage and pathogenic micro-organisms. *Lett. Appl. Microbiol.* 18, 319–322. doi: 10.1111/j.1472-765X.1994.tb00878.x

Lanciotti, R., Vannini, L., Patrignani, F., Iucci, L., Vallicelli, M., Ndagijimana, M., et al. (2006). Effect of high pressure homogenisation of milk on cheese yield and microbiology, lipolysis and proteolysis during ripening of Caciotta cheese. *J. Dairy Res* 73, 216–226. doi: 10.1017/S0022029905001640

Lanciotti, R., Vannini, L., Pittia, P., and Guerzoni, M. E. (2004a). Suitability of high dynamic-pressure-treated milk for the production of yogurt. *Food Microbiol.* 21, 753–760. doi: 10.1016/j.fm.2004.01.014

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *Int. J. Food Microbiol.* 55, 181–186. doi: 10.1016/S0168-1605(00)00161-6

López-Pedemonte, T., Brinez, W. J., Roig-Sagues, A. X., and Guamis, B. (2006). Fate of *Staphylococcus aureus* in cheese treated by ultrahigh pressure homogenization and high hydrostatic pressure. *J. Dairy Sci.* 89, 4536–4544. doi: 10.3168/jds.S0022-0302(06)72502-4

Maresca, P., Donsì, F., and Ferrari, G. (2011). Application of a multi-pass high-pressure homogenization treatment for the pasteurization of fruit juices. *J. Food Eng.* 104, 364–372. doi: 10.1016/j.jfoodeng.2010.12.030

Muramalla, T., and Aryana, K. J. (2011). Some low homogenization pressures improve certain probiotic characteristics of yogurt culture bacteria and *Lactobacillus acidophilus* LA-K. *J. Dairy Sci* 94, 3725–3738. doi: 10.3168/jds.2010-3737

Navarro, J. L., Izquierdo, L., Carbonell, J. V., and Sentandreu, E. (2014). Effect of pH, temperature and maturity on pectinmethylesterase inactivation of citrus juices treated by high-pressure homogenization. *LWT-Food Sci. Technol.* 57, 785–788. doi: 10.1016/j.lwt.2014.01.033

Panozzo, A., Manzocco, L., Calligaris, S., Bartolomeoli, I., Maifreni, M., Lippe, G., et al. (2014). Effect of high pressure homogenisation on microbial inactivation, protein structure and functionality of egg white. *Food Res. Int.* 62, 718–725. doi: 10.1016/j.foodres.2014.04.051

Pathanibul, P., Taylor, T. M., Davidson, P. M., and Harte, F. (2009). Inactivation of *Escherichia coli* and *Listeria innocua* in apple and carrot juices using high pressure homogenization and nisin. *Int. J. Food Microbiol.* 129, 316–320. doi: 10.1016/j.ijfoodmicro.2008.12.020

Patrignani, F., Burns, P., Serrazanetti, D., Vinderola, G., Reinheimer, J., Lanciotti, R., et al. (2009b). Suitability of high pressure-homogenized milk for the production of probiotic fermented milk containing *Lactobacillus paracasei* and *Lactobacillus acidophilus*. *J. Dairy Res.* 76, 74–82. doi: 10.1017/S0022029908003828

Patrignani, F., Serrazanetti, D. I., Maina Mathara, J., Siroli, L., Gardini, F., Holzapfel, W. H., et al. (2015a). Use of homogenisation pressure to improve quality and functionality of probiotic fermented milks containing Lactobacillus rhamnosus BFE 5264. *Int. J. Daity Technol.* 60, 1–10. doi: 10.1111/1471-0307.12251

Patrignani, F., Siroli, L., Serrazanetti, D. I., Gardini, F., and Lanciotti, R. (2015b). Innovative strategies based on the use of essential oils and their components to improve safety, shelf-life and quality of minimally processed fruits and vegetables. *Trends Food Sci. Technol.* 46, 311–319. doi: 10.1016/j.tifs.2015.03.009

Patrignani, F., Tabanelli, G., Siroli, L., Gardini, F., and Lanciotti, R. (2013a). Combined effects of high pressure homogenization treatment and citral on microbiological quality of apricot juice. *Int. J. Food Microbiol.* 160, 273–281. doi: 10.1016/j.ijfoodmicro.2012.10.021

Patrignani, F., Vannini, L., Sado Kamdem, S. L., Hernando, I., Marco-Molés, R., Guerzoni, M. E., et al. (2013b). High pressure homogenization vs heat treatment: safety andfunctional properties of liquid whole egg. *Food Microbiol* 36, 63–69. doi: 10.1016/j.fm.2013.04.004

Patrignani, F., Vannini, L., Sado Kamdem, S. L., Lanciotti, R., and Guerzoni, M. E. (2009a). Effect of high pressure homogenization on *Saccharomyces cerevisiae* inactivation and physico-chemical features in apricot and carrot juices. *Int. J. Food Microbiol.* 136, 26–31. doi: 10.1016/j.ijfoodmicro.2009.09.021

Patrignani, F., Vannini, L., Sado Kamdem, S. L., Lanciotti, R., and Guerzoni, M. E. (2010). Potentialities of high-pressure homogenization to inactivate *Zygosaccharomyces bailii* in fruit juices. *J. Food Sci.* 75, M116. doi: 10.1111/j.1750-3841.2009.01508.x

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., and Trujillo, A. J. (2009). Heat damage evaluation in ultra-high pressure homogenized milk. *Food Hydrocoll.* 23, 1974–1979. doi: 10.1016/j.foodhyd.2009.02.010

Pinho, C. R. G., Franchi, M. A., Tribstm, A. A. L., and Cristianini, M. (2011). Effect of ultra high pressure homogenization on alkaline phosphatise and lactoperoxidase activity in raw skim milk. *Procedia Food Sci.* 1, 874–878. doi: 10.1016/j.profoo.2011.09.131

Reineke, K., Mathys, A., Heinzm, V., and Knorr, D. (2013). Mechanisms of endospore inactivation under high pressure. *Trends Microbiol.* 21, 296–304. doi: 10.1016/j.tim.2013.03.001

Roig-Sagues, A. X., Asto, E., Engers, I., and Hernandez-Herrero, M. M. (2015). Improving the efficiency of ultra-high pressure homogenization treatments to inactivate spores of *Alicyclobacillus* spp. in orange juice controlling the inlet temperature. *LWT-Food Sci. Technol.* 63, 866–871. doi: 10.1016/j.lwt.2015.04.056

Roig-Sagues, A. X., Velazques, R. M., Montealegre-Agramount, P., et al. (2009). Fat content increases of ultra-high pressure homogenization on *Listeria monocytogenes* in milk. *J. Dairy Sci.* 92, 5396–5402.

Ross, A. I. V., Griffiths, M. W., Mittal, G. S., and Deeth, H. C. (2003). Combining nonthermal technologies to control foodborne microorganisms. *Int. J. Food Microbiol.* 89, 125–138. doi: 10.1016/S0168-1605(03)00161-2

Sandra, S., and Dalgleish, D. G. (2005). Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder. *Int. Dairy J.* 15, 1095–1104. doi: 10.1016/j.idairyj.2004.11.015

Schubert, W. W., and Beaudet, R. A. (2011). Determination of lethality rate constants and D-values for heat-resistant *Bacillus spores* ATCC 29669 exposed to dry heat from 125°C to 200°C. *Astrobiol.* 11, 1–11. doi: 10.1089/ast.2010.0502

Setlow, P. (2000). "Resistance of bacterial spores," in *Bacterial Stress Responses*, eds G. Storz and R. Hengge-Aronis (Washington, DC: American Society for Microbiology), 217–230.

Shirgaonkar, I. Z. S., Lothe, R. R., and Pandit, A. B. (1998). Commentson the mechanism of microbial cell disruption in high-pressure and high-speed devices. *Biotechnol. Prog.* 14, 657–660. doi: 10.1021/bp980052g

Siroli, L., Patrignani, F., Serrazanetti, D. I., Gardini, F., and Lanciotti, R. (2015). Innovative strategies based on the use of bio-control agents to improve the safety, shelf-life and quality of minimally processed fruits and vegetables. *Trends Food Sci Technol.* 46, 302–310. doi: 10.1016/j.tifs.2015.04.014

Tabanelli, G., Burns, P., Patrignani, F., Gardini, F., Lanciotti, R., Reinheimer, J., et al. (2012). Effect of a non-lethal high pressure homogenization treatment on the in vivo response of probiotic lactobacilli. *Food Microbiol.* 32, 302–307. doi: 10.1016/j.fm.2012.07.004

Tabanelli, G., Patrignani, F., Gardini, F., Vinderola, C. G., Reinheimer, J. A., Grazia, L., et al. (2014). Effect of a sub-lethal high pressure homogenization treatment on the fatty acid membrane composition of probiotic lactobacilli. *Lett. Appl. Microbiol.* 58, 109–117. doi: 10.1111/lam.12164

Tabanelli, G., Patrignani, F., Vinderola, G. C., Reinheimer, J. A., Gardini, F., and Lanciotti, R. (2013). Effect of sub-lethal high pressure homogenization treatments on in vitro functional and biological properties of lactic acid bacteria. *Food Sci. Technol* 53, 580–586.

Tahiri, I., Makhlouf, J., Paquin, P., and Fliss, I. (2006). Inactivation of food spoilage bacteria and *Escherichia coli* O157:H7 in phosphate buffer and orange juice using dynamic high pressure. *Food Res. Int.* 39, 98–105. doi: 10.1016/j.foodres.2005.06.005

Tapia de Daza, M. S., Alzamora, S. M., and Chanes, J. W. (1996). Combination of preservation factors applied to minimal processing of foods. *Crit. Rev. Food Sci. Nutr.* 36, 629–659. doi: 10.1080/10408399609527742

Taylor, T. M., Roach, A., Black, D. G., Davidson, P. M., and Harte, F. (2007). Inactivation of *Escherichia coli* K-12 exposed to pressures in excess of 300 mpa in a high-pressure homogenizer. *J. Food Prot.* 70, 1007–1010.

Vachon, J. F., Kheadr, E. E., Giasson, J., Paquin, P., and Fliss, I. (2002). Inactivation of foodborne pathogens in milk using dynamic high pressure. *J. Food Prot.* 65, 345–352.

Vannini, L., Lanciotti, R., Baldi, D., and Guerzoni, M. E. (2004). Interactions between high pressure homogenization and antimicrobial activity of lizozyme and lactoperoxidase. *Int. J. Food Microbiol.* 94, 123–135. doi: 10.1016/j.ijfoodmicro.2004.01.005

Vannini, L., Patrignani, F., Iucci, L., Ndagijimana, M., Lanciotti, R., vallicelli, M., et al. (2008). Effect of a pre-treatment of milk with high pressure homogenization on yield as well as on microbiological, lipolytic and proteolytic patterns of "Pecorino" cheese. *Int. J. Food Microbiol.* 128, 329–335.

Velazquez-Estrada, R. M., Hernandez-Herrero, M. M., Lopez-Pedemonte, T., Guamis-Lopez, B., and Roig Sagues, A. X. (2008). Inactivation of *Salmonella enterica* serovar senftenberg 775W in liquid whole egg by ultrahigh pressure homogenization. *J. Food Prot.* 71, 2283–2288.

Wuytack, E. Y., Diels, A. J., and Michiels, C. W. (2002). Bacterial inactivation by high pressure homogenization and high hydrostatic pressure. *Int. J. Food Microbiol.* 77, 205–212. doi: 10.1016/S0168-1605(02)00054-5

Zamora, A., and Guamis, B. (2015). Opportunities for ultra-high-pressure homogenisation (UHPH) for the food industry. *Food. Eng. Rev.* 7, 130–142. doi: 10.1007/s12393-014-9097-4

Zhao, L., Wang, Y., Wang, S., Li, H., Huang, W., and Liao, X. (2014). Inactivation of naturally occurring microbiota in cucumber juice by pressure treatment. *Int. J. Food Microbiol.* 174, 12–18. doi: 10.1016/j.ijfoodmicro.2013.12.023

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 July 2016
doi: 10.3389/fmicb.2016.01124

Detection of *Cronobacter* Genus in Powdered Infant Formula by Enzyme-linked Immunosorbent Assay Using Anti-*Cronobacter* Antibody

Xinjie Song[1†], Shruti Shukla[1†], Gibaek Lee[1], Sunhyun Park[2] and Myunghee Kim[1]*

[1] Department of Food Science and Technology, Yeungnam University, Gyeongsan, South Korea, [2] Food Standard Research Center, Korea Food Research Institute, Seongnam, South Korea

Cronobacter species (*Cronobacter* spp.) are hazardous foodborne pathogens associated with baby food, powdered infant formula (PIF). To develop a rapid and sensitive method for simultaneous detection of seven *Cronobacter* spp. in PIF, an indirect non-competitive enzyme-linked immunosorbent assay (INC-ELISA) was developed based on a novel immunoglobulin G (IgG), anti-*Cronobacter* IgG. The developed INC-ELISA was able to detect seven *Cronobacter* spp. at concentrations ranging from $(5.6 \pm 0.30) \times 10^3$ to $(2.1 \pm 0.01) \times 10^5$ colony forming unit (CFU)/mL in pure culture. Further, INC-ELISA employing anti-*Cronobacter* IgG was applicable for analysis of PIF samples contaminated with less than <10 cells of *Cronobacter* spp. per 25 g of PIF in 36 h. The developed antibody showed slight cross-reactivity with *Franconibacter pulveris* (LMG 24057) at high concentration (10^8 CFU/mL). The INC-ELISA method displayed excellent specificity without compromising cross-reactivity with other foodborne pathogens. The INC-ELISA assay method developed in this study using a novel anti-*Cronobacter* IgG facilitated highly sensitive, efficient, and rapid detection of *Cronobacter* spp. in baby food.

Keywords: *Cronobacter* species, immunoglobulin G, indirect non-competitive enzyme-linked immunosorbent assay, genus-specificity, powdered infant formula

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Zhao Chen, Clemson University, USA
Gonçalo Nieto Almeida, Catholic University of Portugal, Portugal

***Correspondence:**
Myunghee Kim
foodtech@ynu.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: *26 April 2016*
Accepted: *06 July 2016*
Published: *21 July 2016*

Citation:
Song X, Shukla S, Lee G, Park S and Kim M (2016) Detection of Cronobacter Genus in Powdered Infant Formula by Enzyme-linked Immunosorbent Assay Using Anti-Cronobacter Antibody. Front. Microbiol. 7:1124. doi: 10.3389/fmicb.2016.01124

INTRODUCTION

Bacteria of the genus *Enterobacter* are increasingly important human pathogens that cause neonatal meningitis and sepsis (Hunter and Bean, 2013). Enterobacterial infections have become a worldwide health problem partially due to the lack of development of preventive treatments and the emergence of new multi-drug resistant strains. *Enterobacter sakazakii* has recently been reclassified as a *Cronobacter* (Strydom et al., 2012). *Cronobacter* species (*Cronobacter* spp.), previously known as *E. sakazakii*, are foodborne pathogens that pose a high risk of infection to neonates as well as immuno-compromised individuals (World Health Organization [WHO], 2007). *Cronobacter* spp. affect the central nervous system of infants, and survivors often suffer from severe neurological impairments such as hydrocephalus, quadriplegia, and developmental delays (Lai, 2001; Strydom et al., 2012; Jaradat et al., 2014). Species level classification of the *Cronobacter* genus, which includes *C. sakazakii*, *C. muytjensii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. universalis*, and *C. condimenti*, was recently defined by Iversen et al. (2008)

and Joseph et al. (2012b). *Cronobacter* spp. have been isolated from plant-based food products, including cereal, fruits, vegetables, legumes, herbs, and spices, as well as from animal-based food materials such as milk, meat, and fish (Friedemann, 2007; Lee et al., 2012).

All *Cronobacter* spp., except *C. condimenti*, have been associated with human infections (Cruz-Córdova et al., 2012). Historically, *Cronobacter* spp. have been implicated in newborn and infant infections, causing meningitis, necrotizing enterocolitis, and bacteremia (Healy et al., 2010). However, not all *Cronobacter* spp. are linked to infantile infections, and it is thought that virulence among strains may vary. *C. sakazakii*, *C. malonaticus,* and *C. turicensis* are most often isolated from infantile cases (Joseph and Forsythe, 2011). Recent research based on international microbiological standards suggested that all species of *Cronobacter* must be absent in 10 grams of powdered infant formula (PIF) (Odeyemi and Sani, 2016). For an understanding of recent unresolved issues persisting with respect to taxonomy, sources and clinical relevance, and for suggestions on how to safely feed premature neonates (Holy and Forsythe, 2014), it is plausible that virulence determinants have evolved in certain lineages (Joseph et al., 2012a).

Although reservoirs of *Cronobacter* spp. and their modes of transmission are still unknown, *Cronobacter* spp. have been suggested as a source of food contamination, with rodents and flies serving as a secondary route of contamination (Jung and Park, 2006; World Health Organization [WHO], 2007). According to a report by Jung and Park (2006), 20% of PIF samples were found contaminated with *Cronobacter* in the Republic of Korea. Lee et al. (2012) also reported similar results in which *Cronobacter* spp. were isolated from 18.6% of detected food samples. Although a number of reported cases of *Cronobacter* infection are quite low, sequelae can occur with high mortality rates (Lai, 2001; United States Food and Drug Administration [USFDA], 2002; Friedemann, 2007). World Health Organization [WHO] (2007), classified *Cronobacter* together with *Salmonella* as group A pathogens associated with PIF with clear evidence of illness in infants. These categories of organisms were based on their risk of illness to infants.

Culture-dependent isolation and assay methods for complete analysis of *Cronobacter* spp. from PIF usually require 5 to 7 days (United States Food and Drug Administration [USFDA], 2002). A further method was recommended by the International Organization for Standardization and the International Dairy Federation as ISO 22964 (Anonymous, 2006). This method includes a pre-enrichment in buffered peptone water (BPW), a selective enrichment in modified lauryl sulfate tryptose broth containing vancomycin and isolation of presumptive *C. sakazakii* colonies colored in blue-green on *E. sakazakii* isolation agar. These colonies should be streaked on tryptic soy agar and resulting yellow colonies are indicatory for *C. sakazakii*. However, this method was also time- and labor-consuming, as it usually requires 5 to 6 days to obtain a positive result (Norberg et al., 2012).

Lampel and Chen (2009) previously described a new method for the isolation and detection of *Cronobacter* spp. from PIF using a real-time PCR-based assay and chromogenic agar. In their study, suspended cells were isolated from enrichment culture, streaked onto chromogenic agar, and confirmed by real-time PCR assay. Mullane et al. (2006) also developed a method using cationic-magnetic beads to capture *Cronobacter* spp., and subsequent identification was performed after washing off bound cells from the capture phase and plating them onto Druggan–Forsythe–Iversen formulation agar to detect 1 to 5 colony forming unit (CFU)/500 g of PIF within 24 h. However, these PCR-based methods have significant technical requirements of ultra-pure reagents and chemicals along with high equipment costs. Therefore, a rapid, sensitive, and inexpensive method is needed for the detection of *Cronobacter* spp.

Polyclonal antibody can be obtained within a short time (4 to 8 weeks) with minimal financial investment, whereas it takes about 3 to 6 months to produce monoclonal antibodies (Leenaars and Hendriksen, 2005). Polyclonal antibody is commonly used in immunological methods, including enzyme-linked immunosorbent assay (ELISA), for the detection of foodborne pathogens (Brigmon et al., 1992; Kumar et al., 2008; Velusamy et al., 2010). It is also critical for developing a rapid and genus-specific method for identification of *Cronobacter* spp. in the context of food safety issues. Previously, a sandwich ELISA method was developed for the detection of *C. muytjensii* with a detection limit of 6.3×10^4 CFU/mL using a species-specific anti-*C. muytjensii* immunoglobulin G (IgG) (Park et al., 2012). In addition, Xu et al. (2014) also developed a polyclonal and monoclonal antibodies-based indirect ELISA and a sandwich ELISA for the detection of *Cronobacter* spp. The indirect ELISA detected all species of *Cronobacter* assayed, and the limit of detection (LOD) was established as 10^5 CFU/mL. In contrast, sandwich ELISA was specific for *C. sakazakii* and exhibited greater sensitivity than indirect ELISA (LOD of 2×10^4 CFU/mL). Following 10 h of enrichment, *Cronobacter* spp. were detected using either of the two analytical methods in samples inoculated with 1 CFU/100 g PIF. The results from this study demonstrated that both of these novel ELISAs were specific, sensitive, and rapid assays for the screening of pathogenic *Cronobacter* spp. in PIF.

A widely available polyclonal antibody capable of detecting *Cronobacter* spp. could be used to avoid expensive and time-consuming methods. Thus, we decided to produce and characterize a polyclonal antibody for seven *Cronobacter* spp. To our knowledge, there has been no report on the ELISA-based detection of multiple *Cronobacter* spp. In the current work, an indirect non-competitive ELISA (INC-ELISA) method based on anti-*Cronobacter* IgG was developed in pure culture and applied for the detection of seven *Cronobacter* spp. in PIF.

MATERIALS AND METHODS

Strains and Reagents

In the present study, seven *Cronobacter* strains were used to produce antibody and to assess the cross-reactivity of developed method, while eight other bacterial strains belonging to different genera were selected to check the cross-reactivity

of developed assay due to their partial gene sequence similarity and to confirm the accurate detection of pathogen contaminants such as *Salmonella* spp., *Citrobacter* spp., and *Bacillus cereus* (Iversen et al., 2004; World Health Organization [WHO], 2007; Pinto et al., 2013). In the present study, *B. cereus* and *S.* Enteritidis were selected since it was found that the chances of contamination in PIF were highly concerned due to the presence of *B. cereus* and *S.* Enteritidis (Giammanco et al., 2011; Pinto et al., 2013). Giammanco et al. (2011) also reported that *Salmonella* spp. and *Cronobacter* spp. (formerly *E. sakazakii*) are the microorganisms of greatest concern in PIF. In addition, regarding the use of *Citrobacter freundii*, Giammanco et al. (2011) observed that molecular epidemiological survey of *C. freundii* was misidentified as *Cronobacter* spp. isolated from PIF. The phylogenetic tree also showed that *C. freundii* and *Cronobacter* spp. are very closely related species based on their smaller amounts of mahalanobis distances than other species. Hence, it was suggested that *C. freundii* may also be an under-reported cause of bacterial infection, especially in high risk neonates, due to misidentification. *B. cereus* (KCCM 40935), *Buttiauxella noackiae* (ATCC 51713), *C. condimenti* (LMG 26250), *C. dublinensis* (LMG 23823), *C. malonaticus* (LMG 23826), *C. muytjensii* (CDC 3523-75), *C. turicensis* (LMG 23827), *C. muytjensii* (ATCC 51329), *C. sakazakii* (ATCC 29544), *C. sakazakii* (ATCC 29004), *C. universalis* (LMG 26249), *C. freundi* (ATCC 8090), *Escherichia coli* (ATCC 39418), *Franconibacter helveticus* (LMG 23732), *Franconibacter pulveris* (LMG 24057), and *Salmonella* Typhimurium (ATCC 13311) were used in this study. *C. muytjensii* (CDC 3523–75) was donated by Dr. Carol Iversen from University College Dublin, Ireland. Other strains used in this study were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). Strains indicated with LMG were purchased from the Belgian Coordinated Collections of Microorganisms (BCCM; Gent, Belgium). All strains were cultured in nutrient broth (NB) for 18 h at 37°C in a shaking incubator (150 rpm).

NB, nutrient agar (NA), peptone, and skim milk were purchased from Difco (Franklin Lakes, NJ, USA). *Enterobacteriaceae* enrichment (EE) broth and violet red bile glucose (VRBG) agar were purchased from MB cell (Seoul, Korea). Sodium carbonate, sodium bicarbonate, sodium azide, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, caprylic acid, ammonium sulfate, and alkaline phosphatase yellow liquid substrate *p*-nitrophenyl phosphate (pNPP) were purchased from Sigma (St. Louis, MO, USA). Phosphatase-labeled goat anti-rabbit IgG was purchased from Kierkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). Lipopolysaccharide (LPS) extraction kit was purchased from Intron Biotechnology (Seongnam, Korea).

Animal Care Ethics

Animal use protocol was reviewed by the committee members of Yeungnam University and approved by Korea Food and Drug Administration, Republic of Korea (*Animal Ethics License No. 2013-012 and 2012-010*).

Preparation of Immunogen and Immunization

Three types of immunogens were prepared for the development of a genus-specific antibody against *Cronobacter* spp. A sonicated cell protein (SCP) mixture of seven *Cronobacter* spp. was used in this study. The seven *Cronobacter* spp. were separately cultured in NB at 37°C for 18 h to a concentration of 1×10^9 CFU/mL. Each culture (30 mL) was centrifuged at 3,000 × *g* at 4°C for 30 min. The pellets were washed three times with 30 mL of 0.01 M phosphate-buffered saline (PBS) and then suspended in 10 mL of 0.01 M PBS for sonication under 20 KHz power for 5 min on ice. Supernatant was collected after centrifugation at 12,000 × *g*, 4°C for 20 min. Protein concentrations of supernatants were checked with a Bradford kit, after which SCP solutions were adjusted to a concentration of 0.5 mg/L with 0.01 M PBS. SCP solutions were stored at −20°C before use. For immunization, equal volumes of the seven SCP solutions were mixed for use as an immunogen in New Zealand white rabbits.

LPS mixture of the seven *Cronobacter* spp. was prepared using an LPS extraction kit following the instructions of the manufacturer. Each *Cronobacter* spp. culture was centrifuged at 10,000 × *g* at room temperature for 20 min to harvest bacterial cells. Lysis buffer was then added and vortexed vigorously, followed by addition of chloroform, vortexing for 20 s, and incubated at room temperature for 5 min. The mixture was then centrifuged at 10,000 × *g* for 10 min at 4°C. An aliquot of supernatant was transferred to a new tube, mixed well with purification buffer, and kept at −20°C for 10 min. The solution was then centrifuged at 10,000 × *g* for 15 min at 4°C, after which the upper layer was removed to obtain an LPS pellet. The pellet was washed three times with 1 mL of ethanol, centrifuged for 3 min at 10,000 × *g* at 4°C, and then collected and dried at room temperature. For the LPS solution, 50 μL of Tris-HCl buffer (10 mM, pH 8.0) was used to dissolve the LPS pellet. LPS solution of the seven *Cronobacter* spp. was prepared by mixing an equal volume of each LPS solution for use as an immunogen in New Zealand white rabbits.

Formalin-killed cell (FKC) mixture was also prepared as an immunogen. Cultures of the seven *Cronobacter* spp. were applied as treatments with 0.5% formalin for 24 h, followed by centrifugation (3,000 × *g*, 4°C, for 30 min) according to the method of Song and Kim (2013). First injection was administered with a mixture of immunogen and Freund's complete adjuvant (1:1, v/v). Second and third injections were administered using the same mixture of immunogen and Freund's incomplete adjuvant (1:1, v/v) at 4 weeks after the first injection. Immunogen was injected into the back of rabbits at four sites at a concentration of 0.25 mL/site. Blood samples were taken every week until 18 weeks after the first injection.

Preparation and Purification of IgG

Blood samples were centrifuged (10,000 × *g*) for 30 min at 4°C to separate the anti-sera. Anti-*Cronobacter* IgG

was purified from the anti-sera by caprylic acid and ammonium sulfate precipitation as described by McKinney and Parkinson (1987) and according to further modified methods of Shukla et al. (2012) and Song and Kim (2013).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Purified Anti-*Cronobacter* IgG

The purity of various anti-*Cronobacter* IgGs preparations was checked using SDS-PAGE (Atto, Tokyo, Japan) under reduced conditions as described by Park et al. (2012) with some modifications.

Development of INC-ELISA for Detection of *Cronobacter* spp.

Rabbit anti-*Cronobacter* IgG was used to develop an INC-ELISA method for detection of *Cronobacter* spp. Standard response curve of the seven *Cronobacter* spp. was constructed using the following procedure. First, fresh cultures of all tested *Cronobacter* spp. were diluted decimally with 0.05 M carbonate buffer (pH 9.5) in order to coat 96-well plates (SPL Life Sciences, Gyeonggi-do, Korea), whereas only carbonate buffer was used as a negative control. Simultaneously, coated cultures of *Cronobacter* spp. were counted on NA and VRBG agar plates for determination of concentration. After coating at 4°C overnight, 96-well plates were washed three times with 0.01 M PBS (pH 7.0) and blocked with 200 μL of 5% skim milk at 37°C for 2 h. Ninety six-well plates were then washed with 0.01 mol/L PBS-0.05% Tween 20 (PBST), added with rabbit anti-*Cronobacter* IgG, and incubated at 37°C for 1 h. Then, plates were washed again with 0.01 M PBST, after which 100 μL of phosphatase-labeled goat anti-rabbit IgG was added and the plates incubated at 37°C for 1 h. Plates were washed again with 0.01 M PBST, after which 50 μL of pNPP liquid substrate was added to each well for a 30 min enzyme-substrate reaction. Finally, 50 μL of 0.01 M NaOH solution was added to stop the reaction, after which the final reaction mixture was analyzed using an Infinite M200 (Tecan; Seestrasse, Switzerland). A detection limit of the developed INC-ELISA was determined by using a data linearity function and standard deviation of responses for the calibration standard and samples, respectively (Hubaux and Vos, 1970).

Specificity and Sensitivity of INC-ELISA Assay

Seven common *Cronobacter* foodborne pathogens (**Table 1**) were used for the specificity test of the developed INC-ELISA. Cultures of tested foodborne pathogens were serially diluted to final concentrations of 10^0 to 10^8 CFU/mL and then used to check sensitivity of the developed INC-ELISA. All experiments were repeated three times.

Food Trial Using *Cronobacter*-Spiked PIF Sample (Artificial Inoculation of *Cronobacter* into PIF Sample)

Firstly, the seven *Cronobacter* strains were separately cultured in NB at 37°C for 20 h, after which the cultures were serially diluted with BPW to obtain the lowest final concentration (<10 CFU/mL) of *Cronobacter* live cells for use in further experiments. To examine the applicability of the developed anti-*Cronobacter* IgG against each *Cronobacter* strain, 25 g of PIF sample was aseptically placed into a flask, mixed with 225 mL of BPW as a pre-enriched broth, and separately spiked with 1 mL (less than 10 cells/25 g of PIF) of each *Cronobacter* species, followed by pre-enrichment incubation at 37°C for 8 h. Following this, 1 mL of the pre-enrichment culture was inoculated into EE broth and incubated for 8 h of enrichment (Korea Food and Drug Administration [KFDA], 2014). Further, enriched aliquots of pre-treated food samples were tested using the developed INC-ELISA and standard USFDA methods. For control, non-spiked PIF samples were analyzed using the developed INC-ELISA and standard microbiological USFDA methods (United States Food and Drug Administration [USFDA], 2002). All experiments were performed in three replicates in order to maintain reliability of the work.

Statistical Analysis

The detection limit was calculated as the average value of absorbance at zero concentration with three standard deviations (Park et al., 2012). All experiments and results were analyzed in at least three trials for proving reliable and reproducible data.

TABLE 1 | Detection of *Cronobacter* species in pure culture using the developed indirect non-competitive enzyme-linked immunosorbent assay.

Species	Results	Detection limits		Positive/Negative values
		CFU/mL	Log CFU/mL	
Cronobacter condimenti	+	$(2.3 \pm 0.08) \times 10^4$	4.36	18.9
Cronobacter dublinensis	+	$(5.7 \pm 0.15) \times 10^4$	4.75	10.9
Cronobacter malonaticus	+	$(4.9 \pm 0.24) \times 10^4$	4.69	10.8
Cronobacter muytjensii (ATCC 51329)	+	$(1.0 \pm 0.05) \times 10^4$	4	13.9
Cronobacter sakazakii (ATCC 29544)	+	$(5.6 \pm 0.30) \times 10^3$	3.74	12.68
Cronobacter turicensis	+	$(2.9 \pm 0.16) \times 10^4$	4.46	14.7
Cronobacter universalis	+	$(2.1 \pm 0.01) \times 10^5$	5.32	6.0

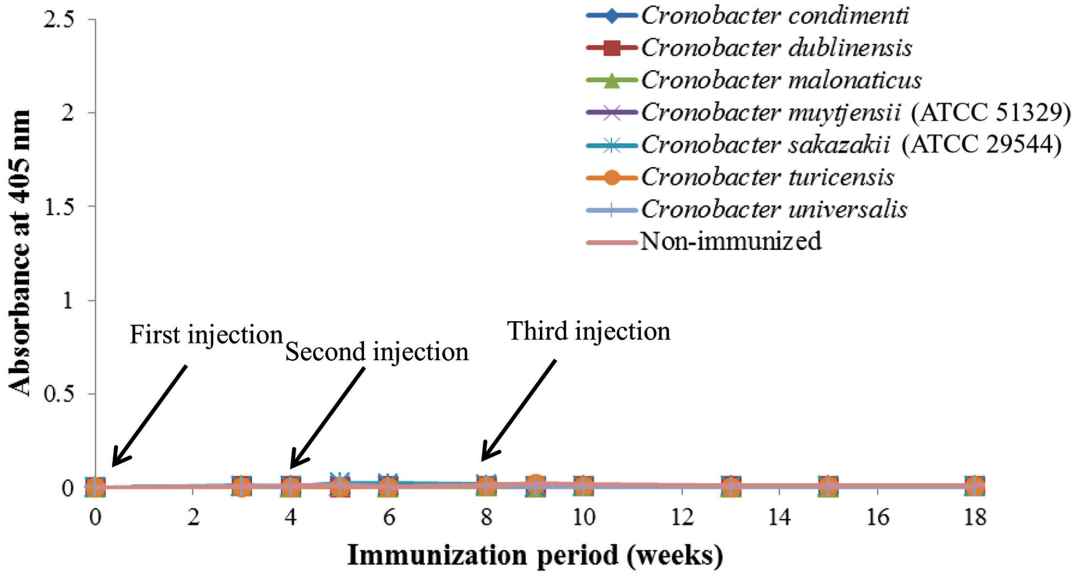


FIGURE 1 | Titers of rabbit anti-serum developed by lipopolysaccharide against seven *Cronobacter* species. All experiments were conducted three times, and data represent mean ± standard deviation.

RESULTS

Characteristics of Developed Antibodies

As shown in **Figure 1**, anti-serum containing anti-*Cronobacter* IgG developed by the LPS mixture showed no titer for any *Cronobacter* species. **Figure 1** provides the fact that LPS antigen of *Cronobacter* is not appropriate for the production of anti-*Cronobacter* antibody. For antibodies developed using FKC and SCP mixtures of *Cronobacter* spp., titers of the developed rabbit anti-*Cronobacter* serum containing IgG were determined by INC-ELISA after 18 weeks of immunization, as shown in **Figures 2** and **3**. Titers of the developed anti-*Cronobacter* seru containing IgG increased after the first injection as well as gradually increased after the second and third injections. Titers of anti-*Cronobacter* serum containing IgG developed from the FKC mixture of *Cronobacter* spp. (**Figure 2**) were in the following order: *C. muytjensii*, *C. turicensis*, *C. malonaticus*, *C. condimenti*, *C. sakazakii*, *C. dublinensis*, and *C. universalis*. Comparatively,

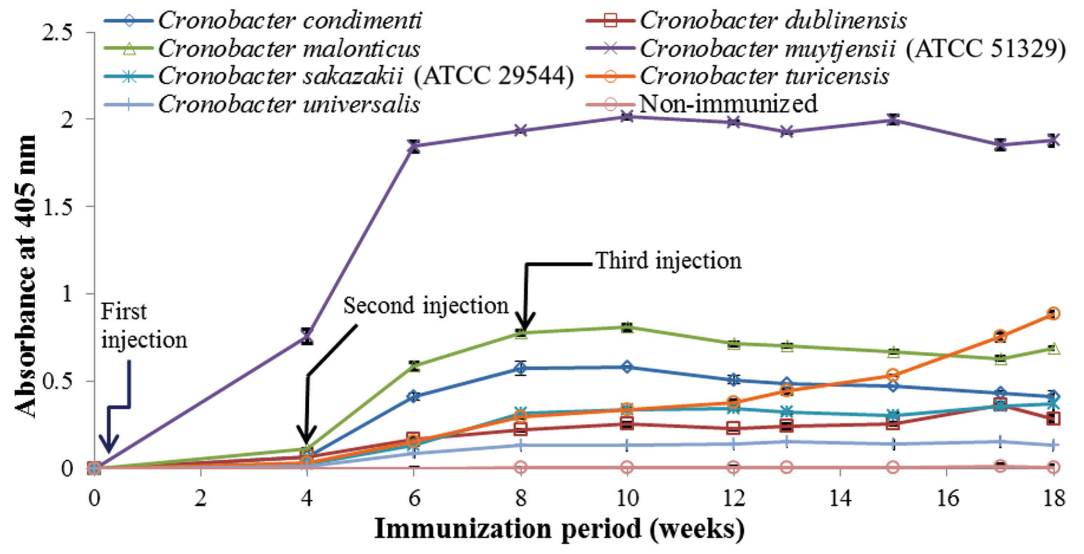


FIGURE 2 | Titers of rabbit anti-serum developed from formalin killed cells mixture against seven *Cronobacter* species. All experiments were conducted three times, and data represent mean ± standard deviation.

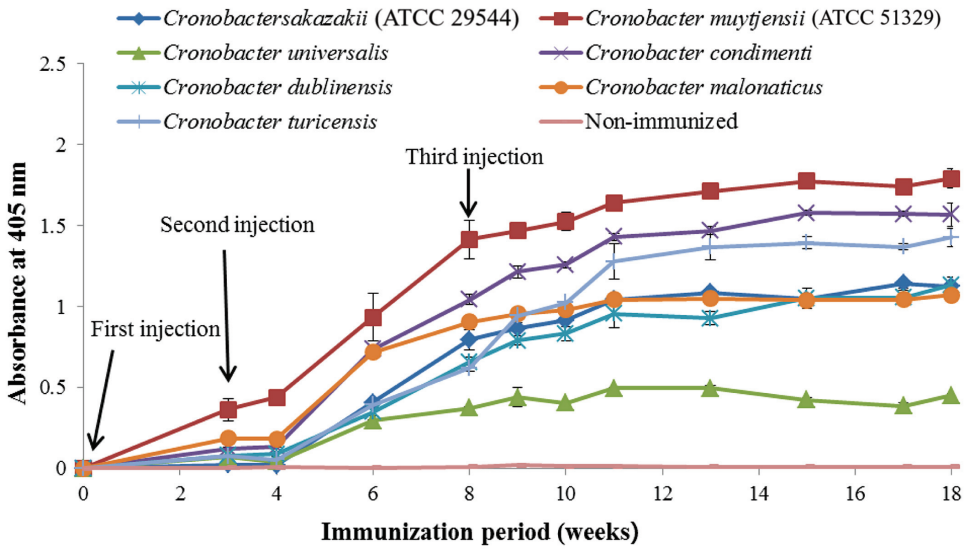


FIGURE 3 | Titers of rabbit anti-serum developed from sonicated cell protein mixture against seven *Cronobacter* species. All experiments were conducted three times, and data represent mean ± standard deviation.

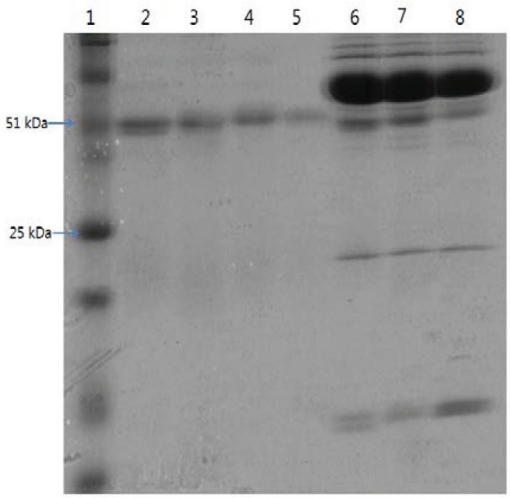


FIGURE 4 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of developed rabbit anti-*Cronobacter* IgG and anti-serum. Lane 1: standard protein marker, lane 2: commercial rabbit IgG, lane 3: anti-*Cronobacter* IgG developed from sonicated cell protein mixture of *Cronobacter* spp., lane 4: anti-*Cronobacter* IgG developed from formalin killed cells mixture of *Cronobacter* spp., lane 5: anti-*Cronobacter* IgG developed from lipopolysaccharide mixture of *Cronobacter* spp., lane 6: anti-serum developed from sonicated cell protein mixture of *Cronobacter* spp., lane 7: anti-serum developed from formalin killed cells mixture of *Cronobacter* spp., lane 8: anti-serum developed from lipopolysaccharide mixture of *Cronobacter* spp.

anti-*Cronobacter* serum containing IgG developed from an SCP mixture of the seven *Cronobacter* spp. showed higher titers against the seven *Cronobacter* spp. (**Figure 3**), which were in the following order: *C. muytjensii*, *C. condimenti, C. turicensis*, *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, and *C. universalis*. It is well known that an increased titer of developed antibody results in effective performance during immunoassay. The developed and purified anti-*Cronobacter* IgG showed very high purity as compared to the commercial rabbit IgG (**Figure 4**). Developed antibody as well as commercial rabbit IgG (lanes 2, 3, 4, and 5) showed a strong band around 51 kDa and a light band around 25 kDa, respectively. These results confirm that our developed rabbit anti-*Cronobacter* IgG with high purity is comparable to commercial rabbit IgG.

INC-ELISA for Detection of *Cronobacter* spp. Using Developed Anti-*Cronobacter* IgG

An INC-ELISA method was developed to detect the seven *Cronobacter* spp. using rabbit anti-*Cronobacter* IgG developed from the aforementioned SCP mixture. The developed INC-ELISA method showed excellent sensitivity toward *Cronobacter* spp., including *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis*, confirming its ability to detect all seven *Cronobacter* spp. in a single test (**Table 1**). Furthermore, the developed INC-ELISA was highly sensitive to all seven different *Cronobacter* spp. with a detection limit ranging from $(5.6 \pm 0.30) \times 10^3$ to $(2.1 \pm 0.01) \times 10^5$ CFU/mL (**Table 1**).

Specificity of Developed INC-ELISA

According to **Table 2**, the developed INC-ELISA showed no cross-reactivity with any of the other tested bacterial genera. The recognition difference between targeted bacteria (positive value; *P* value) and non-targeted bacteria (negative value; *N* value) is an important parameter affecting the results of the

TABLE 2 | Cross-reactivity of the developed indirect non-competitive enzyme-linked immunosorbent assay with non-*Cronobacter* species in pure culture.

Species	Result	Positive/Negative values
Escherichia coli	–	1.002
Salmonella Enterica	–	1.109
Salmonella Typhimurium	–	1.603
Bacillus cereus	–	1.076
Citrobacter freundi	–	1.594
Buttiauxella noackiae	–	1.067
Franconibacter pulveris	±	1.942
Franconibacter helveticus	–	1.010

immunoassay. In the present study, non-targeted bacteria (non-*Cronobacter*) showed a *P*/*N* value of <2 (**Table 2**), which is considered as a negative result, whereas targeted strains showed a *P*/*N* value of >2, which is considered as a positive result (**Table 1**). In support of this, the *P*/*N* value for *F. pulveris* was around 1.942 (**Table 2**) where a *P*/*N* value >2 is considered as a positive result. Compared to several other methods, our newly developed INC-ELISA based on rabbit anti-*Cronobacter* IgG is less expensive and easier to perform since it does not require expensive reagents or probes and has an assay time of only 36 h comprising 8 h of pre-enrichment, 8 h of enrichment, and 20 h of INC-ELISA. During assay development, accuracy of the rapid detection method was confirmed by standard plate colony counting techniques. Moreover, the developed rapid assay is accurate enough to avoid repeated colony counting steps to save detection time.

Detection of *Cronobacter* spp. in Spiked PIF Samples

To detect small amounts of *Cronobacter* spp. cells using the developed method, PIF samples were separately spiked with each of the seven *Cronobacter* spp. at low concentrations (<10 cells/25 g). All PIF samples spiked separately with *C. condimenti*, *C. dublinesis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis* showed the presence of *Cronobacter* spp. in both the INC-ELISA and USFDA/KFDA methods, whereas unspiked samples showed the absence of *Cronobacter* spp. Specific enrichment was carried out in EE broth as per KFDA standard methods (Korea Food and Drug Administration [KFDA], 2014). In the present study, the detection limit of the developed INC-ELISA against *Cronobacter* spp. was found to be <10 cells/25 g of PIF after 16 h of enrichment (**Table 3**).

Further, to confirm the results obtained using the developed antibody and INC-ELISA for *Cronobacter* detection, conventional standard plate count technique using the spreading method on NA and VRBG agar plates was carried out. The PIF samples artificially contaminated with each *Cronobacter* spp. separately (<10 cells/25 g) after 8 h of pre-enrichment and 8 h of enrichment in EE broth showed similar bacterial counts for each *Cronobacter* spp. in the range of 1.1×10^5 – 2.9×10^5 CFU/mL on specific VRBG agar plates (**Table 3**). These results can give an idea to reconfirm the detection limit obtained in pure culture of *Cronobacter* spp. using the developed antibody and INC-ELISA assay (**Table 1**).

DISCUSSION

In this study, an INC-ELISA method based on our laboratory-produced anti-*Cronobacter* IgG was developed and applied for the detection of seven *Cronobacter* spp. in PIF. This study employed three types of immunogens (LPS, FKC, and SCP) to produce three types of antibodies in rabbit for the detection of seven *Cronobacter* spp. As recommended by the Canadian Council on Animal Care [CCAC] (2002), a second injection of immunogen should be performed 3 to 6 weeks after the first immunization. Therefore, in the present work, two immunogen injections were made after the first injection at an interval of 4 weeks during the immunization period. As a result, IgG developed by LPS immunogen showed no titer for any *Cronobacter* spp. These results agree with previous findings of Luk and Lindberg (1991) and Jaradat et al. (2011), who were

TABLE 3 | Food trial for detection of *Cronobacter* and indirect non-*Cronobacter* species in artificially contaminated PIF using the developed non-competitive enzyme-linked immunosorbent assay.

Strains used for inoculation	Spiked level (CFU/25 g)	Results			
		Developed INC-ELISA	USFDA/KFDA	Colony counting on VRBG agar plate after 8 h enrichment	
Control	**0**	**–**	**–**	**CFU/mL**	**Log CFU/mL**
Cronobacter condimenti	<10	+	+	1.9×10^5	5.27
Cronobacter dublinensis	<10	+	+	1.7×10^5	5.23
Cronobacter malonaticus	<10	+	+	1.9×10^5	5.27
Cronobacter muytjensii (ATCC 51329)	<10	+	+	2.9×10^5	5.46
Cronobacter sakazakii (ATCC 29544)	<10	+	+	2.4×10^5	5.38
Cronobacter turicensis	<10	+	+	1.5×10^5	5.17
Cronobacter universalis	<10	+	+	1.1×10^5	5.04

+: Cronobacter spp. detected; –: Cronobacter spp. not detected.

unable to obtain stable antibodies against LPS from *Cronobacter* and *Salmonella* due to the structure and composition of LPS obtained from bacterial cells of *Cronobacter* and *Salmonella*.

Previously, Hochel and Skvor (2009) developed a serotype-specific polyclonal antibody against *E. sakazakii* (*Cronobacter* spp.) capable of detecting 13 *E. sakazakii* spp. from different isolations. Compared to the results of Hochel and Skvor (2009), the developed rabbit anti-*Cronobacter* IgG in this study showed a higher titer, purity, and specificity, which makes it suitable for developing a fast and simple immunoassay method for the detection of *Cronobacter* spp. Therefore, anti-*Cronobacter* IgG developed from an SCP mixture of the seven *Cronobacter* spp. was chosen to develop an INC-ELISA method for genus-specific detection of *Cronobacter* spp. Similarly, Liang et al. (2001) developed a monoclonal antibody for the detection of *Bartonella* spp. capable of specifically detecting all tested species of *Bartonella*.

In addition, Huang et al. (2013) designed two pairs of primers based on *gyr*B sequences for specific identification of *C. sakazakii* and *C. dublinensis*. Although these PCR-based methods can be used to rapidly analyze *Cronobacter* spp., their strict requirements in terms of technique, equipment, and probes limit their application (Ruan et al., 2013). Further, Zhang et al. (2010) reported a cross-priming amplification method combined with immunoblotting analysis for genus-specific detection of *Cronobacter* spp. under isothermal conditions.

Similarly, we developed a polyclonal antibody for *C. muytjensii* as well as fluorescence-based liposome immunoassay for the detection of *C. muytjensii* in our previous study (Song et al., 2015). In this study, the developed antibody was also able to detect other strains of *C. muytjensii* such as *C. muytjensii* ATCC 51329 and *C. muytjensii* CDC3523–75 (Song et al., 2015). Furthermore, as shown in **Table 2**, *F. pulveris* (LMG 24057) showed slight cross-reactivity with INC-ELISA at a high bacterial concentration (10^8 CFU/mL), which might be due to close resemblance between multi-locus sequence typing loci for *Cronobacte*r genus members and *Franconibacter* genera (previously recognized in *Enterobacter* genus) (Forsythe et al., 2014). Recently, Xu et al. (2014) developed a polyclonal antibody for *Cronobacter* spp. by using heat-killed antigen preparation and evaluated its cross-reactivity against various *Cronobacter* strains of the same species. Their results confirmed cross-reactivity for all *Cronobacter* strains of the same species based on positive signals. The detection limit was observed in the range of 10^4–10^5 CFU/mL (Xu et al., 2014). Therefore, it can be hypothesized that the developed antibody and assay in the present study could be applicable for the detection of several *Cronobacter* strains of similar species (Xu et al., 2014).

The above findings confirm that the developed assay method in this study can detect *Cronobacter* spp. in PIF samples with high sensitivity within 36 h. Similarly, Blazkova et al. (2011) developed a method using an immunochromatographic strip for the detection of *Cronobacter* spp. with a detection limit less than 10 cells/10 g of PIF. In the present study, all PIF samples artificially contaminated with *Cronobacter* spp. were positively detected using the developed genus-specific anti-*Cronobacter* IgG antibody in 25 g of PIF after 16 h of sample pre-treatment (8 h of pre-enrichment and 8 h of enrichment) and 20 h of assay time. In contrast, detection and confirmation of this pathogen by the USFDA culture-based method takes up to 3–5 days.

However, the developed method has limited differentiation ability for detection of each *Cronobacter* spp., which must be overcome to validate its practical and industrial usefulness. Hence, development of additional immunoassays based on the produced *Cronobacter* genus-specific antibody multiplexing format such as ELISA are in progress in our laboratory for the purpose of developing a novel assay method with reduced detection time, high sensitivity, and multiplexing detection ability.

In summary, this study focused on the development of a simple, quick, and sensitive genus-specific detection assay for *Cronobacter* spp. using rabbit anti-*Cronobacter* IgG. For this purpose, three immunogens, LPS mixture of *Cronobacter* spp., FKC mixture of *Cronobacter* spp., and SCP mixture of *Cronobacter* spp., were prepared and used to develop antibodies against *Cronobacter* spp. The newly developed rabbit anti-*Cronobacter* IgG was purified using caprylic acid and ammonium sulfate precipitation from anti-sera and showed high purity similar to the commercial rabbit IgG. The rabbit anti-*Cronobacter* IgG developed from the SCP mixture was used to develop a new INC-ELISA method for the detection of *Cronobacter* spp. The developed INC-ELISA showed excellent reactivity with all seven *Cronobacter* spp., excluding other non-*Cronobacter* foodborne pathogens. These results confirm that the developed INC-ELISA is highly sensitive, efficient, and rapid for the detection of *Cronobacter* spp. with a markedly reduced total detection time (from 5 days to 36 h), costs, and handling procedure. This is the first report of a genus-specific antibody and immunoassay method for the detection of *Cronobacter* spp. with the goal of reducing risk of *Cronobacte*r spp. contamination in food.

AUTHOR CONTRIBUTIONS

XS, GL, and SP performed experiments and drafted manuscript; SS contributed interpretation, analyzed data, and wrote paper, MK contributed for conception, designed experiment, analyzed data, and provided technical support.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation, Republic of Korea (NRF), funded by the Ministry of Education (NRF-2014R1A2A1A11053211).

REFERENCES

Anonymous (2006). *Milk and Milk Products – Detection of Enterobacter Sakazakii. Technical Specification ISO/TS 22964. ISO/TS 22964:2006(E) and IDF/RM 210:2006(E)*, 1st Edn. (Geneva: International Organization for Standardization).

Blazkova, M., Javurkova, B., Fukal, L., and Rauch, P. (2011). Immunochromatographic strip test for detection of genus *Cronobacter*. *Biosens. Bioelectron.* 26, 2828–2834. doi: 10.1016/j.bios.2010.10.001

Brigmon, R. L., Zam, S. G., Bitton, G., and Farrah, S. R. (1992). Detection of *Salmonella enteritidis* in environmental samples by monoclonal antibody-based ELISA. *J. Immunol. Method* 152, 135–142. doi: 10.1016/0022-1759(92)90096-C

Canadian Council on Animal Care [CCAC] (2002). *CCAC guidelines on: antibody production*. Ottawa, Canada: The Canadian Council on Animal Care.

Cruz-Córdova, A., Rocha-Ramrez, L. M., Ochoa, S. A., González-Pedrajo, B., Espinosa, N., Eslava, C., et al. (2012). Flagella from five *Cronobacter* species induce pro-inflammatory cytokines in macrophage derivatives from human monocytes. *PLoS ONE* 7:e52091. doi: 10.1371/journal.pone.0052091

Forsythe, S. J., Dickens, B., and Jolley, K. A. (2014). *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age, MLST and whole genome sequence analysis. *BMC Genome* 15:1115–1121. doi: 10.1186/1471-2164-15-1121

Friedemann, M. (2007). *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). *Int. J. Food Microbiol.* 116, 1–10. doi: 10.1016/j.ijfoodmicro.2006.12.018

Giammanco, G. M., Aleo, A., Guida, I., and Mamminia, 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

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

Hochel, I., and Skvor, J. (2009). Characterisation of antibodies for the immunochemical detection of *Enterobacter sakazakii*. *Czech J. Food Sci.* 27, 66–74.

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

Huang, C. H., Chang, M. T., and Huang, L. (2013). Use of the novel species-specific PCR primers targeted to DNA gyrase subunit B (gyrB) gene for species identification of the *Cronobacter sakazakii* and *Cronobacter dublinensis*. *Mol. Cell Probes* 27, 15–18. doi: 10.1016/j.mcp.2012.08.004

Hubaux, A., and Vos, G. (1970). Decision and detection limits for linear calibration curves. *Anal. Chem.* 42, 849–855. doi: 10.1021/ac60290a013

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., McCardell, B., and Tall, B. D. (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

Jaradat, Z. W., Al Nabulsi, A., and Tall, B. D. (2014). *Cronobacter* spp. – opportunistic food-borne pathogens. A review of their virulence and environmental-adaptive traits. *J. Med. Microbiol.* 63, 1023–1037. doi: 10.1099/jmm.0.073742-0

Jaradat, Z. W., Rashdan, A. M., Ababneh, Q. O., and Jaradat, S. A. (2011). Characterization of surface proteins of *Cronobacter muytjensii* using monoclonal antibodies and MALDI-TOF mass spectrometry. *BMC Microbiol.* 11:148. doi: 10.1186/1471-2180-11-148

Joseph, S., Cetinkaya, E., Drahovska, H., and Levican, A. (2012a). *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel 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., and Shih, R. (2012b). Comparative analysis of genome sequences covering the seven *Cronobacter* species. *PLoS ONE* 7:e49455. doi: 10.1371/journal.pone.0049455

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, M. K., and Park, J. H. (2006). Prevalence and thermal stability of *Enterobacter sakazakii* from unprocessed ready-to-eat agricultural products and powdered infant formulas. *Food Sci. Biotechnol.* 15, 152–157.

Korea Food and Drug Administration [KFDA] (2014). *Korea Food and Drug Administration [KFDA]*. Available at: http://www.foodsafetykorea.go.kr/foodcode/menu_01_03.jsp?idx=394

Kumar, S., Balakrishna, K., and Batra, H. V. (2008). Enrichment-ELISA for detection of *Salmonella* typhi from food and water samples. *Biomed. Environ. Sci.* 21, 137–143. doi: 10.1016/S0895-3988(08)60019-7

Lai, K. K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. *Medicine* 80, 113–122. doi: 10.1097/00005792-200103000-00004

Lampel, K. A., and Chen, Y. (2009). Method for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula. *Int. J. Food Microbiol.* 136, 179–184. doi: 10.1016/j.ijfoodmicro.2009.08.016

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

Leenaars, M., and Hendriksen, C. F. M. (2005). Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. *ILAR J.* 46, 269–279. doi: 10.1093/ilar.46.3.269

Liang, Z., Scola, B. L., Lepidi, H., and Raoult, D. (2001). Production of *Bartonella* genus-specific monoclonal antibodies. *Clin. Diag. Lab. Immunol.* 4, 847–849. doi: 10.1128/CDLI.8.4.847-849.2001

Luk, J. M., and Lindberg, A. A. (1991). Rapid and sensitive detection of *Salmonella* by immunomagnetic monoclonal antibody-based assays. *J. Immunol. Method* 137, 1–8. doi: 10.1016/0022-1759(91)90387-U

McKinney, M. M., and Parkinson, A. (1987). A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascetic fluid. *J. Immunol. Method* 96, 271–278. doi: 10.1016/0022-1759(87)90324-3

Mullane, N. R., Murry, J., Drudy, D., and Prentice, N. (2006). Detection of *Enterobacter sakazakii* in dried infant milk formula by cationic-magnetic-bead capture. *Appl. Environ. Microbiol.* 72, 6325–6330. doi: 10.1128/AEM.03056-05

Norberg, S., Stanton, C., Ross, R. P., Hill, C., Fitzgerald, G. F., and Cotter, P. D. (2012). *Cronobacter* spp. in powdered infant formula. *J. Food Prot.* 75, 607–620. doi: 10.4315/0362-028X.JFP-11-285

Odeyemi, O. A., and Sani, N. A. (2016). The prevention of *Cronobacter* infections in hospital neonatal intensive care units. *J. Infect. Public Health* 9, 110–112. doi: 10.1016/j.jiph.2015.06.012

Park, S., Shukla, S., Kim, Y., Oh, S., Kim, S. H., and Kim, M. (2012). Development of sandwich enzyme-linked immunosorbent assay for the detection of *Cronobacter muytjensii* (formerly called *Enterobacter sakazakii*). *Microbiol. Immunol.* 56, 472–479. doi: 10.1111/j.1348-0421.2012.00466.x

Pinto, A., Bonerba, E., and Bozzo, G. (2013). Occurrence of potentially enterotoxigenic *Bacillus cereus* in infant milk powder. *Euro. Food Res. Technol.* 237, 275–279. doi: 10.1007/s00217-013-1988-8

Ruan, J., Li, M., Liu, Y. P., and Li, Y. Q. (2013). Rapid and sensitive detection of *Cronobacter* spp. *(*previously *Enterobacter sakazakii)* in food by duplex PCR combined with capillary electrophoresis-laser-induced fluorescence detector. *J Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 922, 15–20. doi: 10.1016/j.jchromb.2013.01.008

Shukla, S., Bang, J., Heu, S., and Kim, M. (2012). Development of immunoliposome-based assay for the detection of *Salmonella typhimurium*. *Eur. Food Res. Technol.* 234, 53–59. doi: 10.1007/s00217-011-1606-6

Song, X., and Kim, M. (2013). Development of an indirect non-competitive enzyme-linked immunosorbent assay for the detection of *Cronobacter muytjensii* in infant formula powder. *Korean J. Food Nutri.* 26, 936–944. doi: 10.9799/ksfan.2013.26.4.936

Song, X., Shukla, S., Oh, S., Kim, Y., and Kim, M. (2015). Development of fluorescence-based liposome immunoassay for detection of *Cronobacter muytjensii* in pure culture. *Curr. Microbiol.* 70, 246–252. doi: 10.1007/s00284-014-0708-3

Strydom, A., Cawthorn, D. M., Cameron, M., and Witthuhn, R. C. (2012). Species of *Cronobacter*-A review of recent advances in the genus and their significance in infant formula milk. *Int. Dairy J.* 27, 3–12. doi: 10.1016/j.idairyj.2012.06.005

United States Food and Drug Administration [USFDA] (2002). *Isolation and Enumeration of Enterobacter sakazakii from Dehydrated Infant Formula*. Available at: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/ucm114665.htm

Velusamy, V., Arshak, K., Korostynska, O., and Oliwa, K. (2010). An overview of foodborne pathogen detection: in the perspective of biosensors. *Biotechnol. Adv.* 28, 232–254. doi: 10.1016/j.biotechadv.2009.12.004

World Health Organization [WHO] (2007). *Enterobacter sakazakii and Salmonella in Powdered Infant Formula*. Geneva: WHO.

Xu, X., Zhang, Y., Shi, M., and Sheng, W. (2014). Two novel analytical methods based on polyclonal and monoclonal antibodies for the rapid detection of *Cronobacter* spp.: development and application in powdered infant formula. *LWT Food Sci. Technol.* 56, 335–340. doi: 10.1016/j.lwt.2013.11.028

Zhang, Y. L., Zhang, X., Zhang, H. W., and Liu, W. (2010). Rapid and sensitive detection of *Enterobacter sakazakii* by cross-priming amplification combined with immuno-blotting analysis. *Mol. Cell. Probes* 24, 396–400. doi: 10.1016/j.mcp.2010.09.001

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 July 2016
doi: 10.3389/fmicb.2016.01105

Structural Analysis and Biological Toxicity of Aflatoxins B1 and B2 Degradation Products Following Detoxification by *Ocimum basilicum* and *Cassia fistula* Aqueous Extracts

Wajiha Iram[1,2], Tehmina Anjum[1]*, Mazhar Iqbal[2]*, Abdul Ghaffar[3], Mateen Abbas[4] and Abdul Muqeet Khan[4]*

[1] Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan, [2] Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan, [3] Department of Chemistry, University of Engineering and Technology, Lahore, Pakistan, [4] Quality Operating Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Universitá degli Studi di Teramo, Italy

Reviewed by:
Robert Lawrence Brown,
United States Department of Agriculture – Agricultural Research Service, USA
Rosalba Lanciotti,
University of Bologna, Italy

***Correspondence:**
Tehmina Anjum
tehminaanjum@yahoo.com
Wajiha Iram
wajiha_mppl@yahoo.com
Mazhar Iqbal
hamzamgondal@yahoo.com

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

Received: 25 April 2016
Accepted: 01 July 2016
Published: 14 July 2016

Citation:
Iram W, Anjum T, Iqbal M, Ghaffar A, Abbas M and Khan AM (2016) Structural Analysis and Biological Toxicity of Aflatoxins B1 and B2 Degradation Products Following Detoxification by Ocimum basilicum and Cassia fistula Aqueous Extracts. Front. Microbiol. 7:1105. doi: 10.3389/fmicb.2016.01105

This study showed the comparison between *Ocimum basilicum* and *Cassia fistula* (leaves and branch) aqueous extracts for their ability to detoxify of aflatoxins B1 and B2 (AFB1; 100 $\mu g\ L^{-1}$ and AFB2; 50 $\mu g\ L^{-1}$) by *In Vitro* assays and decontamination studies. Results indicated that *O. basilicum* leaves extract was found to be highly significant ($P < 0.05$) in degrading AFB1 and AFB2, i.e., 90.4 and 88.6%, respectively. However, *O. basilicum* branch, *C. fistula* leaves and branch extracts proved to be less efficient in degrading these aflatoxins, under optimized conditions, i.e., pH 8, temperature 30°C and incubation period of 72 h. Moreover the antifungal activity of these plants extracts were also tested. The findings depicted that *O. basilicum* leaves extract showed maximum growth inhibition of aflatoxigenic isolates, i.e., 82–87% as compared to other tested plants extracts. The structural elucidation of degraded toxin products by LCMS/MS analysis showed that nine degraded products of AFB1 and AFB2 were formed. MS/MS spectra showed that most of the products were formed by the removal of double bond in the terminal furan ring and modification of lactone group indicating less toxicity as compared to parent compounds. Brine shrimps bioassay further confirmed the low toxicity of degraded products, showing that *O. basilicum* leaves extract can be used as an effective tool for the detoxification of aflatoxins.

Keywords: plant extract, aflatoxin, degradation, LCMS/MS, toxicity

INTRODUCTION

Mycotoxins are chemically and biologically active secondary metabolites (of molecular weight ≤ 700), produced by filamentous fungi that readily colonize crops in the field or after harvest (Richard, 2007; Turner et al., 2010). Currently, more than 400 compounds are recognized as mycotoxins, among them aflatoxins have assumed significance due to their deleterious effects on humans, poultry, and livestock (Sur and Celik, 2003; Wild and Montesano, 2009). Aflatoxins are group of heterocyclic, oxygen containing mycotoxins that possess the bisdifuran ring system.

About 18 different types of aflatoxins are identified among them most commonly occurring ones are aflatoxins B1, B2, G1, G2, M1, and M2 which are mostly produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxins were first identified as the portable toxin that destroyed more than 100,000 turkey poults (turkey X disease) in England in the early 1960s (Kuhn and Ghannoum, 2003). In Kenya, (April 2004) an outbreak of jaundice with a high fatality rate was reported in humans due to aflatoxin poisoning from eating contaminated home grown maize, and resulted in 317 cases and 125 deaths (Baumgartner et al., 2005). There are strict regulations limiting mycotoxins levels in 77 countries, with specific regulatory levels for aflatoxins in food and feed stuffs (Bhatnagar et al., 2006). This shows that contamination of agricultural commodities with aflatoxin has been the subject of serious concern on an international level. Such contamination occurs by the invasion of aflatoxigenic strains ubiquitously found before and during harvesting, or by improper storage of agricultural commodities (Hwan and Choi, 2007). The food and agriculture organization (FAO) estimates that about 1000 million metric tons of foodstuff could be contaminated with mycotoxins each year (Bhat et al., 2010).

In recent years, the need to develop disease control measure as alternative to chemicals has been a priority of scientists worldwide (Reddy et al., 2009). Therefore it is important to find a practical, cost effective, and non-toxic method to prevent fungal and mycotoxin contamination in foods and feeds. Natural plant products are of interest as a source of safer or more effective substitutes for synthetic antimicrobial agents and may provide alternative way to prevent food or feed from fungal or mycotoxin contamination. Large-scale application of plant products have attracted the attention of several microbiologists as they are biodegradable, biologically safe, cost effective, renewable in nature and at the same time, conveniently used as eco-friendly technique to detoxify mycotoxins (Varma and Dubey, 1999). Previously documented literature showed that powder and extracts of various herbs and medicinal plants found to be effective in detoxifying aflatoxins and growth inhibition of toxigenic fungi. Krishnamurthy and Shashikala (2006) worked on inhibition of aflatoxin B1 production by *Aspergillus flavus*, isolated from soybean seeds by incubation with certain natural plant products at different time interval. The outcome of their study showed that captan, neem cake, leaf powder of *Withania somnifera* (Linn.), peel powder of *Camellia sinensis* (Linn.), *Citrus medica* (Linn.), and pongamia cake controlled the aflatoxin B1 production. They concluded that all the natural product treatments applied were significantly effective in inhibiting aflatoxin B1 production. In a more comprehensive study in Satish et al. (2007) scrutinized aqueous extract of 52 plants for their antifungal potential against eight important species of *Aspergillus*. They observed that among tested plants, aqueous extracts of *Acacia nilotica* (Linn.), *Eucalyptus globules* Labill, *Achras zapota* (Linn.), *Datura stramonium* (Linn.), *Emblica officinalis* (*Gaertn.*), *Lawsonia inermis* L. (henna), *Mimusops elengi* (Linn.), *Peltophorum pterocarpum* (DC.) Baker ex. K. Heyne, *Polyalthia longifolia* Benth. and Hook, *Prosopis juliflora* (Sw.) DC, *Punica granatum* (Linn.), and *Syzygium cumini* (Linn.) exhibited significant antifungal activity against *Aspergillus* species. Correspondingly Reddy et al. (2009) explored the potential of certain plant extracts and biocontrol agents for the reduction of aflatoxin B1 (AFB1) in stored rice. Among the plant extracts tested, *Syzygium aromaticum* (L.) Merr. Et Perry, *Curcuma longa* (L.), *Allium sativum* (L.), and *Ocimum sanctum* (Linn.) significantly inhibited the *A. flavus* growth and AFB1 production. Velazhahan et al. (2010) evaluated various medicinal plants extracts for their ability to detoxify aflatoxin G1 (AFG1) by thin-layer chromatography and enzyme-linked immunosorbent assay (ELISA). Of the various plant extracts, the seeds extract of *Trachyspermum ammi* showed maximum degradation of AFG1 after 24 h of incubation. Another study by Vijayanandraj et al. (2014) also demonstrated the effect of different parameters on aqueous extracts of various medicinal plants for detoxification of aflatoxin B1 (AFB1). They concluded that leaf extracts of *Adhatoda vasica* Nees showed the maximum degradation of AFB1 ($\geq$98%) after incubation for 24 h at 37°C. Moreover, Kannan and Velazhahan (2014) explored the potential of some indigenous medicinal plants extracts for detoxification of aflatoxins. The study showed that among several tested plants, *Barleria lupulina* Lindl. leaves extract exhibited maximum detoxification of aflatoxins B1, B2, G1, and G2 at pH 10 whereas detoxification percentage decreased at pH 7 and 3. Time course study of aflatoxin detoxification by *B. lupulina* extract showed that degeneration of aflatoxin occurred within 10 min and this percentage was increased with increase in incubation period.

In current investigation, *Ocimum basilicum* L. (Family: Lamiaceae, Common name: Sweet basil) and *Cassia fistula* L. (Family: Fabaceae, Common name: Amaltas) were used to evaluate their aflatoxin detoxifying potential. *Ocimum basilicum* is a common herb that is known for its ornamental and therapeutic importance while *C. fistula* is a moderate size deciduous tree, commonly known as golden shower. Both plants have been reported to possess hepatoprotective, antitumor, antitoxic, anti-inflammatory, antibacterial, and antifungal effects. Their Phytochemical studies revealed the presence of terpenoids, alkaloids, flavonoids, tannins, saponin, glycosides, and ascorbic acid (Neelam et al., 2011; Khair-ul-Bariyah et al., 2012). In this present study, these plants were selected to develop a cost effect and environment friendly strategy for aflatoxin detoxification.

MATERIALS AND METHODS

Chemical and Reagents

Aflatoxins B1 and B2 purified from *A. flavus* were prepared in laboratory under optimized conditions and compared through HPLC with standard AFB1 and AFB2 purchased from (Sigma–Aldrich, St. Louis, MO, USA). Stock solutions of AFB1 (1000 $\mu g\ L^{-1}$) and AFB2 (500 $\mu g\ L^{-1}$) were prepared in methanol and stored at 4°C. The working solutions of AFB1 (100 $\mu g\ L^{-1}$) and AFB2 (50 $\mu g\ L^{-1}$) were prepared by diluting the stock solution.

Plant Extract Preparation

Ocimum basilicum and *Cassia fistula* samples (leaves and branches) were collected from Quaid-e-Azam campus, University

TABLE 1 | Effect of temperature and incubation period on aflatoxin detoxification by *O. basilicum* and *C. fistula* aqueous extracts.

Treatments	Temperature	Percentage reduction in AFB1			Percentage reduction in AFB2		
		6 h	24 h	72 h	6 h	24 h	72 h
Toxin	25	$0.71^{p\ B}$	$1.71^{m\ AB}$	$2.86^{o\ A}$	$0.53^{s\ BC}$	$0.84^{t\ B}$	$1.02^{w\ A}$
	30	$0.88^{p\ B}$	$2.30^{m\ AB}$	$3.81^{o\ A}$	$0.68^{s\ BC}$	$0.99^{t\ B}$	$1.16^{w\ A}$
	35	$2.54^{p\ C}$	$3.01^{m\ B}$	$4.49^{o\ A}$	$0.82^{s\ B}$	$1.14^{t\ AB}$	$1.31^{w\ A}$
	40	$3.87^{p\ C}$	$4.33^{m\ B}$	$5.15^{o\ A}$	$0.97^{s\ B}$	$1.29^{t\ AB}$	$1.46^{w\ A}$
	45	$5.19^{p\ AB}$	$5.66^{m\ AB}$	$5.82^{o\ A}$	$1.12^{s\ B}$	$1.44^{t\ AB}$	$1.61^{w\ A}$
	50	$6.48^{op\ AB}$	$6.51^{m\ AB}$	$6.98^{o\ A}$	$1.27^{s\ B}$	$1.59^{t\ AB}$	$1.76^{w\ A}$
	55	$7.14^{op\ BC}$	$7.84^{m\ B}$	$8.30^{o\ A}$	$1.42^{s\ B}$	$1.73^{t\ AB}$	$1.91^{w\ A}$
	60	$7.80^{op\ B}$	$9.16^{m\ AB}$	$9.63^{o\ A}$	$1.57^{s\ BC}$	$1.88^{t\ B}$	$2.06^{w\ A}$
Toxin + water	25	$1.48^{p\ B}$	$3.36^{m\ AB}$	$3.38^{o\ A}$	$0.47^{s\ C}$	$1.47^{t\ B}$	$2.25^{w\ A}$
	30	$2.64^{p\ C}$	$3.56^{m\ B}$	$4.19^{o\ A}$	$1.14^{s\ B}$	$2.04^{t\ AB}$	$2.41^{w\ A}$
	35	$3.44^{p\ C}$	$4.80^{m\ B}$	$5.45^{o\ A}$	$1.23^{s\ B}$	$2.73^{t\ AB}$	$2.87^{w\ A}$
	40	$4.76^{p\ A}$	$6.13^{m\ AB}$	$6.48^{o\ A}$	$1.98^{s\ B}$	$3.84^{t\ AB}$	$3.40^{w\ A}$
	45	$6.08^{op\ B}$	$7.45^{m\ AB}$	$7.47^{o\ A}$	$2.72^{s\ C}$	$3.92^{t\ B}$	$4.44^{w\ A}$
	50	$7.41^{op\ B}$	$8.46^{m\ AB}$	$8.77^{o\ A}$	$3.46^{s\ C}$	$4.43^{t\ B}$	$6.07^{w\ A}$
	55	$8.73^{op\ C}$	$9.46^{m\ B}$	$10.10^{o\ A}$	$4.21^{s\ AB}$	$4.96^{t\ B}$	$7.19^{w\ A}$
	60	$10.05^{op\ BC}$	$10.45^{m\ B}$	$11.42^{o\ A}$	$4.95^{s\ C}$	$5.48^{t\ B}$	$8.31^{w\ A}$
T + *C. fistula* leaves	25	$23.81^{lmn\ C}$	$30.00^{kl\ B}$	$36.65^{lmn\ A}$	$32.20^{pqr\ C}$	$41.13^{p–s\ B}$	$48.94^{r–u\ A}$
	30	$27.68^{k–n\ C}$	$30.74^{jkl\ B}$	$37.84^{k–n\ A}$	$33.94^{opq\ C}$	$43.36^{o–r\ B}$	$50.80^{p–t\ A}$
	35	$32.15^{j–n\ C}$	$34.32^{i–l\ B}$	$40.52^{j–n\ A}$	$36.17^{nop\ C}$	$44.85^{m–r\ B}$	$52.29^{o–s\ A}$
	40	$39.59^{h–k\ C}$	$44.74^{f–l\ B}$	$47.67^{h–l\ A}$	$38.40^{mno\ C}$	$46.34^{k–q\ B}$	$53.78^{n–q\ A}$
	45	$41.99^{g–k\ C}$	$45.93^{f–l\ B}$	$51.24^{hij\ A}$	$40.64^{lmn\ C}$	$47.83^{j–p\ B}$	$55.27^{mno\ A}$
	50	$43.16^{f–j\ C}$	$46.82^{f–l\ B}$	$52.43^{hi\ A}$	$42.87^{klm\ C}$	$49.32^{i–o\ B}$	$56.76^{lmn\ A}$
	55	$45.25^{d–j\ C}$	$50.10^{e–h\ B}$	$52.73^{hi\ A}$	$45.10^{jkl\ C}$	$50.80^{h–n\ B}$	$58.24^{klm\ A}$
	60	$46.74^{d–j\ C}$	$50.69^{e–h\ B}$	$53.92^{h\ A}$	$47.33^{ijk\ C}$	$52.29^{g–l\ B}$	$59.73^{jkl\ A}$
T + *C. fistula* branch	25	$19.87^{no\ C}$	$25.06^{l\ B}$	$30.30^{n\ A}$	$27.24^{r\ C}$	$36.17^{s\ B}$	$43.98^{v\ A}$
	30	$23.15^{mn\ C}$	$25.80^{l\ B}$	$31.49^{n\ A}$	$28.98^{qr\ C}$	$38.40^{rs\ B}$	$45.84^{uv\ A}$
	35	$27.62^{k–n\ C}$	$29.38^{kl\ B}$	$34.17^{mn\ A}$	$31.21^{pqr\ C}$	$39.89^{qrs\ B}$	$47.33^{tuv\ A}$
	40	$35.06^{j–m\ C}$	$39.80^{h–k\ B}$	$41.31^{i–n\ A}$	$33.44^{opq\ C}$	$41.38^{p–s\ B}$	$48.82^{stu\ A}$
	45	$36.67^{i–m\ C}$	$40.99^{g–k\ B}$	$44.89^{h–m\ A}$	$35.67^{nop\ C}$	$42.87^{o–s\ B}$	$50.31^{q–t\ A}$
	50	$38.04^{i–l\ C}$	$41.88^{g–k\ B}$	$46.08^{h–l\ A}$	$37.90^{mno\ C}$	$44.35^{n–r\ B}$	$51.79^{o–s\ A}$
	55	$40.12^{g–k\ C}$	$45.16^{f–l\ B}$	$47.87^{h–l\ A}$	$40.14^{lmn\ C}$	$45.84^{l–q\ B}$	$53.28^{n–r\ A}$
	60	$41.61^{g–k\ C}$	$45.75^{f–l\ B}$	$49.06^{h–k\ A}$	$42.37^{klm\ C}$	$47.33^{k–p\ B}$	$54.77^{m–p\ A}$
T + *O. basilicum* leaves	25	$54.41^{c–g\ C}$	$66.62^{bcd\ B}$	$77.91^{de\ A}$	$70.08^{f\ C}$	$74.55^{e\ B}$	$76.78^{f\ A}$
	30	$58.29^{b–e\ C}$	$67.37^{bcd\ B}$	$79.10^{cde\ A}$	$71.82^{ef\ C}$	$76.78^{de\ B}$	$79.01^{ef\ A}$
	35	$62.75^{abc\ C}$	$70.94^{abc\ B}$	$81.78^{a–d\ A}$	$74.05^{def\ C}$	$78.27^{cde\ B}$	$80.50^{def\ A}$
	40	$69.0^{ab\ C}$	$78.09^{ab\ B}$	$86.54^{a–d\ A}$	$76.28^{cde\ C}$	$79.76^{b–e\ B}$	$81.99^{cde\ A}$
	45	$72.64^{a\ C}$	$79.28^{ab\ B}$	$90.12^{abc\ A}$	$78.52^{bcd\ C}$	$81.99^{a–d\ B}$	$83.48^{bcd\ A}$
	50	$73.47^{a\ C}$	$79.57^{ab\ B}$	$91.31^{ab\ A}$	$80.75^{abc\ C}$	$83.48^{abc\ B}$	$84.96^{abc\ A}$
	55	$74.81^{a\ C}$	$82.85^{a\ B}$	$91.60^{ab\ A}$	$82.98^{ab\ C}$	$84.96^{ab\ B}$	$86.45^{ab\ A}$
	60	$76.30^{a\ C}$	$83.45^{a\ B}$	$92.80^{a\ A}$	$85.21^{a\ B}$	$87.20^{a\ AB}$	$87.94^{a\ A}$
T + *O. basilicum* branch	25	$35.42^{j–m\ C}$	$43.70^{f–j\ B}$	$65.73^{g\ A}$	$40.32^{lmn\ C}$	$49.25^{i–o\ B}$	$57.06^{lmn\ A}$
	30	$39.29^{ijk\ C}$	$44.44^{f–l\ B}$	$66.93^{fg\ A}$	$42.06^{klm\ C}$	$51.48^{h–m\ B}$	$58.92^{klm\ A}$
	35	$43.76^{d–j\ C}$	$48.02^{e–l\ B}$	$69.61^{efg\ A}$	$44.29^{jkl\ C}$	$52.97^{g–k\ B}$	$60.41^{jkl\ A}$
	40	$51.20^{c–l\ C}$	$55.16^{d–g\ B}$	$76.75^{def\ A}$	$46.52^{ijk\ C}$	$54.46^{f–j\ B}$	$61.90^{ijk\ A}$
	45	$54.03^{c–h\ C}$	$56.36^{def\ B}$	$80.32^{b–e\ A}$	$48.75^{hij\ C}$	$55.95^{f–l\ B}$	$63.39^{hij\ A}$
	50	$54.78^{c–g\ C}$	$57.25^{def\ B}$	$81.52^{a–d\ A}$	$50.99^{ghi\ C}$	$57.43^{fgh\ B}$	$64.87^{ghi\ A}$
	55	$56.86^{b–f\ C}$	$60.52^{cde\ B}$	$81.80^{a–d\ A}$	$53.22^{gh\ C}$	$58.92^{fg\ B}$	$66.36^{gh\ A}$
	60	$58.35^{bcd\ C}$	$61.12^{cde\ B}$	$83.00^{a–d\ A}$	$55.45^{g\ C}$	$60.41^{f\ B}$	$67.85^{g\ A}$

Values are mean of three replicates. Small alphabetic letters with different values indicate significant differences ($p < 0.05$) in toxin reduction at different temperature and incubation periods in each column. Capital alphabetic letters with different values indicate significant differences ($p < 0.05$) among controls and tested plant extracts at different temperature and incubation period in each row.

of the Punjab, Lahore. Samples were surface-sterilized using 1% sodium hypochlorite for 10 min and washed several times with sterile distilled water. After surface sterilization, aqueous plants extract were prepared according to the method described by Velazhahan et al. (2010) with some modifications. For extract preparation, 10 g of leaves/branches were homogenize with 10 mL of sterile distilled water. Then homogenate was filtered through muslin cloth and centrifuged at 14,000 rpm for 20 min. Supernatant was sterilized using syringe filter assembly and used for further detoxification studies.

Fungal Growth Inhibition Assay

Antifungal activity of plants extracts against toxigenic isolates of *A. flavus* was determined by fungal growth inhibition assay as described by Fiori et al. (2000). To 80 ml of malt extract agar medium 20 ml of 10% each filter-sterilized plant extract was added. Then aflatoxigenic isolates of *A. flavus* from 5 days old culture were inoculated centrally into the plates and incubated at room temperature (28 ± 2°C). The colony diameter of test fungi was measured after 4 days by calculating the average radial growth. The percentage inhibition of mycelial growth in relation to the control treatment was calculated by following equation. Three replicates of each treatment were experimented. In control, isolates were inoculated in MEA plates without any plant extract and incubated as described above.

$$\text{Percent growth inhibition} = ((a - b)/a) \times 100$$

Where,

a = diameter of fungal colony (mean) in control

b = diameter of fungal colony (mean) with plant extract

Detoxification of Aflatoxins (B1 and B2) by Medicinal Plants Extracts (*In Vitro* Studies)

For *In Vitro* detoxification assay, 50 μL of working solution containing (100 μg L^{-1}) AFB1 and (50 μg L^{-1}) AFB2 was mixed with 250 μL of plants extract and incubated for various intervals of time. Subsequently, the reaction was terminated by adding 250 μL of Chloroform to the above mixture. For recovery of residual toxin mixture was vortex thoroughly. Low speed centrifugation was done for the separation of Chloroform fraction. After that organic phase was withdrawn, evaporated to dryness under gentle stream of nitrogen and redissolved in methanol. Control comprised of toxin: water (50 μL: 250 μL) and incubated under same conditions. All experiments were conducted in triplicate.

Optimization of Analytical Parameters for Detoxification Assay (*In Vitro*)

Temperature and Incubation Time

Plants extracts were incubated with toxins at various intervals of temperature and incubation periods, i.e., 25, 30°C. . .. . .60°C for 6, 24, and 72 h, respectively. Afterward, the toxin content in the reaction mixture was determined as described above.

Optimum pH

The original pH of plants extracts was modified in the range of 2.0–8.0 by using 1 N HCl or 1 N NaoH and then assayed for toxin detoxification activity. Control consists of distilled water with same pH range as well as untreated extract.

Decontamination of Maize Samples Using Plants Extracts

Maize samples were decontaminated according to method described by Das and Mishra (2000) with some modifications. Ten grams of maize samples were kept in each 250 ml Erlenmeyer flask and spiked with 3 ml of aflatoxins (with concentration B1 100 μg L^{-1} and B2 50 μg L^{-1}). These samples were then incubated with 10 ml of aforesaid plants extracts at 30°C for 72 h. After that aflatoxin was extracted according to the modified method of Stroka et al. (2000). Maize samples were incubated with water: acetonitrile (15: 85%) on shaking water bath for 2 h. Afterward the extracts were filtered through filter paper (Whatman, Inc., Clifton, NJ, USA). Then filtrate was passed through afla immunoaffinity column in solid phase extraction assembly. Toxins were slowly eluted from the column with 1 ml of methanol in a glass vial, which was further analyzed by TLC and HPLC. Control comprised of untreated maize sample, sample with plant extract without toxin and sample with toxin without plant extract. Each experiment was performed in triplicate.

TLC and HPLC Analysis of Aflatoxin

The qualitative analysis of residual toxin was determined by thin layer chromatography (TLC) according to the method described by Ramesh et al. (2013) with some modifications. Twenty-five microliters of methanolic fraction of treated and untreated samples were spotted on 0.25 mm silica gel $60F_{254}$ (20 cm × 20 cm, Merck). Then plates were developed on chloroform: acetone (92: 8 v/v) and viewed under UV light at 365 nm.

Before quantitative analysis by HPLC, treated and untreated toxins were derivatized by the method described by Hernandez-Hierro et al. (2008) with some modifications. For this purpose, eluted toxin was evaporated to dryness with gentle stream of nitrogen, redissolved in 200 μL *n*-hexane, vortexed and 50 μL of trifluoroacetic acid (TFA) was added in it. Then, 950 μL of acetonitrile–water (1:9) was added to above solution and filtered by using syringe filter assembly. The filtrate was analyzed by HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse-phase C18 column (Merck, Darmstadt, Germany), and a fluorescence detector. Water:methanol:acetonitrile (60:20:20 v/v/v) were used as mobile phase at a flow rate of 1 mL $min^{-1.}$ Toxins were detected at excitation and emission wavelengths of 360 nm and 440 nm, respectively. For HPLC method validation, calibration curves were drawn using series of calibration solutions in methanol. Each standard solution was chromatographed in duplicate.

Identification of Degraded Toxin Products

Mass spectral studies were carried out for the identification degraded toxin products.

LCMS Analysis of Degraded Toxin

Degraded toxin products were analyzed by using surveyor LC system equipped with mass spectrophotometer and PDA plus detectors (Thermo Fisher Scientific). System was validated with known standards individually and in mixture form. All analysis were performed in triplicate using luna phenomenex C_{18} column (150 mm × 4.6 mm, 3 μm), in isocratic mode. Following are the LC-MS conditions for Aflatoxins. Injection volume was 10 μL. Mobile phase consisted of methanol:acetonitrile:water (22.5:22.5:55.0 v/v). Column temperature was maintained at 30°C. The total operation time was 25 min with the flow rate of 0.5 mL min^{-1}. MS conditions were as follows: capillary temperature was 335°C, sheath gas flow and auxiliary gas flow was 20 L min^{-1} and 4 L min^{-1}, respectively. Source voltage, capillary voltage, and tube lens voltage was 5 KV, 49 V, and 120 V, respectively. Toxins incubated with water under optimized conditions (i.e., temp 30°C and pH 8) were run as a control.

Electrospray Ionization Mass Spectrometery (ESI – MS/MS) of Aflatoxins through Direct Insertion Pump

In order to predict the molecular formulae as well as elemental composition of AFB1 and AFB2 degradation products, samples were further analyzed by electrospray ionization mass spectrometer. MS/MS was performed on was performed on a Thermo Scientific LTQ XL System fitted with electrospray ionization (ESI) source operating in positive ionization mode with optimum conditions set as follows: capillary voltage to 49.0 V, source voltage to 5.0 KV, Tube lens voltage to 110 V, and capillary temperature to 275°C. Sheath and auxiliary gas flow were adjusted to get stable spray, i.e., 3 L min^{-1} and 0.4 L min^{-1}, respectively. Data was collected in positive mode within the range of 100 to 500 m/z. The final identification of an unknown compound was based on the accurate measurement of mass of parent ions and fragments, as well as other useful MS/MS spectrum information (Wang et al., 2011). In MS/MS experiments, untreated aflatoxins and water treated toxins were run as a control.

Brine Shrimps Bioassay for Toxicity Assessment of Degraded Products

Brine shrimps (*Artemia salina*) bioassay was carried out for testing the biological toxicity of degraded toxin products by the method developed by Solis et al. (1993) with some modifications.

Shrimp eggs (100–200 mg) were hatched in artificial sea water (34 g sea salt/L of deionized water) by incubation under 60 W lamp, providing direct light and warmth (26°C). Throughout hatching period, the same conditions of light sensitivity and temperature were maintained. After an incubation period, the hatched nauplii were separated from shells and transferred to fresh sea water.

Three hundred microliters of treated and untreated AFB1 and AFB2 (100 and 50 μg L^{-1}) were added to 96 well plate separately, dried and redissolved in 200 μL of sea water. After that, 200 μL of sea water containing 40–45 organisms were pipetted into each well, resulting in a final volume of 400 μL and incubated for 24–96 h at 26°C. Percentage mortality was determined by counting the immobile (dead) larvae under stereo microscope. Toxicity of each solution was evaluated in triplicate.

Statistical Analysis

Statistical analysis was carried out by using DSSTAT software. Data were analyzed by analysis of variance (ANOVA) and differences among the means were determined for significance at $P \leq 0.05$ using Tukey's multiple range test.

RESULTS

Inhibitory Effects of Aqueous Plants Extracts on the Growth of Aflatoxigenic Isolates of *Aspergillus flavus*

Ocimum basilicum and *Cassia fistula* aqueous extracts were tested to determine their inhibitory effect on growth of aflatoxigenic isolates of *Aspergillus flavus*. **Table 2** depicted that *O. basilicum* leaves extract showed 82.8–87.7% inhibition of growth of tested isolates. This was followed by *O. basilicum* branch extract with percentage inhibition of 57–68.3% in growth of all tested isolates. While 23.6–49.8% and 16.4–42.3% inhibition in mycelial growth was recorded by aqueous extracts of *C. fistula* leaves and branch, respectively.

Influence of Temperature and Incubation Period on Toxin Detoxification by Plants Extracts

The aflatoxins (B1 and B2) detoxifying efficacy of *Ocimum basilicum* and *Cassia fistula* (leaves and branches) aqueous extracts were tested at different temperature and incubation time. The degree of detoxification was compared with control experimented under same conditions. Results showed that percentage of detoxification increased with increase in incubation time to 6–72 h (**Table 1**). At lowest tested temperature (25°C), maximum detoxification of AFB1 and AFB2 was shown by *O. basilicum* leaves extract, i.e., 77.9 and 76.7%, respectively, after 72 h of incubation. At this temperature, 36.6–65.7% and 48.9–57.0% reduction in AFB1 and AFB2 level was recorded in samples treated with *C. fistula* leaves and *O. basilicum* branch extract. While least significant detoxification of AFB1 and AFB2 was observed after treatment with *C. fistula* branch extracts, i.e., 30.3 and 43.9%, respectively.

Similarly, at 30°C most significant detoxification of AFB1 and AFB2 was shown by *O. basilicum* leaves extract which was followed by *O. basilicum* branch, *C. fistula* leaves and branch extracts, after 72 h of incubation. In control samples at 30°C only

TABLE 2 | Effects of aqueous plants extracts on mycelial growth inhibition of *Aspergillus flavus* isolates.

Aqueous plant extracts	Percentage of mycelial growth inhibition of *Aspergillus flavus* isolates				
	Isolate 24	Isolate 31	Isolate 37	Isolate 44	Isolate 42
C. fistula branch	35.4^{d}	42.3^{cd}	16.4^{d}	32.8^{cd}	39.3^{cd}
C. fistula leaves	41.5^{c}	49.8^{c}	23.6^{c}	37.4^{c}	44.8^{c}
O. basilicum branch	67.7^{b}	65.2^{b}	57.0^{b}	64.9^{b}	68.3^{b}
O. basilicum leaves	87.7^{a}	87.6^{a}	84.8^{a}	82.8^{a}	87.4^{a}

Values are mean of three replicates. Values with different alphabetic letters indicate significant differences among tested plants extract as determined by Tukey's multiple range test.

TABLE 3 | Effect of pH on detoxification of AFB1 and AFB2 by *C. fistula* and *O. basilicum* aqueous extracts after 72 h of Incubation.

Treatments	% Detoxification AFB1				% Detoxification AFB2			
	pH 2	pH 4	pH 6	pH 8	pH 2	pH 4	pH 6	pH 8
Toxin + water	12.67$^{c\ C}$	13.88$^{d\ C}$	14.87$^{d\ B}$	16.49$^{d\ A}$	5.43$^{d\ D}$	9.69$^{d\ C}$	11.00$^{d\ B}$	12.86$^{d\ A}$
C. fistula leaves + toxin	40.12$^{bc\ C}$	39.23$^{c\ BC}$	40.58$^{c\ B}$	54.37$^{c\ A}$	36.68$^{c\ D}$	48.67$^{c\ C}$	51.03$^{bc\ B}$	53.05$^{c\ A}$
C. fistula branch + toxin	34.83$^{bc\ C}$	35.48$^{c\ BC}$	36.85$^{c\ B}$	40.73$^{c\ A}$	32.95$^{c\ D}$	44.35$^{c\ C}$	47.25$^{c\ B}$	49.47$^{c\ A}$
O. basilicum leaves + toxin	78.81$^{a\ D}$	80.62$^{a\ C}$	83.58$^{a\ B}$	90.43$^{a\ A}$	72.77$^{a\ C}$	76.08$^{a\ BC}$	77.37$^{a\ B}$	88.69$^{a\ A}$
O. basilicum branch + toxin	46.67$^{b\ D}$	52.34$^{b\ C}$	63.45$^{b\ B}$	74.97^{b}	44.72$^{b\ D}$	55.86$^{b\ C}$	59.50$^{b\ B}$	62.57$^{b\ A}$

Values are mean of three replicates. Small alphabetic letters with different values indicate significant differences ($p < 0.05$) among tested plant extracts. Capital alphabetic letters with different values indicate significant differences ($p < 0.05$) among pH treatments.

4.19 and 2.41% AFB1 and AFB2 were found to be inactivated (**Table 1**).

The findings of this study depicted that the percentage detoxification of AFB1 and AFB2 by plants extracts was gradually increased with the consequential increase in temperature to 35–55°C. Similarly highest detoxification of AFB1 and AFB2 was observed at 60°C but this detoxification may be due to synergistic effect of heat and moisture. In present study, *O. basilicum* leaves extracts showed highest detoxification of aflatoxins at all tested temperatures and incubation periods. However, further experimentations were carried out at 30°C because it is more or less near to room temperature and also found to be prevailing temperature in most of the storehouses of Punjab especially in summer, which may provide a cost effective approach to detoxify these toxins.

Effect of pH on Detoxification of Aflatoxins by Plants Extracts

This study was aimed to observe the detoxifying effect of pH using *O. basilicum* and *C. fistula* aqueous extracts. Distilled water with pH adjusted to 2, 4, 6, and 8 was used as a control. Data revealed that least significant detoxification was observed at pH 2. At this pH, maximum detoxification AFB1 and AFB2 was observed in samples treated with *O. basilicum* leaves extract, i.e., 78.8 and 72.7%, respectively (**Table 3**). This was followed by *O. basilicum* branch, *C. fistula* leaves and branch extracts.

The percentage of detoxification increases with increase in pH from acidic to alkaline range. Hence, the highest degradation of AFB1 and AFB2 was recorded at pH 8 after treatment with *O. basilicum* leaves extracts, i.e., 90.4 and 88.6%, respectively. While 40.7–74.9 and 49.4–62.5% detoxification of AFB1 and AFB2 was shown by *O. basilicum* branch, *C. fistula* leaves and branch extracts, respectively. At this pH respective control showed 16.4 and 12.8% degradation of AFB1 and AFB2 (**Table 3**). So, further studies were done at pH 8 as the results clearly depicted that the efficacy of plants extracts to detoxify AFB1 and AFB2 significantly enhanced at this pH.

TABLE 4 | Decontamination of maize samples by *C. fistula* and *O. basilicum* aqueous extracts under optimized conditions.

	Toxin recovery $\mu g\ L^{-1}$ (% detoxification)	
	AFB1	AFB2
Control		
Unspiked maize	0.5	0.3
Unspiked maize + *C. fistula* leaves extract	0.0	0.0
Unspiked maize + *C. fistula* branch extract	0.0	0.0
Unspiked maize + *O. basilicum* leaves extract	0.0	0.0
Unspiked maize + *O. basilicum* branch extract	0.0	0.0
Spiked maize sample (AFB1: 100 $\mu g\ L^{-1}$; AFB2: 50 $\mu g\ L^{-1}$)	97.3	47.6
Treatment		
Maize + *C. fistula* leaves extract	56.9 (43.1^{c})	25.2 (49.6^{c})
Maize + *C. fistula* branch extract	62.3 (37.7^{d})	28.6 (42.7^{d})
Maize + *O. basilicum* leaves extract	13.1 (86.9^{a})	8.3 (83.5^{a})
Maize + *O. basilicum* branch extract	32.7 (67.3^{b})	21.9 (56.1^{b})

Values are mean of three replicates. Figures in the parenthesis are detoxification % age. Values with different alphabetic letters indicate significant differences ($P < 0.05$) as determined by Tukey's multiple range test.

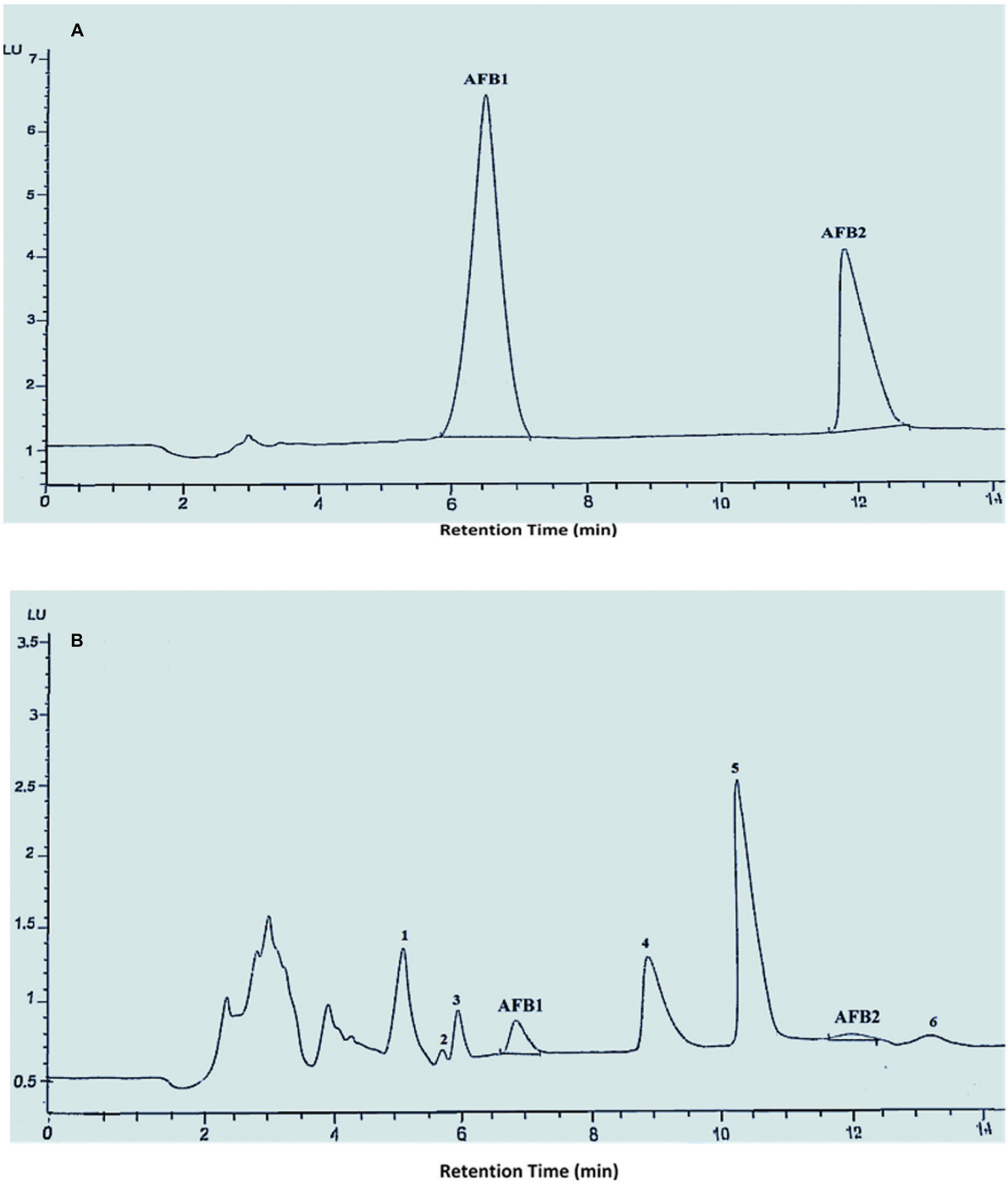


FIGURE 1 | HPLC chromatogram of AFB1 and AFB2. Whereas **(A)** untreated toxins; **(B)** toxin treated with *O. basilicum* leaves extract at 30°C and pH 8.

Decontamination of Maize Samples by Plants Extracts

This study was conducted under optimized condition of *In Vitro* assays, i.e., pH (8), temp (30°C) and incubation time (72 h). According to results, only 0.5–0.3 μg L^{-1} AFB1 and AFB2 were found to be present in unspiked control maize samples. However, 97.3 μg L^{-1} of AFB1 and 47.6 μg L^{-1} of AFB2 were recovered from control maize samples spiked with 100 μg L^{-1} AFB1 and 50 μg L^{-1} AFB2 (**Table 4**).

The findings of this study depicted a similar detoxifying trend as that was recorded in *In Vitro* studies. Results showed that aqueous extracts of *O. basilicum* leaves found as most significant

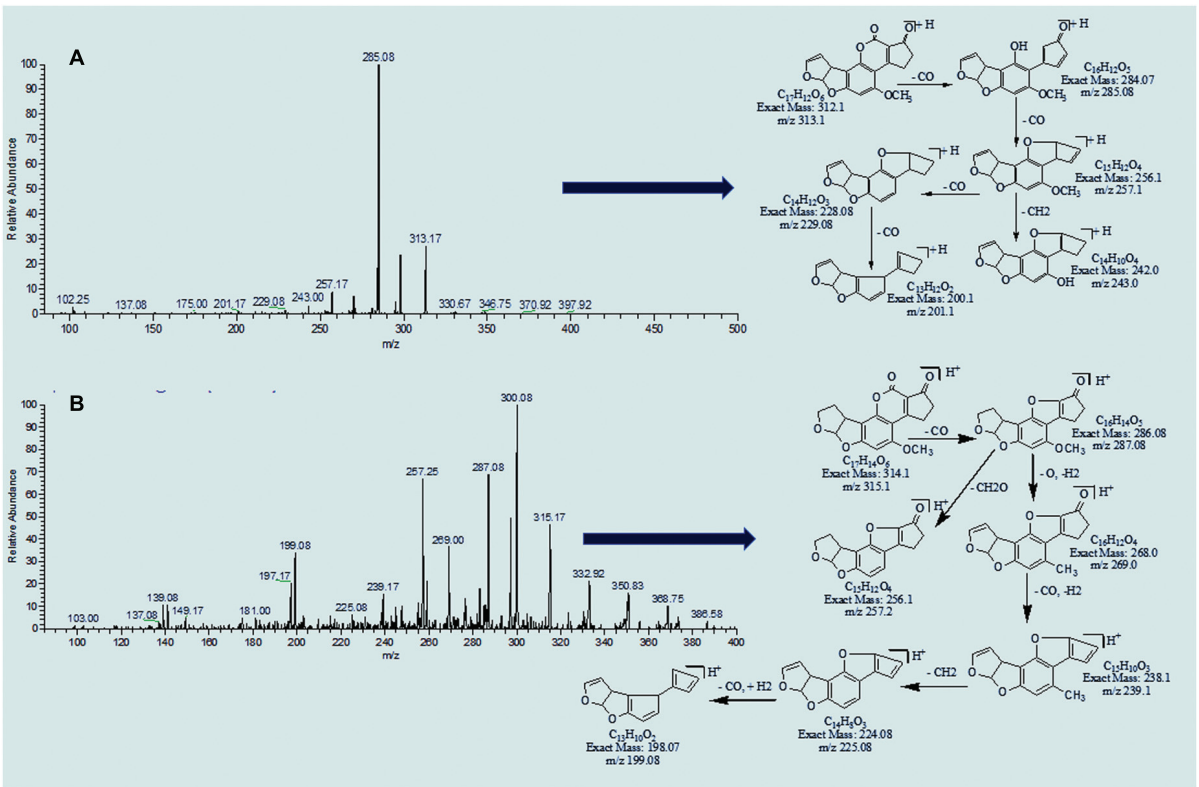


FIGURE 2 | MS/MS Spectra and fragmentation pathway. (A) AFB1 and **(B)** AFB2.

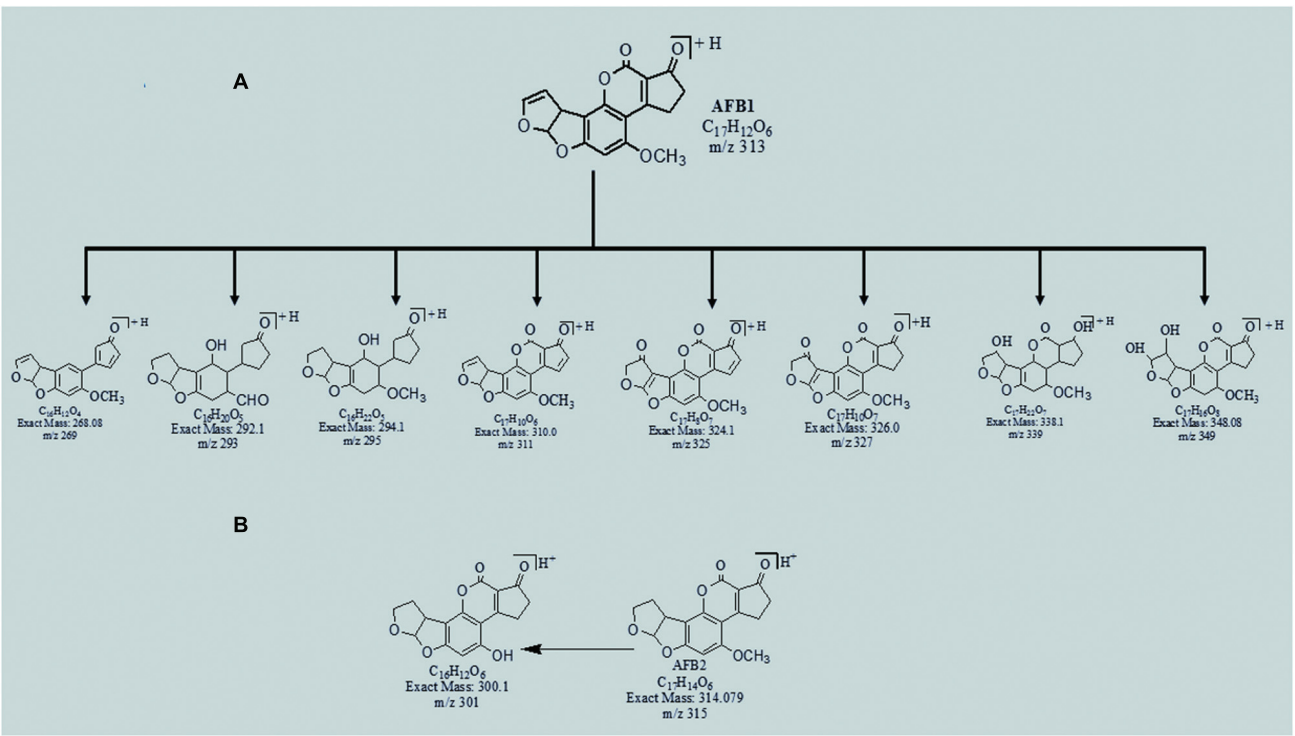


FIGURE 3 | Possible degraded products of (A) AFB1 and (B) AFB2 after treatment with *O. basilicum* leaves extracts at 30°C and pH 8.

source to detoxify AFB1 and AFB2 as they showed detoxification percentage of 86.9 and 83.5%, respectively in spiked maize samples. While in spiked samples, 37.7–67.3% and 42.7–56.1% detoxification of AFB1 and AFB2 was recoded after treatment with *O. basilicum* branch, *C. fistula* leaves and branch extracts (**Table 4**).

In this present investigation, overall results of various assays demonstrated that *O. basilicum* leaves extracts degraded AFB1 and AFB2 most significantly under optimized conditions as compared to *O. basilicum* branch, *C. fistula* leaves and branch extracts.

Moreover HPLC chromatograms that obtained after treatment with *O. basilicum* leaves extracts, showed the presence of various peaks whose footprints were not found in chromatogram of control which may be attributed to toxin degradation products (**Figure 1**). So, aflatoxins degraded with *O. basilicum* leaves extracts were used in further structural characterization studies for the identification of degradation products.

Structural Characterization of Aflatoxin and Their Degradation Products

Aflatoxins showed good ESI ionization in positive ion mode. A molecular base ion at m/z 313.17 and m/z 315.17 was detected for protonated adduct $[M+ H]^+$ of aflatoxins B1 and B2 while sodium adduct $[M+ Na]^+$ was formed at m/z 335 and m/z 337, respectively. The identity of parent ion was validated by its fragmentation into daughter ions. The protonated molecule was chosen as the precursor ion for aflatoxins in the product ion scan mode because the sodium adduct did not exhibit specific fragmentation for any compound. The fragmentation pattern of AFB1 and AFB2 has an important reference value in analyzing fragmentation pathway of degraded products.

MS/MS Analysis of AFB1 and AFB2

AFB1 is composed of 17 carbon atoms, 6 oxygen, and 12 hydrogen atoms while AFB2 contains 14 hydrogen atoms in their formulae along with 17 carbon and 6 oxygen atoms. MS/MS spectrum revealed that continuous loss of carbon monoxide (CO) was the main fragmentation pathway of AFB1. Methyl and methanol losses occurred on methoxy group located on side chain of benzene. The double bond equivalence (DBE) of AFB1 was 12 (**Figure 2A**). However, MS/MS fragmentation pathway of AFB2 illustrated that daughter ions were formed by loss of carbon monoxide, oxygen, hydrogen, and methyl group (**Figure 2B**). The DBE of AFB2 was 11. Identification of

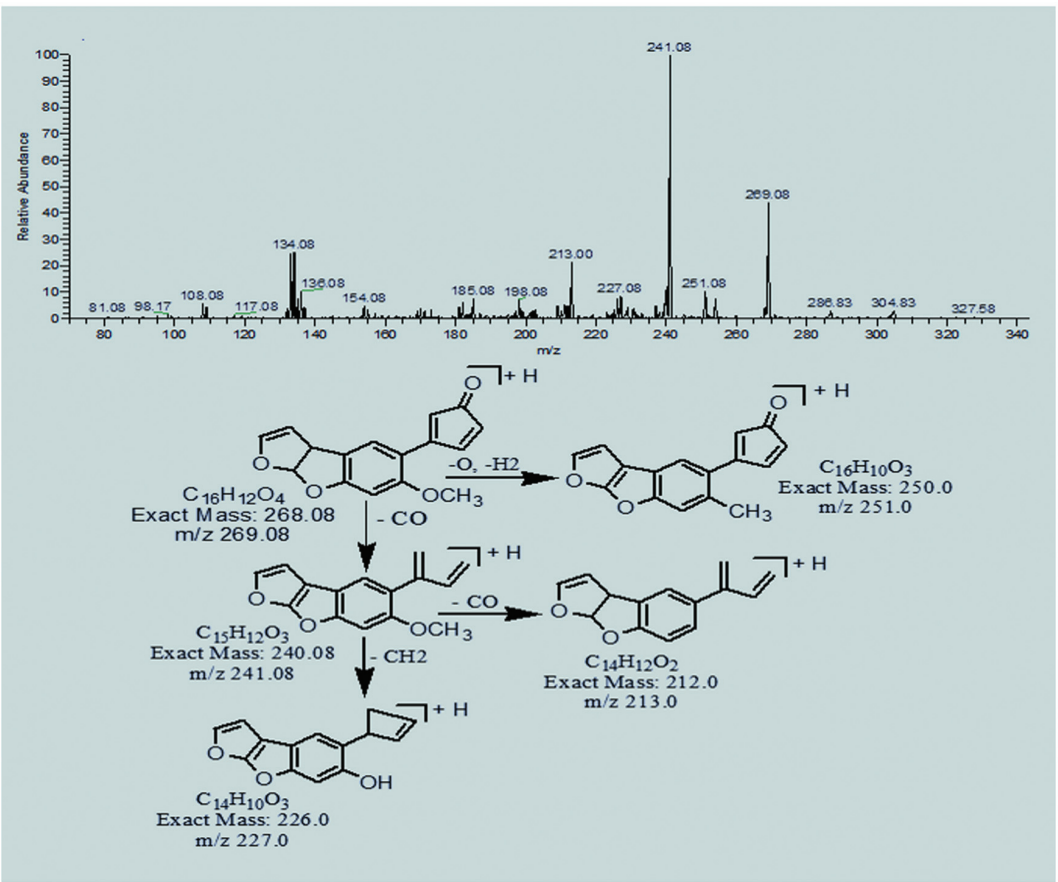


FIGURE 4 | MS/MS spectra and fragmentation pathway of degradation product with 269.08 m/z.

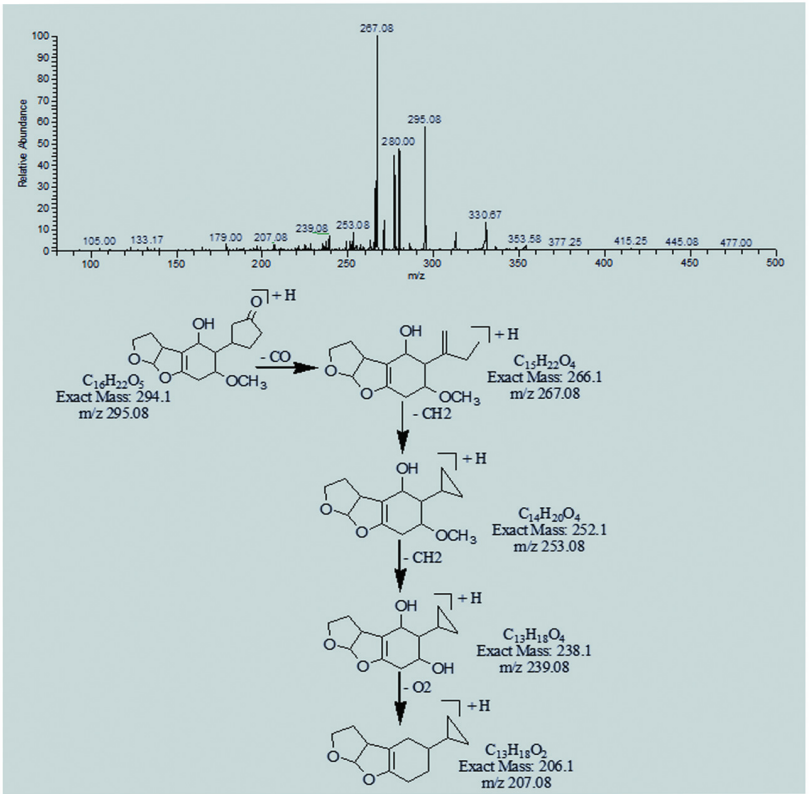


FIGURE 5 | MS/MS spectra and fragmentation pathway of degradation product with 295.08 m/z.

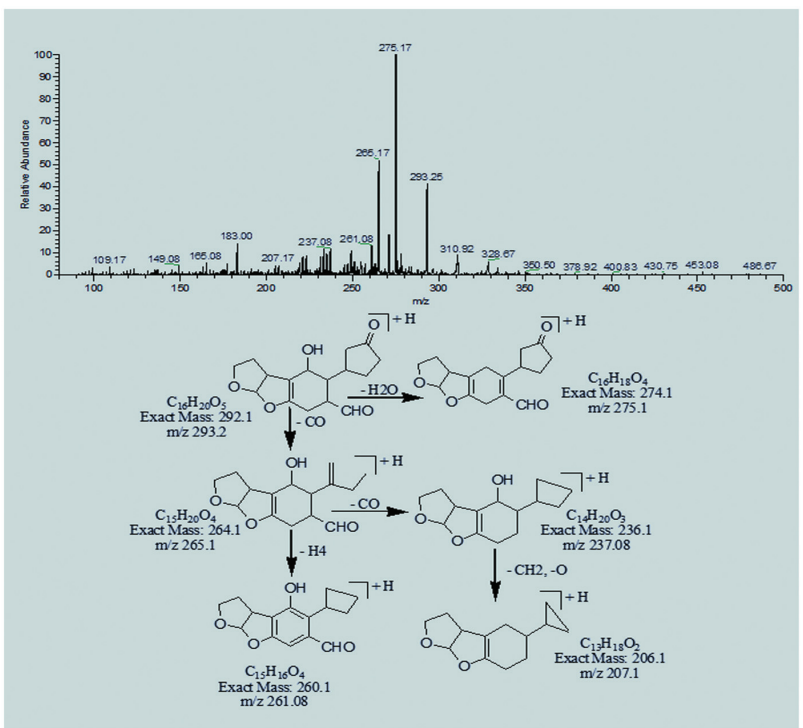


FIGURE 6 | MS/MS spectra and fragmentation pathway of degradation product with 293.25 m/z.

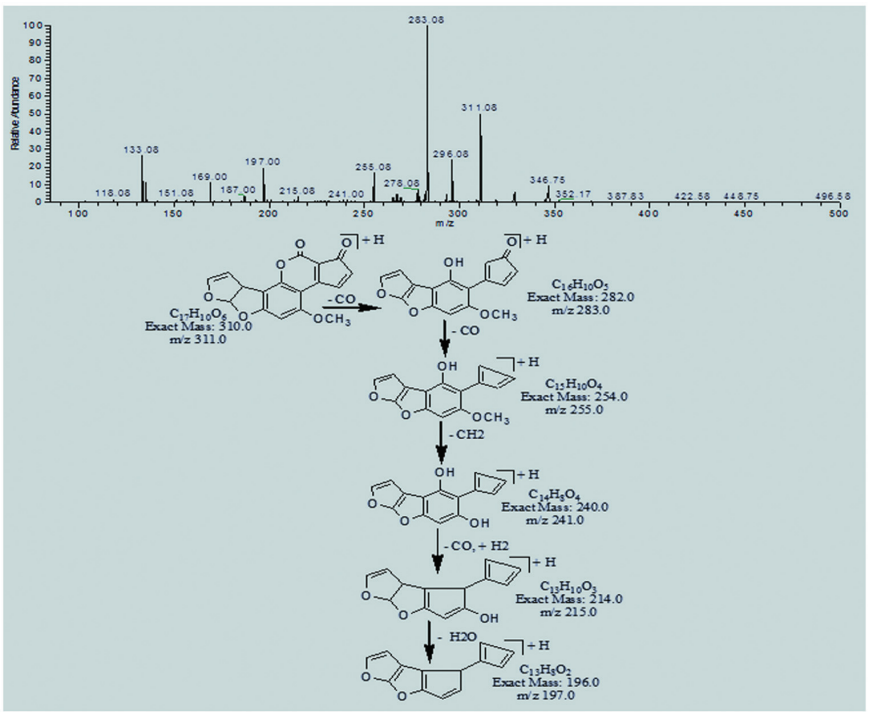


FIGURE 7 | MS/MS spectra and fragmentation pathway of degradation product with 311.08 m/z.

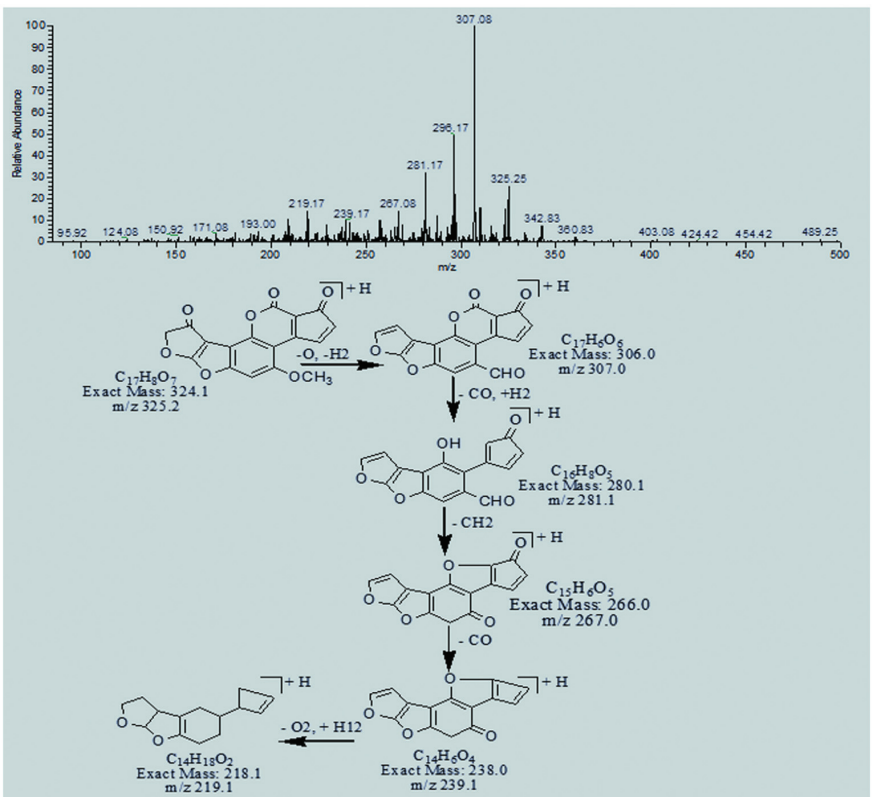


FIGURE 8 | MS/MS spectra and fragmentation pathway of degradation product with 325.25 m/z.

degradation products were based on accurate mass measurement of ions and similar fragmentation pathways with that of AFB1 and AFB2.

It is evidenced from the results that nine degraded products were obtained after detoxification of AFB1 and AFB2 by using *O. basilicum* leaves extract. Among them, some were formed by the modification of lactone ring while others were produced as a result of addition reaction in furan rings of aflatoxins. Structural formulas of possible degraded products of AFB1 and AFB2 are shown in **Figures 3A,B**.

MS/MS Analysis of Degraded Products of AFB1

The degradation product at m/z 269.08 corresponded to molecular formula $C_{16}H_{12}O_4$ formed by the loss of carbon dioxide from lactone ring of AFB1. The DBE of $C_{16}H_{12}O_4$ was one less than AFB1. Loss of oxygen, methyl group and carbon monoxide was the main fragmentation pathway (**Figure 4**).

The degradation products $C_{16}H_{22}O_5$ (m/z 295.08) and $C_{16}H_{20}O_5$ (m/z 293.25) were formed due to the loss of carbon monoxide by the opening of lactone ring and addition of hydrogen atom to AFB1 molecule. The DBE content of both products was same, i.e., six while difference between them was of two hydrogen atoms. Losses of CO, CH_2, and O were the main fragmentation pathway of both products (**Figures 5** and **6**).

Degradation product $C_{17}H_{10}O_6$ with ion peak at m/z 311.08 was formed by the loss of hydrogen atoms implying greater DBE content than that of AFB1. The fragmentation pathway of $C_{17}H_{10}O_6$ was different from that of AFB1. The precursor ion yielded a series of product ions represented by 283.08$[M\text{-}CO]^+$, 255.08$[M\text{-}C_2O_2]^+$, 241.0$[M\text{-}C_3H_2O_2]^+$, 215.08$[M\text{-}C_4O_3]^+$, and 197.0$[M\text{-}C_4H_2O_4]^+$ (**Figure 7**).

The degradation products $C_{17}H_8O_7$ (with m/z 325.25) and $C_{17}H_{10}O_7$ (with m/z 327.0) were formed by the addition of oxygen atom on the double bond of terminal furan ring on the left side. The difference between these two products was only of two hydrogen atoms. The DBE content of both products was greater than AFB1, i.e., 14 and 13, respectively. The precursor ion $C_{17}H_8O_7$ yielded a series of product ions represented by 307.08$[M\text{-}H_2O]^+$, 281.17$[M\text{-}CO_2]^+$, 267.08$[M\text{-}C_2H_2O_2]^+$, and 239.17$[M\text{-}C_3H_2O_3]^+$. While the fragmentation pathway of

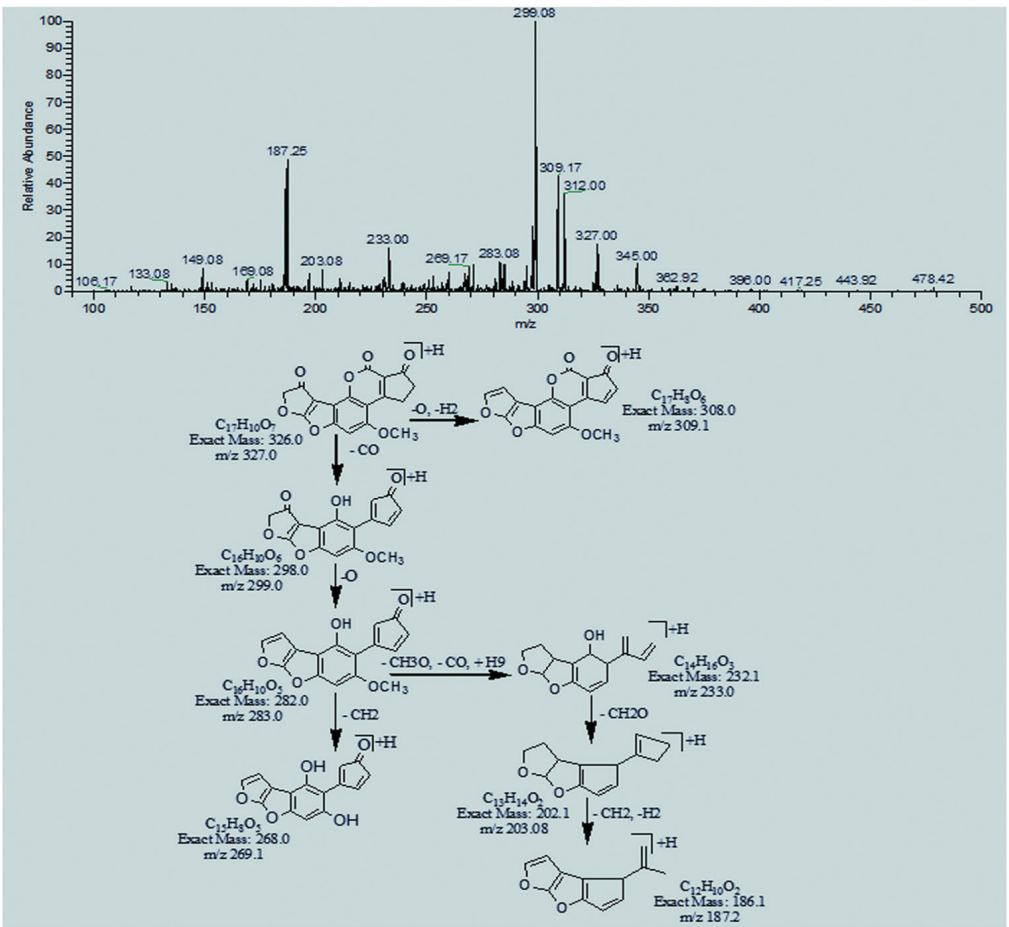


FIGURE 9 | MS/MS spectra and fragmentation pathway of degradation product with 327.0 m/z.

$C_{17}H_{10}O_7$ showed losses of O, CO, CH_2, and CH_3O groups (**Figures 8** and **9**).

The degradation product $C_{17}H_{22}O_7$ at m/z 339.17 produced as a result of addition reaction on furan rings. Double bonds were replaced by the addition of hydrogen atoms with lower DBE content than that of AFB1 i.e., seven. The fragmentation pathway was different from that of AFB1. Fragments of $C_{17}H_{22}O_7$ showed losses of carbon monoxide, water, oxygen, and methyl groups. More details on fragmentation pathway are shown in **Figure 10**.

The degradation product $C_{17}H_{16}O_8$ (with 349.0 m/z) had one more H_4O_2 molecule and less DBE content than AFB1, i.e., 10. The result is most likely caused by additional reaction of two hydroxyl groups on the double bond of terminal furan ring. The precursor ion yielded a series of product ions represented by 331.17$[M\text{-}H_2O]^+$, 321.33$[M\text{-}CO]^+$, 305.25$[M\text{-}CO_2]^+$, 287.25$[M\text{-}CH_2O_3]^+$, 261.08$[M\text{-}C_2O_4]^+$, 257.33$[M\text{-}C_2H_4O_4]^+$, 231.00 $[M\text{-}C_3H_2O_5]^+$, and 217.08$[M\text{-}C_3O_6]^+$ (**Figure 11**).

MS/MS Analysis of AFB2 Degradation Product

The degradation product at m/z 301.25 with molecular formula $C_{16}H_{12}O_6$ was formed by the replacement of methoxy group with hydroxyl group on the side chain of benzene ring. The DBE of $C_{16}H_{12}O_6$ was same as that of AFB2. Fragmentation pathway involves the loss of carbon dioxide, carbon monoxide, and water molecules (**Figure 12**).

Estimation of Toxicity of Degradation Products

The biological toxicity of untreated and treated aflatoxins B1 and B2 were compared through brine shrimps (*Artemia salina*) bioassay to confirm the safety of degraded products. Hatched shrimps larvae were incubated with treated and untreated aflatoxins at 26°C for 24–96 h to observe the mortality response

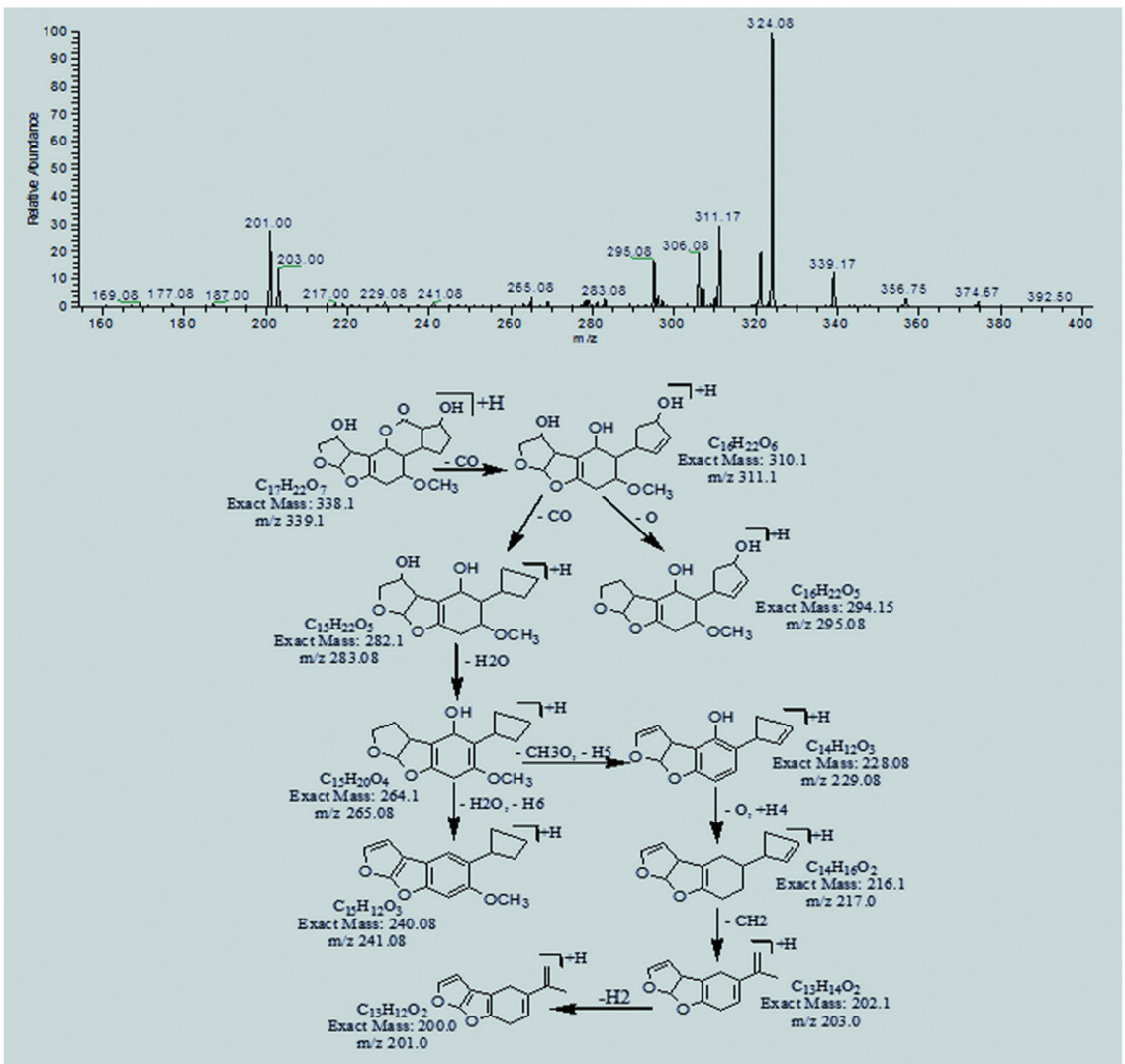


FIGURE 10 | MS/MS spectra and fragmentation pathway of degradation product with 339.1 m/z.

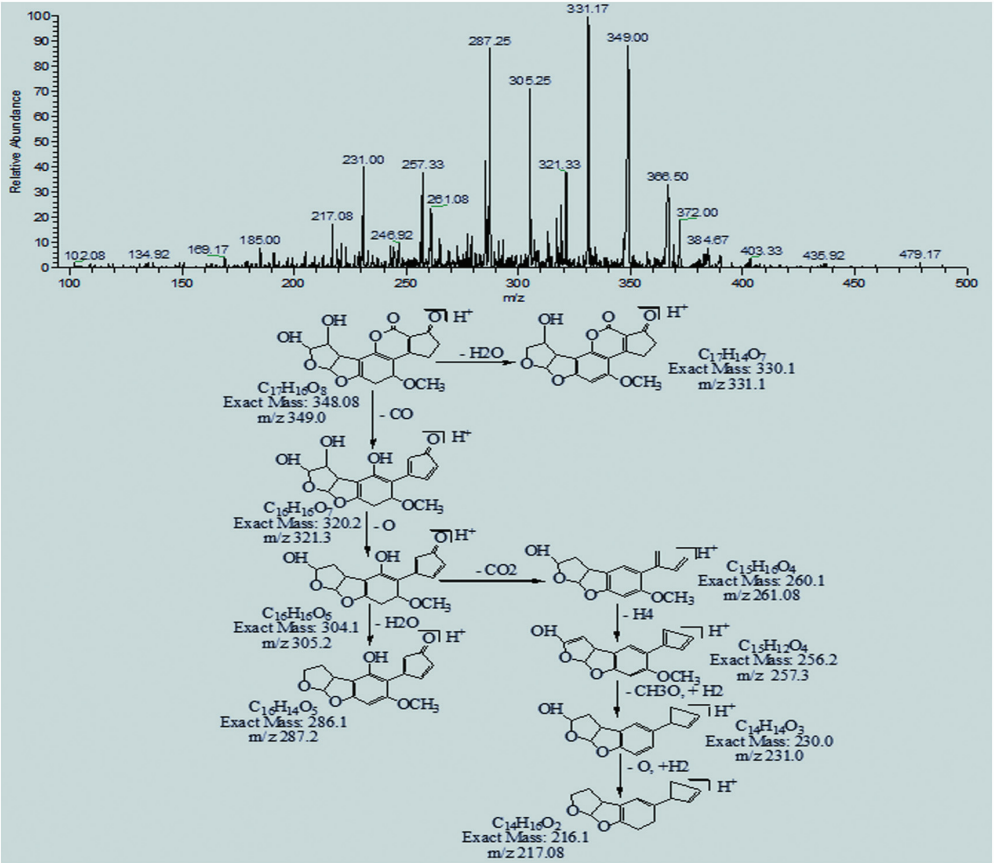


FIGURE 11 | MS/MS spectra and fragmentation pathway of degradation product with 349.0 m/z.

of larvae. Natural mortalities were determined in blank sea water and in well treated with solvent only. The percentage of mortality was calculated from average number of dead larvae per concentration.

Larvae incubated with untreated aflatoxins showed significant increase in larval mortality as compared to control, i.e., 83%. This percentage of mortality was increased to 91.7% with increase in incubation time. As compared to untreated toxins, significant reduction in larval mortality was recorded after incubation with treated toxins. Data derived from tests conducted with AFB1 (100 $\mu g\ L^{-1}$) and AFB2 (50 $\mu g\ L^{-1}$) degraded by *O. basilicum* leaves extracts showed only 9.2–22.5% mortality of shrimps larvae after 96 h of incubation (**Table 5**). The outcome of this study clearly implies that aflatoxins degradation products showed much lower toxicity toward brine shrimps larvae than untreated aflatoxins

DISCUSSION

Use of certain plant extracts and biocontrol agents as a source of safer and more effective control on the growth of aflatoxigenic fungi and aflatoxin production have been under investigation by many authors (Gowda et al., 2004; Joseph et al., 2005; Suleiman et al., 2008; Reddy et al., 2009). Much emphasis was given to herbal, medicinal, and aromatic plants for their antifungal activities against food spoilage and aflatoxigenic fungi (Soliman and Badeaa, 2002; Maraqa et al., 2007; Gandomi et al., 2009; El-Nagerabi et al., 2012). Plants are rich source of bioactive secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids, reported to have antifungal properties (Nwachukwu and Umchuruba, 2001).

In the present study, aqueous extracts of *O. basilicum* and *Cassia fistula* were tested for their antifungal potential against aflatoxigenic isolates of *A. flavus*. All tested plants extracts exhibited diverse degree of antifungal activity against *A. flavus* isolates. The maximum antifungal activity was shown by aqueous extract of *O. basilicum* leaves followed by *O. basilicum* branch, *C. fistula* leaves and branch extract. Similarly a study conducted by Tanackov et al. (2011) also showed that extracts of *O. basilicum* significantly inhibit the growth of the *Fusarium oxysporum*, *F. proliferatum*, *F. subglutinans,* and *F. verticillioides*. Antifungal potential of aforementioned plants used in this study, were also described by many scientists (Abo et al., 1999; Phongpaichit et al., 2004; Pujo et al., 2009; Bhalodia and Shukla, 2011).

According to previously documented literature, essential oils and extracts of various spices and herbs like cinnamon,

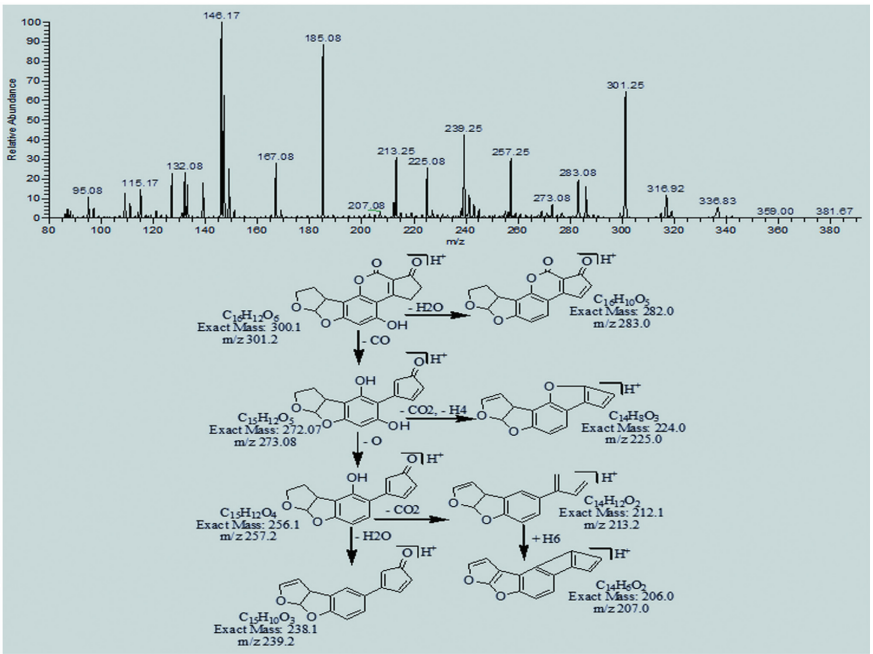


FIGURE 12 | MS/MS spectra and fragmentation pathway of degradation product with 301.2 m/z.

TABLE 5 | Effect of detoxified toxins on percent mortality of Brine shrimp larvae at 26°C after 24–96 h after incubation.

Treatments	Incubation period (h)	No. of living shrimps	No. of dead shrimps	% Mortality
Control				
Sea water + shrimps	24	40	0	0
	48	40	0	0
	72	40	0	0
	96	39	1	2.5
Methanol + shrimps	24	38	1	2.5
	48	38	2	5
	72	37	3	7.5
	96	36	3	7.5
Untreated toxins + shrimps	24	7	33	83.0
	48	5	35	86.7
	72	4	36	89.2
	96	3	37	91.7
Treatment				
Treated toxin with *O. basilicum* leaves extract + shrimps	24	36	4	9.2
	48	35	5	13.3
	72	32	8	20.0
	96	31	9	22.5

Values are mean of three replicates. Data was analyzed by analysis of variance (ANOVA).

peppermint, basil, and lemongrass can be suggested as plant based safe food additive in protecting food and feed from fungal and mycotoxin contamination (Montes-Belmont and Carvajall, 1998; Burt, 2004; Yang et al., 2007). In this study, *In Vitro* assays were performed with *O. basilicum* and *Cassia fistula* aqueous extracts under optimized conditions of temperature, pH and incubation time. In case of temperature, highest detoxification percentage was observed at 60°C but this detoxification could be due to synergistic action of heat and moisture (Basappa and Shantha, 1996; Rustom, 1997). Similar findings were recorded in a study conducted by Hajare et al. (2005) who worked on aflatoxin inactivation by using Ajwain seeds extract under optimized conditions. Their results showed that highest inactivation was obtained at 60°C but further studies were conducted on 45°C to eliminate the effect of heat and moisture on toxin inactivation. Likewise in this present investigation 30°C was selected for further studies.

The results of present study depicted that percentage of detoxification increase with increase in incubation time to 72 h. These observations are in close agreement to the findings of earlier workers (Hajare et al., 2005; Velazhahan et al., 2010; Kannan and Velazhahan, 2014; Vijayanandraj et al., 2014). Furthermore in *In Vitro* assays, pH of reaction mixture was optimized and found that percentage of detoxification increases as the pH change from acidic to alkaline range. In this study highest detoxification of AFB1 and AFB2 was shown by *O. basilicum* leaves extract at pH 8. Similar finding were obtained by Kannan and Velazhahan (2014) who explored the potential of *Barleria lupulina* leaf extract on detoxification of aflatoxins. In addition, Méndez-Albores et al. (2004) found that aflatoxin florescence, attributed to the coumarin moiety, diminish or even disappear in alkaline treatment.

In the past, numerous researchers observe the structural changes in aflatoxin molecule after detoxification by various means, i.e., micro-organisms, physical and chemical agents, ultraviolet (UV) rays, Gamma rays, and plant products

(Alberts et al., 2006; Albores et al., 2008; Guan et al., 2010; Velazhahan et al., 2010; Wang et al., 2011; Farzaneh et al., 2012; Inoue et al., 2013; Luo et al., 2013; Samuel et al., 2014; Vijayanandraj et al., 2014). Lee et al. (1981) observed that lactone ring plays an important role in fluorescence of aflatoxin molecule. On its cleavage, the molecule becomes non-florescent with subsequent significant reduction in toxicity. The toxicity of aflatoxins has been studied by various scientists (Guengerich, 2001, 2008; Hussein and Brasel, 2001). Their toxicity data demonstrated that aflatoxins have cyclopentene ring and furan moiety in their chemical structure. In AFB1 presence of double bond in the terminal furan ring is key factor for its toxic and carcinogenic activities (Wang et al., 2011). As compare to AFB1, the toxicity of AFB2 is hundreds times less due to presence of saturated furan ring (Dvorackova, 1990). The degraded products of AFB2 may be active but were less potent than that of parent compound. Thus removing the double bond of terminal furan ring and modification of lactone ring are major aims of detoxification.

In this present study, data recovered from HPLC revealed that after treatment with *O. basilicum* leaves extracts AFB1 and AFB2 were degraded into number of other compounds whose properties are different from parent toxins. So, the presence of these degraded products was further confirmed by LCMS/MS studies. It is evidenced from the results that eight degraded products were obtained after detoxification of AFB1 by using *O. basilicum* leaves extract. Among them, 50% of degraded products (with m/z 339, 325, 349, and 327) were formed by removal of double bond in furan ring whereas in 25% of products obtained at m/z 293 and 295 modification of lactone ring and removal of double bond in furan ring was occurred. Other studies in literature also supported the similar findings. Velazhahan et al. (2010) reported detoxification of aflatoxin G1 by seed extract of Ajowan (*T. ammi*) and suggested the modification of lactone ring structure of AFG1 as mechanism of detoxification. Similar findings were observed by Vijayanandraj et al. (2014) after detoxification of aflatoxin B1 by an aqueous extract from leaves of *Adhatoda vasica* Nees. A study conducted by Wang et al. (2011) on structure elucidation of radiolytic products of aflatoxin B1 in methanol water solution revealed that in most of radiolytic products addition reaction occurred on the double bond of terminal furan ring, resulting significantly reduced toxicity as compared to that of AFB1. Experiments by Luo et al. (2013) showed that aflatoxin B1 can be effectively degraded by using ozone in aqueous system. According to them, due to conjugate addition reaction on the double bond of terminal furan ring for AFB1, the toxicity of degradation products was significantly decreased compared with that of AFB1.

Brine shrimps (*Artemia salina*) larvae appears to be as susceptible as biological indicator of toxicity of some mycotoxins in foods and feeds (Hartl and Humpf, 2000; Favilla et al., 2006). Several studies in the past were conducted on brine shrimps larvae to evaluate the toxic effects of aflatoxins (Harwing and Scott, 1971; Schmidt, 1989; Logrieco et al., 1996; Durakovic et al., 2002; Moretti et al., 2007). In this study hatched shrimps larvae were incubated with treated and untreated aflatoxins at 26°C for 24 to 96hrs to observe the mortality response of larvae. As compared to untreated toxins, significant reduction in larval mortality was recorded after incubation with treated toxins. Therefore, toxicity of most of degraded products compared with that of aflatoxin was reduced to a much lower level. The reason behind this is that most of the degradation products obtained after treatment with *O. basilicum* leaves extracts were formed as a result of addition reaction on the double bond of terminal furan ring and modification of lactone ring as indicated by mass spectral analysis which is most significant determinant of toxic and carcinogenic activities of aflatoxins. These results were in close agreement with those of Samuel et al. (2014) who worked on detoxification of aflatoxin B1 by *Pseudomonas putida*. He compared the toxicity of treated and untreated AFB1 toward HeLa cells and concluded that degraded products are non-toxic (D1) or much less toxic (D2 and D3) than AFB1 to the cells at the tested concentrations.

CONCLUSION

The efficient detoxification of aflatoxins by using *O. basilicum* leaves extracts under optimized conditions. These extracts are easily available, cost effective, biologically safe and provide an excellent alternative source of toxin detoxification instead of physical and chemical methods. Direct spray of aqueous plant extract is easy to prepare and convenient for the farmers as no technical knowledge is involved but the formulations may vary and cause errors in treatment. This trait should be properly investigated. Another most important aspect is the shelf life of the plant extract preparations that should sustain variations in environmental conditions and remain feasible for the effective detoxification of aflatoxins. Future implications of this approach are quite promising in order to overcome world food hunger improving the quality of food items.

AUTHOR CONTRIBUTIONS

TA is the supervisor and MA is co-supervisor of this work. WI has worked as research scholar for this project. AG and MI has provided technical guidance for various analyses. AMK has helped in data interpretation.

FUNDING

This work was not funded by any agency and was done using indigenous resources of Fungal biotechnology lab, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.

ACKNOWLEDGMENTS

Authors are extremely thankful to MI (National Institute of Biotechnology and Genetic Engineering), AG (University of Engineering and Technology), and MA and AM (University of Veterinary and Animal Sciences) for the provision of specialized apparatus and technical guidance for successful accomplishment of this study.

REFERENCES

Abo, K. A., Lasaki, S. W., and Deyemi, A. A. (1999). Laxative and antimicrobial properties of Cassia species growing in Ibadan. *Nig. J. Nat Prod. Med* 3, 47–50.

Alberts, J. F., Engelbrecht, Y., Steyn, P. S., Holzapfel, W. H., and Zyl, W. H. (2006). Biological degradation of aflatoxin B1 by *Rhodococcus erythropolis* cultures. *Int. J. Food Microbiol* 109, 121–126. doi: 10.1016/j.ijfoodmicro.2006.01.019

Albores, M. A., Vazquez, N. I., Ruvalcaba, M. R., and Martinez, E. M. (2008). Mass spectrometery/mass spectrometry study on the degradation of B-Aflatoxins in maize with aqueous citric acid. *Am. J. Agric. Biol. Sci.* 3, 482–489. doi: 10.3844/ajabssp.2008.482.489

Basappa, S. C., and Shantha, T. (1996). Methods for detoxification of aflatoxin in foods and feeds – a critical appraisal. *J. Food Sci. Technol.* 33, 95–107.

Baumgartner, A. E., Lindblade, K., Gieseker, K., Rogers, S. H., Kieszak, S., Njapau, H., et al. (2005). Case control study of an acute aflatoxicosis outbreak-Kenya-2004. *Environ. Health Perspect.* 113, 1779–1783. doi: 10.1289/ehp.8384

Bhalodia, N. R., and Shukla, V. J. (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula*: an ethnomedicinal plant. *J. Adv. Pharm. Technol. Res.* 2, 104–109. doi: 10.4103/2231-4040.82956

Bhat, R., Rai, R. V., and Karim, A. A. (2010). Mycotoxins in Food and Feed: Present Status and Future Concerns. *Compre Rev. Food Sci. Food Safety* 9, 57–81. doi: 10.1111/j.1541-4337.2009.00094.x

Bhatnagar, D., Cary, J. W., Ehrlich, K., Yu, J., and Cleveland, T. E. (2006). Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. *Mycopathologia* 162, 155–166. doi: 10.1007/s11046-006-0050-9

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

Das, C., and Mishra, H. N. (2000). In vitro degradation of aflatoxin B1 in groundnut (*Arachis hypogea*) meal by horse radish peroxidase. *Lebens. Wiss. Technol.* 33, 308–312. doi: 10.1006/fstl.2000.0655

Durakovic, S., Delas, F., and Durakovic, L. (2002). "Microbial indicators of safety and quality of foods," in *'Modern microbiology of food'*, ed. S. Durakovic (London: Kugler), 249–282.

Dvorackova, I. (1990). *Aflatoxins and Human Health*. Boca Raton, FL: CRC Press, 135–150.

El-Nagerabi, S. A. F., Al-Bahry, S. N., Elshafie, A. E., and Al-Hilali, S. (2012). Effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil on the growth and aflatoxin B1 production of *Aspergillus flavus* and *Aspergillus parasiticus* strains. *Food Control* 25, 59–63. doi: 10.1016/j.foodcont.2011.09.033

Farzaneh, M., Shi, Z., Ghassempour, A., Sedaghat, N., Ahmadzadeh, M., Mirabolfathy, M., et al. (2012). Aflatoxin B1 degradation by *Bacillus subtilis* UTBSP1 from pistachio nuts of Iran. *Food control* 23, 100–106. doi: 10.1016/j.foodcont.2011.06.018

Favilla, M., Macchia, L., Gallo, A., and Altomare, C. (2006). Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) intervertebrate bioassays. *Food. Chem. Toxicol.* 44, 1922–1931. doi: 10.1016/j.fct.2006.06.024

Fiori, A. C. G., Schwan-Estrada, K. R. F., Stangarlin, J. R., Vida, J. B., Scapim, C. A., Cruz, M. E. S., et al. (2000). Antifungal activity of leaf extracts and essential oils of some medicinal plants against *Didymella bryoniae*. *J. Phytopathol.* 148, 483–487. doi: 10.1046/j.1439-0434.2000.00524.x

Gandomi, H., Misaghi, A., Basti, A. A., Bokaei, S., Khosravi, A., and Abbasifar, A. (2009). Effect of *Zataria multifora* Bioss. Essential oil on growth and aflatoxin formation by Aspergillus flavus in culture media and cheese. *Food. Chem. Toxicol.* 47, 2397–2400. doi: 10.1016/j.fct.2009.05.024

Gowda, N. K. S., Malathi, V., and Suganthi, R. U. (2004). Effect of some chemical and herbal compounds on growth of *Aspergillus parasiticus* and aflatoxin production. *Ani Feed Sci. Technol.* 116, 281–291. doi: 10.1016/j.anifeedsci.2004.02.008

Guan, S., Zhao, L., Qiugang, M. A., Zhou, T., Wang, N., Xinxu, Hu, et al. (2010). In Vitro efficacy of *Myxococcus fulvus* ANSM068 to biotransform aflatoxin B1. *Int. J. Mol. Sci.* 11, 4063–4079. doi: 10.3390/ijms11104063

Guengerich, F. P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83. doi: 10.1021/tx700079z

Guengerich, F. P. (2008). Cytochrome P450 reactions and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83. doi: 10.1021/tx700079z

Hajare, S. S., Hajare, S. N., and Sharma, A. (2005). Aflatoxin inactivation using aqueous extract of ajowan (*Trachyspermum ammi*) seeds. *J. Food Sci.* 70, 29–34. doi: 10.1111/j.1365-2621.2005.tb09016.x

Hartl, M., and Humpf, H. U. (2000). Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. *Food. Chemi. Toxicol.* 38, 1097–1102. doi: 10.1016/S0278-6915(00)00112-5

Harwing, J., and Scott, P. M. (1971). Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* 21, 1011–1016.

Hernandez-Hierro, J. M., Garcia-Villanova, R. J., Torrero, P. R., and Fonseca, I. M. T. (2008). Aflatoxins and Ochratoxin A in red paprika for retail sale in Spain: occurrence and evaluation of a simultaneous analytical method. *J. Agric. Food Chem.* 56, 751–756. doi: 10.1021/jf073002c

Hussein, H., and Brasel, J. (2001). Toxicity, metabolism, and impact on humans and animals. *Toxicology* 167, 101–134. doi: 10.1016/S0300-483X(01)00471-1

Hwan, D. J., and Choi, D. K. (2007). Aflatoxins: detection, toxicity, and biosynthesis. *Biotech. Bioproc. Engineer* 12, 585–593. doi: 10.1007/BF02931073

Inoue, T., Nagatomi, Y., Uyama, A., and Mochizuki, N. (2013). Degradation of aflatoxin B1 during fermentation of alcoholic beverages. *Toxins* 5, 1219–1229. doi: 10.3390/toxins5071219

Joseph, G. S., Jayaprakasha, G. K., Selvi, A. T., Sena, B. S., and Sakariah, K. K. (2005). Antiaflatoxigenic and antioxidant activities of *Garcinia* extract. *Int. J. Food Microbiol.* 101, 153–160. doi: 10.1016/j.ijfoodmicro.2004.11.001

Kannan, K., and Velazhahan, R. (2014). The potential of leaf extract of *Barleria lupulina* for detoxification of aflatoxins. *Indian Phytopathol.* 67, 298–302.

Khair-ul-Bariyah, S., Ahmed, D., and Ikram, M. (2012). *Ocimum Basilicum*: a review on phytochemical and pharmacological studies. *Pak. J. Chem.* 2, 78–85. doi: 10.15228/2012.v02.i02.p05

Krishnamurthy, Y. L., and Shashikala, J. (2006). Inhibition of aflatoxin B1 production of *Aspergillus flavus*, isolated from soybean seeds by certain natural plant products. *Appl. Microbiol.* 43, 469–474. doi: 10.1111/j.1472-765X.2006.02011.x

Kuhn, D. M., and Ghannoum, M. A. (2003). Indoor mold, toxigenic fungi, and *Stachybotrys chartarum*: infectious disease perspective. *Clin. Microbiol. Rev.* 16, 144–172. doi: 10.1128/CMR.16.1.144-172.2003

Lee, L. S., Dunn, J. J., Delucca, A. J., and Ciegler, A. (1981). Role of lactone ring of aflatoxin B1 in toxicity and mutagenicity. *Experientia* 37, 16–17. doi: 10.1007/BF01965543

Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A., Caraffa, M. F., et al. (1996). Fusaproliferin production by *Fusarium subglutinans* and its toxicity to *Artemia salina* SF-9 insect cells, and IARC / LCL 171 human B limphocytes. *Appl. Environ. Microbiol.* 62, 3378–3384.

Luo, X., Wang, R., Wang, L., Wang, Y., and Chen, Z. (2013). Structural elucidation and toxicity analyses of the degradation products of aflatoxin B1 by aqueous ozone. *Food Control* 31, 331–336. doi: 10.1016/j.foodcont.2012.10.030

Maraqa, A., Alsharoa, N. F., Farah, H., Albjeirami, W. M., Shakya, A. K., and Sallal, A. J. (2007). Effect of *Nigella sativa* extract and oil on aflatoxin production by *Aspergillus flavus*. *Turk. J. Biol.* 31, 155–159.

Méndez-Albores, J. A., Villa, G. A., Del Rio-García, J. C., and Martínez, E. M. (2004). Aflatoxin-detoxification achieved with Mexican traditional nixtamalization process (MTNP) is reversible. *J. Sci. Food Agri.* 84, 1611–1614. doi: 10.1002/jsfa.1853

Montes-Belmont, R., and Carvajall, M. (1998). Control of *Aspergillus flavus* in maize with plant essential oils and their components. *J. Food Prot.* 61, 616–619.

Moretti, A., Mule, G., Ritieni, A., and Logrieco, A. (2007). Further data on the production of beauvericin, enniatins and fusaproliferin and toxicity to *Artemia salina* by *Fusarium* species *Gibberella fujikuroi* species complex. *Int. J. Food Microbiol.* 118, 158–163.

Neelam, C., Ranjan, B., Komal, S., and Nootan, C. (2011). Review on *Cassia fistula*. *Int. J. Res. Ayurveda Pharm.* 2, 426–430.

Nwachukwu, E. O., and Umchuruba, C. I. (2001). Antifungal activities of some leaf extracts on seed-borne fungi of African yam bean seeds, seed germination and seedling emergence. *J. Appl. Sci. Environ. Manag.* 5, 29–32.

Phongpaichit, S., Pujenjob, N., Rukachaisirikul, V., and Ongsakul, M. (2004). Antifungal activity from leaf extracts of *Cassia alata*, *Cassia fistula*, and *Cassia tora* L. Songklanakarin. *J. Sci. Technol.* 26, 741–748.

Pujo, A., Udomsilp, J., Khan, K. P., and Thobunluepop, P. (2009). Antifungal activity of essential oils from basil (*Ocimum basilicum* Linn.) and sweet fennel (*Ocimum gratissimum* Linn.): alternative strategies to control pathogenic fungi in organic rice. *As. J. Food Ag-Ind. (Spec. Issue)* 2, S2–S9.

Ramesh, J., Sarathchandra, G., and Sureshkumar, V. (2013). Analysis of feed samples for aflatoxin B1 contamination by HPTLC – a validated method. *Int. J. Curr. Microbiol. App. Sci.* 2, 373–377.

Reddy, K. R. N., Reddy, C. S., and Muralidharan, K. (2009). Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. *Food Control* 20, 173–178. doi: 10.1016/j.foodcont.2008.03.009

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses- An overview. *Int J. Food Microbiol.* 119, 3–10. doi: 10.1016/j.ijfoodmicro.2007.07.019

Rustom, I. Y. S. (1997). Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chem.* 59, 57–67. doi: 10.1016/S0308-8146(96)00096-9

Samuel, M. S., Sivaramakrishna, A., and Mehta, A. (2014). Degradation and detoxification of aflatoxin B1 by *Pseudomonas putida*. *Int. Biodeter. Biodegr.* 86, 202–209. doi: 10.1016/j.ibiod.2013.08.026

Satish, S., Mohana, D. C., Ranhavendra, M. P., and Raveesha, K. A. (2007). Antifungal activity of some plant extracts against important seed borne pathogens of *Aspergillus* sp. *J. Agri. Technol.* 3, 109–119.

Schmidt, R. (1989). "The application of *Artemia salina* L. bioassay for screening of aflatoxins," in *Aspergillus: Mycotoxins Taxonomy and Pathogenicity,* ed. J. Chelkowski (Amsterdam: Elsevier), 121–130.

Soliman, K. M., and Badeaa, R. I. (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food. Chem Toxicol* 40, 1669–1675. doi: 10.1016/S0278-6915(02)00120-5

Solis, P. N., Wright, C. W., Anderson, M. M., Gupta, M. P., and Phillipson, J. D. (1993). A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). *Planta Med.* 59, 250–252. doi: 10.1055/s-2006-959661

Stroka, J., Otterdijk, R., and Anklam, E. (2000). Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices. *J. Chromatogr.* 904, 251–256. doi: 10.1016/S0021-9673(00)00930-4

Suleiman, M. N., Emua, S. A., and Taiga, A. (2008). Effect of aqueous extracts on a spot fungus (*Fusarium* sp.) isolated from cowpea. *Am-Eurasian J. Sust. Agri.* 2, 261–263.

Sur, E., and Celik, I. (2003). Effects of aflatoxin B1 on the development of bursa of fabricius and blood lymphocyte acid phosphatase of the chicken. *Br. Poult. Sci.* 44, 558–566. doi: 10.1080/00071660310001618352

Tanackov, K. S., Dimic, G., Levic, J., and Tuco, D. (2011). Antifungal activities of basil (*Ocimum basilicum* L.) extract on *Fusarium* species. *Afri J. Biotech.* 10, 10188–10195.

Turner, N. W., Subrahmanyam, S., and Piletsky, S. A. (2010). Analytical methods for determination of mycotoxins: a review. *Anal. Chim. Acta* 632, 168–180. doi: 10.1016/j.aca.2008.11.010

Varma, J., and Dubey, N. K. (1999). Prospective of botanical and microbial products aspesticides of tomorrow. *Curr. Sci.* 76, 172–179.

Velazhahan, R., Vijayanandraj, S., Vijayasamundeeswari, A., Paranidharan, V., Samiyappan, R., Iwamoto, T., et al. (2010). Detoxification of aflatoxins by seed extracts of the medicinal plant. *Food Control* 21, 719–725.

Vijayanandraj, S., Brinda, R., Kannan, K., Adhithya, R., Vinothini, S., Senthil, K., et al. (2014). Detoxification of aflatoxin B1 by an aqueous extract from leaves of *Adhatoda* vasica Nees. *Microbiol. Res.* 169, 294–300. doi: 10.1016/j.micres.2013.07.008

Wang, F., Xie, F., Xue, X., Wang, Z., Fan, B., and Ha, Y. (2011). Structure elucidation and toxicity analysis of the radiolytic produts of aflatoxin B1 in methanol-water solution. *J. Hazard. Mater.* 192, 1192–1202. doi: 10.1016/j.jhazmat.2011.06.027

Wild, C. P., and Montesano, R. (2009). A model of interaction: aflatoxins and hepatitis viruses in liver cancer etiology and prevention. *Cancer Lett.* 286, 22–28. doi: 10.1016/j.canlet.2009.02.053

Yang, W. Z., Benchaar, C., Ametaj, B. N., Chaves, A. V., He, M. L., and McAllister, T. A. (2007). Effects of garlic and juniper berry essential oils on ruminal fermentation, site and extent of digestion in lactating cows. *J. Dairy Sci.* 90, 5671–5681. doi: 10.3168/jds.2007-0369

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.

frontiers in Microbiology

REVIEW
published: 06 July 2016
doi: 10.3389/fmicb.2016.01051

Marine Biotoxins: Occurrence, Toxicity, Regulatory Limits and Reference Methods

Pierina Visciano[1], Maria Schirone[1], Miriam Berti[2], Anna Milandri[3], Rosanna Tofalo[1] and Giovanna Suzzi[1]*

[1] Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy, [2] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy, [3] National Reference Laboratory for Marine Biotoxins, Fondazione Centro Ricerche Marine, Cesenatico, Italy

Harmful algal blooms are natural phenomena caused by the massive growth of phytoplankton that may contain highly toxic chemicals, the so-called marine biotoxins causing illness and even death to both aquatic organisms and humans. Their occurrence has been increased in frequency and severity, suggesting a worldwide public health risk. Marine biotoxins can accumulate in bivalve molluscs and regulatory limits have been set for some classes according to European Union legislation. These compounds can be distinguished in water- and fat-soluble molecules. The first group involves those of Paralytic Shellfish Poisoning and Amnesic Shellfish Poisoning, whereas the toxins soluble in fat can cause Diarrheic Shellfish Poisoning and Neurotoxic Shellfish Poisoning. Due to the lack of long-term toxicity studies, establishing tolerable daily intakes for any of these marine biotoxins was not possible, but an acute reference dose can be considered more appropriate, because these molecules show an acute toxicity. Dietary exposure assessment is linked both to the levels of marine biotoxins present in bivalve molluscs and the portion that could be eaten by consumers. Symptoms may vary from a severe gastrointestinal intoxication with diarrhea, nausea, vomiting, and abdominal cramps to neurological disorders such as ataxia, dizziness, partial paralysis, and respiratory distress. The official method for the detection of marine biotoxins is the mouse bioassay (MBA) showing some limits due to ethical restrictions and insufficient specificity. For this reason, the liquid chromatography–mass spectrometry method has replaced MBA as the reference technique. However, the monitoring of algal blooms producing marine biotoxins should be regularly assessed in order to obtain more reliable, accurate estimates of bloom toxicity and their potential impacts.

Keywords: shellfish poisoning, toxicity, symptoms, human health, detection method

OPEN ACCESS

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Leda Giannuzzi, University of La Plata – Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina
Andrew K. Ottens, Virginia Commonwealth University, USA

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

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

Received: *28 April 2016*
Accepted: *23 June 2016*
Published: *06 July 2016*

Citation:
Visciano P, Schirone M, Berti M, Milandri A, Tofalo R and Suzzi G (2016) Marine Biotoxins: Occurrence, Toxicity, Regulatory Limits and Reference Methods. Front. Microbiol. 7:1051. doi: 10.3389/fmicb.2016.01051

INTRODUCTION

Harmful algal blooms (HAB) are natural phenomena carried out by the overgrowth of marine phytoplankton (Ferreiro et al., 2015). Over the last decades the occurrence and intensity of HAB appear to be increasing on a global scale due to rising ocean temperatures and growing coastal eutrophication (McCarthy et al., 2015). The geographical expansion of HAB can also be associated with ballast waters transporting encysted algae to new environments or massive algae spreading

caused by aquaculture practices (Anderson et al., 2002; Maso and Garcés, 2006; Smayda, 2007). Among the thousands of microalgal species known in nature, about 300 are involved in harmful events and more than 100 (of these species) produce persistent natural toxins that can cause intoxication or even death in humans and animals. Moreover, such toxic outbreaks can have consequences on other components of human wellbeing both in terms of socio-economic impact and costs. The main factors affecting HAB occurrence and their influence on the environment, shellfisheries and consumers are shown in **Figure 1**. The direct impact of HAB on human health is linked to poisoning after consumption of contaminated seafood, skin contact with contaminated water, and/or inhalation of aerosolized biotoxins. Nowadays the increasing of information about human exposure hazards as well as the strategies able to prevent HAB occurrence in seafood need to be understood and improved, because such phenomenon results from complex interactions among physical, chemical, and biological processes in the marine environment (Berdalet et al., 2015). Moreover, even if the classic description of intoxication due to marine biotoxins is represented by acute symptoms, there is also little information about the impacts of long-term low-level exposure together with epidemiological studies regarding the populations at risk (Lefebvre and Robertson, 2010). The toxicological mechanisms of some marine biotoxins are yet incompletely understood and no observations of adverse effects in humans have been reported.

It is well known that marine biotoxins can accumulate in the tissues of some marine organisms, particularly filter-feeding bivalves. In all cases, they are *de novo* produced by certain photo- or mixo-trophic microalgae not by the shellfish, and filter-feeding transfers them to the molluscs (Nielsen et al., 2016). Mussels filter approximately 20 L water/h and during HAB, waters may contain several million algal cells per liter. Some phytoplankton species in these blooms produce phycotoxins that can accumulate through the marine food webs (Ferreiro et al., 2015). Outbreaks of intoxication in humans due to marine biotoxins (**Figure 2**) are caused by the ingestion of contaminated shellfish and can have a wide range of symptoms linked to the specific toxic compound (Richter and Fidler, 2015; Turner and Goya, 2015). Damage to nervous or intestinal system rather than loss of memory have been observed depending on the type of algal bloom. Among them some dinoflagellate and diatom species, such as *Noctiluca scintillans* and *Skeletonema costatum*, are responsible only for discoloration of water and death of fish and other marine organisms, whereas species belonging to the genera *Alexandrium*, *Gymnodinium*, *Dinophysis*, and *Pseudo-nitzschia* are the main producers of marine biotoxins for humans (Berti and Milandri, 2014; Bruce et al., 2015).

Marine biotoxins can be distinguished in water- and fat-soluble according to their solubility. On the basis of their poisoning symptoms, they are also classified as toxins causing paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), diarrheic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and ciguatera fish poisoning (CFP) (Poletti et al., 2003).

According to their own chemical structure, marine biotoxins are classified into eight groups – namely the azaspiracid (AZA), brevetoxin (BTX), cyclic imine (CI), domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX), and yessotoxin (YTX) groups. Two additional groups, palytoxin (PlTX) and ciguatoxin (CTX), are also considered (EFSA, 2009a). Marine biotoxins and toxic effects for consumers after the ingestion of contaminated seafood are reported in **Table 1**.

Human exposure to biotoxins generally refers only to occasional consumption and is characterized by acute and short-term events, so that acute reference doses (ARfD) have been established for these toxic compounds rather than tolerable daily intakes that could not be determined due to the lack of appropriate toxicological data. The ARfD are derived from the corresponding concentration of marine biotoxins per kg of shellfish meat when consuming a large portion (400 g) of shellfish. A crucial issue, when deriving both these values from the most relevant toxicological information, is the size of safety factors. Generally, default values of 10 and 100 on the basis of human and animal data are applied, respectively (FAO/IOC/WHO, 2004). Furthermore, the safety factor is usually increased if, due to the critical effect, there is a lowest observable adverse effect level (LOAEL) instead of a no observable adverse effect level (NOAEL). The ARfD, safety factors, LOAEL and NOAEL for each individual marine biotoxin, as well as regulatory limits and maximum levels based on consumption of 400 g of shellfish are reported in **Table 2**.

The present review focuses on the specific toxicity and epidemiology of marine biotoxins, the regulatory limits set by European Union (EU) legislation for the most toxic compounds as well as the methods of detection and quantification.

PARALYTIC SHELLFISH POISONING

Paralytic shellfish poisoning, caused by 58 closely related compounds based on a tetrahydropurine skeleton (Burrell et al., 2013), is one of the most studied intoxications with serious symptoms in humans. In particular, it is the result of exposure to saxitoxin (STX) and gonyautoxin (GTX). In 1957, a PSP toxin was isolated in clams (*Saxidomus giganteus*) living in Alaska coastal areas and in 1975 the chemical structure was assigned to STX. The main producers of PSP toxins are dinoflagellates of the genus *Alexandrium* occurring along the Atlantic and Pacific coast (Bernd and Bernd, 2008) but also in the Mediterranean Sea, where other species such as *Gymnodinium catenatum* can be present (Berti and Milandri, 2014).

More than 30 STX analogs have been identified and grouped into four subgroups: carbamate, *N*-sulfo-carbamoyl, decarbamoyl, and hydroxylated saxitoxins (EFSA, 2009a).

The intake of biotoxins necessary to cause human poisoning varies greatly due to the differences in susceptibility among individuals. The symptoms are similar to paralytic phenomena (cramp, signs of paralysis, and blocking of respiration) because PSP toxins are potential neurotoxins blocking the excitation current in nerve and muscle cells (Schirone et al., 2011). Human PSP outbreaks can be distinguished in mild, moderately

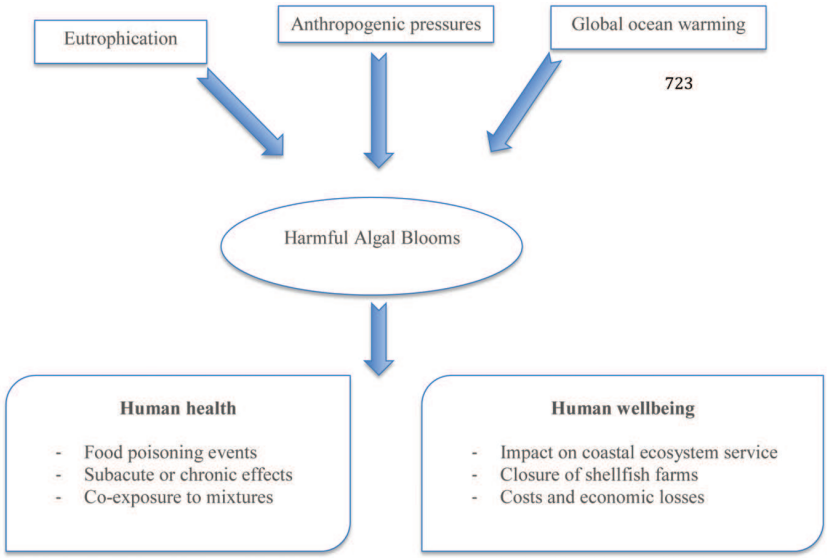


FIGURE 1 | Factors involved in the occurrence of harmful algal blooms and the consequences on human health and wellbeing (modified from Berdalet et al., 2015).

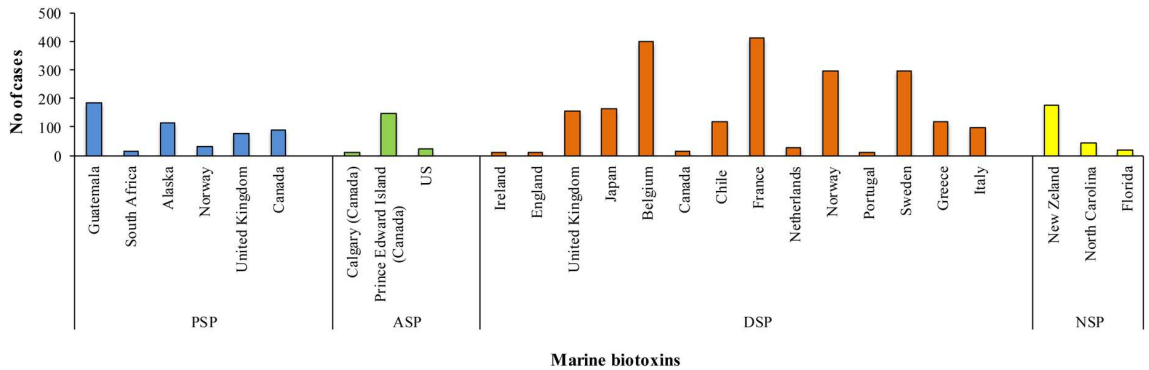


FIGURE 2 | Outbreaks (number of cases) of poisoning due to marine biotoxins occurred from 1970 through 2010.

severe and extremely severe. The symptoms occurring in the mild form include tingling sensation or numbness around the lips, gradually spreading to the face and neck, a prickly sensation in fingertips and toes, headache, dizziness, and nausea. The moderately severe illness is characterized by incoherent speech, progression of prickly sensation to arms and legs, stiffness and non-coordination of limbs, general weakness and feeling of lightness, then slight respiratory difficulty and rapid pulse plus backache as late symptoms. In the extremely severe form muscular paralysis, pronounced respiratory difficulty and a choking sensation may occur. In fatal cases, death is caused by respiratory paralysis occurring within 2–12 h after the consumption of contaminated shellfish, in absence of artificial respiration. Patients who survive PSP for 24 h, with or without mechanical intervention, have a high probability of a full and rapid recovery (FAO/IOC/WHO, 2004).

Blooms of toxic microalgae producing PSP toxins represent an expanding threat to both human health and fishery resources all over the world. In particular, PSP has been recognized for over a century as a clinical entity in the austral part of South America. A study regarding an outbreak in the Patagonia fjords reported that some fishermen were intoxicated by consumption of bivalve *Aulacomya ater* and two of them died after 3–4 h; in that case up to 8575 μg of STX, equivalent/100 g of shellfish meat, were detected by MBA (García et al., 2004).

The highest number of PSP cases (2124 with 120 deaths) was reported in the Philippines from 1983 to 2002 (Ching et al., 2015). The human fatality proportion from PSP varies according to the ability of the local medical system to treat such intoxication. In a short report, the authors referred that 45 people living along the coast of Nicaragua developed symptoms of PSP and a person died. In similar outbreaks in Southeast Asia and Latin America, the case fatality proportion ranged between 2 and 14% whereas

TABLE 1 | Classification of marine biotoxins and main adverse effects in humans.

Marine biotoxin	Group	Source	Symptomatology
Saxitoxin	PSP	*Alexandrium* spp. *Gymnodinium catenatum* *Pyrodinium bahamense*	• Gastrointestinal symptoms • Paralytic phenomena • Recovery or death
Domoic acid	ASP	*Pseudo-nitzschia* spp. *Nitzschia* spp.	• Gastrointestinal and neurological symptoms • Cardiac or respiratory problems • Recovery or death
Okadaic acid		*Prorocentrum lima* *Dinophysis* spp.	• Gastrointestinal symptoms • Recovery within 3 days
Pectenotoxin		*Dinophysis* spp.	• Gastrointestinal symptoms
Yessotoxin	DSP	*Protoceratium reticulatum* *Lingulodinium polyedrum* *Gonyaulax spinifera*	• Lack of observations in humans
Azaspiracid		*Amphidoma languida* *Azadinium spinosum*	• Gastrointestinal symptoms
Brevetoxin	NSP	*Karenia brevis*	• Gastrointestinal and neurological symptoms • Respiratory problems • Recovery or death
Ciguatoxin	CFP	*Gambierdiscus* spp.	• Gastrointestinal symptoms • Cardiovascular or neurological problems
Cyclic imine		*Alexandrium* spp. *Karenia* spp.	• Lack of observations in humans
Palytoxin		*Palythoa* spp. *Ostreopsis* spp.	• Gastrointestinal symptoms • Muscle and cutaneous problems

no death occurred among more than 200 cases in Europe and North America (Callejas et al., 2015).

AMNESIC SHELLFISH POISONING

Domoic acid and other toxic DA isomers, produced mostly by marine diatoms of the genus *Pseudo-nitzschia*, are responsible for ASP. This compound is a cyclic tricarboxylic amino acid with many structural and functional similarities with kainic acid, an analog to glutamic acid. In particular, DA binds with glutamate receptors in central nervous system causing over-stimulation of these receptors with production of reactive oxygen species and sometimes cell death (Schwarz et al., 2014).

In humans, symptoms range from gastrointestinal effects (nausea, vomiting, diarrhea, or abdominal cramps) and/or neurological signs (confusion, lethargy, disorientation, paresthesia, and short-term memory loss) and in extreme cases coma or death.

The accumulation of DA in bivalve tissues depends on several factors, such as the presence of *Pseudo-nitzschia* species and their toxic content as well as on the balance of DA accumulation and depuration by shellfish (Mafra et al., 2010).

There are no reported cases of human illness associated with DA in any European countries or regions other than North America. However, in the absence of formal reporting systems, it cannot be assumed that mild cases have not occurred. Furthermore, the data relating to cases of human poisoning caused by DA are limited, except for a unique ASP outbreak in Canada in 1987. Such event involved 150 people with 19 hospitalization and 4 deaths after consumption of contaminated mussels (Jeffery et al., 2004; Álvarez et al., 2015).

Effective seafood monitoring programs for the detection of DA in the shellfish and coastal waters worldwide have been implemented by many regulatory agencies and therefore, human ASP events have not been documented since the first outbreak in 1987.

Domoic acid has also a toxic effect on marine wildlife and many poisoning events have been described in marine birds and mammals. A chronic DA epileptic syndrome was characterized in sea lions between 1998 and 2006. Therefore, sea lions are used as a sentinel species able to predicting potential hazard for human health (Lefebvre and Robertson, 2010).

DIARRHEIC SHELLFISH POISONING

This syndrome is one of the known intoxications caused by marine biotoxins and represents a frequent concern in shellfish industries, because it can cause a prolonged closure of mussel

TABLE 2 | Regulatory limits, lowest observable adverse effect level (LOAEL), no observable adverse effect level (NOAEL), and acute reference dose for marine biotoxins (modified by FAO/IOC/WHO, 2004; EFSA, 2009b).

Marine biotoxins	Regulatory limits	Exposure after consumption of 400 g portion at the EU limit	LOAEL (1) NOAEL (2) µg/kg b.w.***	Safety factors Human data (H) Animal data (A)	Acute reference dose (ARfD)	Maximum concentration in shellfish meat (400 g portion) not exceeding ARfD
Okadaic acid	160 µg OA eq.*/kg SM**	64 µg OA eq./kg person	1 (1)	3 (H)	0.3 µg OA eq./kg b.w.	45 µg OA eq./kg SM
Azaspiracid	160 µg AZA eq./kg SM	64 µg AZA1 eq./kg person	0.4 (1)	10 (H)	0.2 µg AZA1 eq./kg b.w.	30 µg AZA1 eq./kg SM
Pectenotoxin	160 µg PTX eq./kg SM	64 µg PTX2 eq./kg person	–	–	0.8 µg PTX2 eq./kg b.w.	120 µg PTX2 eq./kg SM
Yessotoxin	3.75 mg YTX eq./kg SM	400 µg YTX eq./kg person	5000 (2)	100 (A)	25 µg YTX eq./kg b.w.	3.75 mg YTX eq./kg SM
Saxitoxin	800 µg PSP/kg SM	320 µg STX eq./kg person	2 (1)	3 (H)	0.5 µg STX eq./kg b.w.	75 µg STX eq./kg SM
Domoic acid	20 mg DA/kg SM	8 mg DA/kg person	1000 (1)	10 (H)	30 µg DA/kg b.w.	4.5 mg DA/kg SM

*eq. = equivalent; **SM = shellfish meat; ***b.w. = body weight; – = not reported.

harvesting activity. The interruption of mussel sales and early public announcements are highly effective in controlling DSP outbreaks (Chen et al., 2013).

The accumulation of DSP toxins in any shellfish species is still little known. These toxins are produced by dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*, even if the first genus is considered the main source, whereas *Prorocentrum* species are benthic and thus unavailable for suspension-feeding mussels (Nielsen et al., 2016).

Diarrheic shellfish poisoning toxins are polyether compounds with distinctive chemical structures grouped into four structural classes (Berti and Milandri, 2014): okadaic acid (OA) and its derivatives (dinophysistoxin or DTX); pectenotoxin (PTX); yessotoxin and its derivatives (YTX) and azaspiracid (AZA). The symptoms caused by OA group include diarrhea, nausea, vomiting, and abdominal pain, starting 30 min to a few hours after consumption of contaminated shellfish, with complete recovery within 3 days (Trainer et al., 2013). Among the other mentioned classes, PTX and YTX were first believed to be relevant in DSP syndrome due to their co-occurrence in shellfish with OA group, but they have not be implicated in human illness (Li et al., 2012), whereas AZA causes a form of poisoning characterized by nausea, vomiting, diarrhea, and stomach cramps.

Regarding to OA group toxins, it has been reported that they are soluble in fats and easily cross the cell membrane determining an inhibition of serine and threonine phosphoprotein phosphatases (EFSA, 2008a).

Pectenotoxins are cyclic polyethers of marine origin and PTX-2 is the main toxin of such group. They have been associated with cases of diarrhea and other symptoms similar to those induced by OA and its derivatives (Poletti et al., 2003). They disrupt actin in the cytoskeleton, and may cause cell cycle arrest and cell death (Espiña et al., 2008).

Yessotoxin and its analogs are polyether toxins produced by dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera*. Even if they have been associated with DSP group, they do not cause diarrhea or inhibition of protein phosphatases (Paz et al., 2008) and their symptoms are still unknown in humans (Visciano et al., 2013).

Azaspiracids are nitrogen-containing polyether toxins comprising an unique spiral ring assembly containing a heterocyclic amine and an aliphatic carboxylic acid moiety. Even if 21 different analogs have been identified, AZA1, AZA2, and AZA3 are the most important ones depending on occurrence and toxicity (EFSA, 2008b).

Vale (2012) reported that the contamination of Portuguese shellfish with DSP toxins is an annually recurrent phenomenon, influenced by meteorological parameters affecting the blooming of the causative toxic microalgae, belonging to genus *Dinophysis*.

High levels of DSP toxins were also detected in shellfish harvested along the Chinese coast causing illness in more than 200 people in the year 2011 (Li et al., 2012). In the same year, 57 cases were described from Zheijang Province, China (Chen et al., 2013).

A DSP outbreak involving three people after the consumption of contaminated mussels was reported in Washington State

(Trainer et al., 2013). Coincidentally 62 DSP illnesses occurred in British Columbia due to the ingestion of Pacific coast mussels, representing the first report of DSP in Western Canada (Taylor et al., 2013).

The DSP toxins are the most frequent and abundant marine biotoxins regularly found in shellfish from Southern European coastal areas (Braga et al., 2016). In France 11 DSP outbreaks involving 45 individuals were described in 2009. The contaminated mussels contained OA group toxin concentrations approximately eight times higher than what the European regulatory limits provide (Hossen et al., 2011). In 2010, more than 300 people in Northern Italy were poisoned by OA contaminated mussels (Bacchiocchi et al., 2015).

Symptoms of intoxication caused by YTX in humans are still unknown due to the fact that no human intoxication has been reported to date. However, toxicological studies carried out in rodents showed that YTX can be highly toxic when injected intraperitoneally (Visciano et al., 2013). Furthermore, PTX do not induce DSP-like symptoms even if acute toxicity can be observed in MBA (Wang et al., 2015). High levels of YTX were reported in shellfish farmed along the North Adriatic coast in 2004 correlated with the increasing presence of *G. spinifera* (Bacchiocchi et al., 2015).

Among the most recently identified groups of toxins causing human intoxication, AZA has been associated with shellfish poisoning in several people after consumption of mussels in the Netherlands in 1995. After that first event it was found in mussels harvested all over Europe, North-West Africa and Chile.

Some other outbreaks due to AZA were described in North-Western Ireland, affecting 12 people after the consumption of locally cultivated mussels; 10 individuals became ill with DSP symptoms in Ravenna (Italy) and 400 poisonings were reported in Belgium from mussels cultivated in Denmark (Furey et al., 2010).

NEUROTOXIC SHELLFISH POISONING

The brevetoxins (BTX) produced by the "Florida red tide" dinoflagellate *Karenia brevis* are polyether ladder compounds responsible for massive fish and marine mammal mortality above all in the Gulf of Mexico (Cassell et al., 2015) but also along the East coast of the United States and New Zealand (EFSA, 2010a). In humans, BTX are the causative agents of NSP and asthma-like symptoms through inhalation exposure (Sun et al., 2016).

Neurotoxic shellfish poisoning is characterized by both neurological and gastrointestinal effects which include nausea, vomiting, diarrhea, parasthesia, cramps, bronchoconstriction, paralysis, seizures, coma and, in extreme cases, may lead to death (Watkins et al., 2008).

On the basis of their molecular structures formed of 10–11 *trans*-fused rings, BTX have 2 skeletal backbones: A- and B-type and a variety of side chain substituents on the rings distal to the lactone (Cassell et al., 2015). They bind to and activate the voltage-gated sodium channels in cell membrane causing depolarization of neuronal and muscle cell membranes (EFSA, 2010a).

Brevetoxins have been implicated in the death of large numbers of fish and in morbidity and mortality of marine mammals (Gebhard et al., 2015).

There has been only a small number of sporadic cases of NSP in the United States in humans, with hospitalization but no fatalities. Outbreaks occurred in Florida with 2 cases in 1995, 3 in 1996, 2 in 2001, and 4 in 2005. In last event, 2 out of the 4 patients were children, more seriously affected than the adults. Another outbreak characterized by severity of symptoms was reported in 2006 in Florida (Watkins et al., 2008).

Respiratory effects associated with aerosolized red tide involve sneezing, throat irritation, burning, and itchy; in the case of asthmatics individuals a significantly increase of these symptoms has been observed (Fleming et al., 2007; Bean et al., 2011).

CIGUATERA FISH POISONING

Ciguatera fish poisoning (CFP) is the most common foodborne illness worldwide with 50,000–500,000 incidences *per annum* (Mattei et al., 2014). The causative agents are toxins belonging to the CTX group (Hossen et al., 2015). Such syndrome results from the bioaccumulation and metabolism of precursor toxins along the fish food web. Precursor toxins (named gambiertoxins) are produced by benthic dinoflagellates of the genus *Gambierdiscus*, whose distribution includes tropical and subtropical coral reef areas, and accumulated by large predatory fishes, such as Spanish mackerels, moray eels, barracuda and snappers (Litaker et al., 2009; EFSA, 2010b; Chan, 2015).

Ciguatoxins are lipid-soluble polyether compounds consisting of 13–14 rings fused by ether linkages into a rigid ladder-like structure. More than 20 CTX analogs have been identified (EFSA, 2010b). At the cellular level, CTX activate the sodium ion channels causing cell membrane excitability and cell disruption (Hidalgo et al., 2002).

With regard to symptomatology, the acute period (24 h) is characterized by gastrointestinal problems (nausea, vomiting, abdominal pain, and diarrhea) whereas cardiovascular (bradycardia and hypertension) and neurological complications may occur within a few hours to 2 weeks after exposure, such as paresthesias, disesthesias, and hyperesthesias (Silva et al., 2015).

Even if the occurrence of CTX is generally restricted to some specific areas, in recent years a spread of ciguatoxic fish has been observed near European coasts and in the Mediterranean Sea. Recently, 6 outbreaks involving 28 people between 2010 and 2011 and one event with 20 cases in 2012 were described in New York and Northern Germany, respectively (Mattei et al., 2014).

A survey about the epidemiology of CFP in Asia reported 3 large outbreaks involving 100–200 patients in China, whereas 11 cases occurred between 1991 and 2008 in Taiwan. Among them, there was one death from ciguatera in 1998. In Malaysia 11 individuals were hospitalized in 2010 after the consumption of head and viscera of contaminated fish and 33 outbreaks affecting 103 subjects were described in Japan (Chan, 2015).

CYCLIC IMINES

This group includes spirolides (SPX), gymnodimines (GYM), pinnatoxins (PnTX), and pteriatoxins (PtTX); SPX and GYM are produced by algal species from the genera *Alexandrium* and *Karenia*, whereas *Vulcanodinium rugosum* has been identified as the producer of PnTX (McCarthy et al., 2015). Recent studies suggest that PtTX are biotransformation products of PnTX in shellfish (Selwood et al., 2010). On the basis of their chemical structures, CI are macrocyclic compounds with imine and spiro-linked ether moieties (EFSA, 2010c). These moieties, common to all the members of CI, are thought to be the main structural determinants for their toxicity. Recent studies showed that CI antagonized both muscle type and heteromeric and homomeric neuronal nicotinic acetylcholine receptors as well as muscarinic acetylcholine receptors (Hellyer et al., 2013).

No acute symptoms have been recorded in humans and the toxicity of SPX and GYM is still being explored in mammalian models (Marrouchi et al., 2013); they induce a rapid death (within minutes) in laboratory mice injected intraperitoneally but they are often co-extracted with other lipophilic toxins such as OA and its analogs producing false positive in MBA test (Salgado et al., 2015). The first isolation of SPX was reported by Hu et al. (1995) from shellfish collected along the South Eastern coast of Nova Scotia, Canada, whereas GYM was isolated and characterized in the early 1990s from New Zealand oysters (MacKenzie, 1994).

Pinnatoxins and PtTX were originally identified in Japan in shellfish belonging to the genera *Pinna* and *Pteria*, respectively (Rundberget et al., 2011).

No acute poisoning events have been linked to contamination by PnTX and only one outbreak was reported associated with the bivalve of genus *Pinna*. However, PnTX show fast-acting toxicity when injected intraperitoneally into mice (Hess et al., 2013).

Despite the high acute toxicity in MBA, CI are not currently regulated in seafood due to the fact that acute poisoning in humans have not been directly related to shellfish contamination (Harju et al., 2016).

PALYTOXIN

Palytoxin are potent non-protein marine compounds produced by corals belonging to the genus *Palythoa* and dinoflagellates belonging to the genus *Ostreopsis*. Several analogs have been identified (Biré et al., 2013).

Such group of toxins involves complex polyhydroxylated compounds with both lipophilic and hydrophilic areas.

Several symptoms were described after the consumption of shellfish and included a metallic taste, gastrointestinal malaise, diarrhea, nausea, vomiting, ataxias, dizziness myalgia, dyspnea, convulsion, and bradicardia.

About 200 people showed cutaneous and respiratory problems after exposure to marine aerosols containing PlTX in 2005 in Italy, whereas similar symptoms were reported between 2006 and 2009 in France (Biré et al., 2013). In literature, some cases of intoxication were fatal (Deeds and Schwartz, 2010; Aligizaki et al., 2011).

THE EUROPEAN UNION LEGISLATION

The EU food legislation focuses also on bivalve molluscs, giving space to them in a specific section in the Annexes to the Regulations of the "Hygiene Package". In particular, Regulation (EC) No 853/2004 provides the maximum limits for marine biotoxins and Regulation (EC) No 854/2004 establishes monitoring and sampling plans in the production areas of live bivalve molluscs.

The monitoring of biotoxins in molluscs and health effects due to their consumption are important tasks for seafood control, because marine biotoxins may cause serious diseases in humans. According to the above mentioned Regulation 854/2004/EC, the production areas are periodically monitored to check the presence of toxins-producing plankton and their occurrence in live bivalve molluscs. Such monitoring is generally applied weekly during the periods when harvesting is allowed, but the sampling frequency must be representative for the considered area, taking into account the possible variations in the presence of plankton containing marine biotoxins. Such frequency may be reduced if the risk assessment on toxins or phytoplankton occurrence suggests a very low risk of intoxication. On the contrary, if the results of monitoring exceed the regulatory limits the production area shall be closed by the competent authority and can be re-opened when at least two consecutive results of biotoxin levels in molluscs meet with legislation. The closure of the production area is necessary in order to ensure that molluscs harmful to human health are not placed on the market. In fact the prevention of contaminated seafood reaching the markets is currently an effective way to protect human health (Berdalet et al., 2015).

Regulation (EC) No 2074/2005 highlights that the proposed maximum levels are based on provisional data and should be reassessed once new scientific evidence becomes available. The subsequent regulations amending the last one have provided internationally recognized methods for the different marine biotoxins. The increased complexity of biotoxin classes points out how the development of additional tools in the monitoring of production areas and molluscs is necessary. Testing methods for the detection of marine biotoxins can be implemented and refined as well. Actually these methods can be biological, functional or chemical assays, each of them showing advantages or disadvantages that must be taken into account.

The biological methods use animal models such as rats and/or mice. They are not very sensible and can be subject to interferences. Moreover, they do not give information about the concentration of the different toxins and it is difficult to identify which toxin causes the death of the mice. They are also time consuming and expensive. Even if they can have ethical problems, the MBA allows to detect new or unknown marine biotoxins during the periodic monitoring of production areas.

The functional methods use the capacity of marine biotoxins to trigger specific responses by interacting with a cellular component that selectively recognizes their structures and behaves as a receptor in sensitive systems, thereby transforming the chemical information of the incoming ligands into defined cellular effects. Therefore, the analytical challenge posed by the extreme complexity of toxin profiles is handled by the biological

system, whose responses are specific for any toxin group. The main disadvantages are represented by the employment of viable cell strains, the potential of interferences and a particular expertise of laboratory technicians.

The chemical analytical methods are the current most powerful analytical tools able to identify multiple toxins. They are based on liquid chromatography (LC) to separate marine biotoxins by an extraction step, followed by the toxin-specific detection by UV (LC–UV), fluorescence (LC–FL), or mass spectrometry (LC–MS/MS). Due to the compound specific detection, the obtained results relate to individual concentrations of the investigated compounds and can be transformed into toxic equivalent, using conversion factors.

The MBA used for detecting PSP toxins is an official AOAC method, which has been used for more than 50 years in many Member States. Such method lays down that, initially one, but preferably 2 or 3 mice are injected intraperitoneally with 1 mL of an acid extract of molluscs. The time of death is recorded and if it lasts less than 5 min, dilutions of the extract are necessary, until the time of death becomes equal to 5–7 min. At that point three mice are injected and the average time of death is determined. The toxic concentrations are calculated in Mouse Units (MU) multiplied by the dilution factor and, if necessary, by a weight correcting factor, giving the result in MU/100 g. The conversion from MU into μg of STX equivalent is obtained multiplying by a conversion factor, which is calculated by each laboratory and periodically controlled.

The HPLC chemical method with fluorimetric detection and pre-column oxidation is applicable for the determination and quantification of most PSP toxin groups. Other chemical methods to determine PSP toxins, for instance LC–MS, are still being developed.

Other methods able to detect STX-group toxins are biomolecular methods, suitable however for *screening* purposes only. The biomolecular methodologies for toxins of the STX group are based on three different strategies by means of receptors, characteristics of cytotoxicity and antibodies. With regard to the latter approach, it is important to point out that, although the antibodies are very sensitive, their main problem to detect toxins of the STX group is the lack of good cross-reactivity to all the members of the group. As the differences of toxicity among the compounds of such group might be very high too, not always the toxicity corresponds to the toxin levels quantified by the antibody.

The official tests for the DA group toxins are based on the LC with UV detection. As a *screening* system for that group of toxins, it is possible to use also an LC–MS method that is completely intra-laboratory validated. The Regulation (EC) No 1244/2007 suggested as a *screening* procedure for toxins of the DA group the use of the 2006.02 ASP method based on the *Enzyme-Linked Immunosorbent Assay* (ELISA). It shows some advantages because it is sensitive and rapid, can be automated, requires a minimum training and does not require expansive equipment. However, it must be considered that in case of conflicting results, the HPLC chemical method with UV detector is the only valid reference method.

A Standard Operating Procedure was validated under the coordination of the European Reference Laboratory for marine biotoxins (EURL-MB) in an inter-laboratory validation study carried out by the National Reference Laboratories of Belgium, France, Germany, Ireland, Italy, Netherlands, Sweden, and the United Kingdom. Such method resulted highly specific and sensitive for the direct quantitative detection of all the four groups of regulated liposoluble toxins by the LC–MS/MS, using the certified reference materials for each compound to be investigated. The chromatographic separation of toxins is performed by gradient elution. Results are reported per toxin group as requested by the EU legislation and the total toxicity is calculated using the toxicity equivalence factors (TEF) recommended by CONTAM group of EFSA.

A series of procedures, differing in the portion (hepatopancreas or whole body) to be tested and in the solvents used for extraction and purification, may be carried out to detect marine toxins as referred to in Regulation (EC) No 2074/2005 and further amendments. In particular, two different analytic procedures have been optimized: for species of molluscs with a digestive gland of large dimensions (mussels, oysters, razor-shells, scallops, etc.), the procedure of hepatopancreas (20 g) must be used, whereas for species of molluscs where the digestive gland has small dimensions (clams, tellins, etc.) or is absent (tunicates, echinoderms, marine gastropods, etc.) the procedure of whole body (100 g) must be applied. The standard operating procedure provides a further protocol, whose application is recommended when the presence of YTX in the sample is suspected.

However, the lack of specificity of the biological test for DSP and its inadequacy for satisfying the regulatory limits prompted the ban of the MBA and its replacement with validated alternative methods. The Regulation (EU) No 15/2011 identified the LC–MS/MS as the reference method for liposoluble biotoxins, even if the MBA could be used as a *screening* method for new and unknown toxins.

AUTHOR CONTRIBUTIONS

MS and PV devised and drafted the review; MB, AM, and RT prepared the literature overview; GS contributed to manuscript revision.

ACKNOWLEDGMENT

The authors thank Prof. Francesca Rosati, University of Teramo, for her support in editing the English text.

REFERENCES

Aligizaki, K., Katikou, P., Milandri, A., and Diogène, J. (2011). Occurrence of palytoxingroup toxins in seafood and future strategies to complement the present state of the art. *Toxicon* 57, 390–399. doi: 10.1016/j.toxicon.2010.11.014

Álvarez, G., Uribe, E., Regueiro, J., Martin, H., Gajardo, T., Jara, L., et al. (2015). Depuration and anatomical distribution of domoic acid in the surf clam *Mesodesma donacium*. *Toxicon* 102, 1–7. doi: 10.1016/j.toxicon.2015.05.011

Anderson, D., Glibert, P., and Burkholder, J. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries Coasts* 25, 704–726. doi: 10.1007/BF02804901

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

Bean, J. A., Fleming, L. E., Kirkpatrick, B., Backer, L. C., Nierenberg, K., Reich, A., et al. (2011). Florida red tide toxins (brevetoxins) and longitudinal respiratory effects in asthmatics. *Harmful Algae* 10, 744–748. doi: 10.1016/j.hal.2011.06.008

Berdalet, E., Fleming, L., Gowen, R., Davidson, K., Hess, P., Backer, L., et al. (2015). Marine harmful algal blooms, human health and wellbeing: challenges and opportunities in the 21st century. *J. Mar. Biol. Assoc. U.K.* 96, 61–91. doi: 10.1017/S0025315415001733

Bernd, C., and Bernd, L. (2008). Determination of marine biotoxins relevant for regulations: from the mouse bioassay to coupled LC-MS methods. *Anal. Bioanal. Chem.* 391, 117–134. doi: 10.1007/s00216-007-1778-x

Berti, M., and Milandri, A. (2014). "Le biotossine marine," in *Igiene Degli Alimenti*, eds M. Schirone and P. Visciano (Bologna: Edagricole), 163–198.

Biré, R., Trotereau, S., Lemée, R., Delpont, C., Chabot, B., Aumond, Y., et al. (2013). Occurrence of palytoxins in marine organisms from different trophic levels of the French Mediterranean coast harvested in 2009. *Harmful Algae* 28, 10–22. doi: 10.1016/j.hal.2013.04.007

Braga, A. C., ALves, R. N., Maulvault, A. L., Barbosa, V., Marques, A., and Costa, P. R. (2016). In vitro bioaccessibility of the marine biotoxins okadaic acid in shellfish. *Food Chem. Toxicol.* 89, 54–59. doi: 10.1016/j.fct.2016.01.008

Bruce, K. L., Leterme, S. C., Ellis, A. V., and Lenehan, C. E. (2015). Approaches for the detection of harmful algal blooms using oligonucleotide interactions. *Anal. Bioanal. Chem.* 407, 95–116. doi: 10.1007/s00216-014-8193-x

Burrell, S., Gunnarsson, T., Gunnarsson, K., Clarke, D., and Turner, A. D. (2013). First detection of paralytic shellfish poisoning (PSP) toxins in Icelandic mussels (*Mytilus edulis*): links to causative phytoplankton species. *Food Control* 31, 295–301. doi: 10.1016/j.foodcont.2012.10.002

Callejas, L., Darce, A. C. M., Amador, J. J., Conklin, L., Gaffga, N., Rogers, H. S., et al. (2015). Paralytic shellfish poisonings resulting from an algal bloom in Nicaragua. *BMC Res. Notes* 8:74. doi: 10.1186/s13104-015-1012-4

Cassell, R. T., Chen, W., Thomas, S., Liu, L., and Rein, K. S. (2015). Brevetoxin, the dinoflagellate neurotoxin, localizes to thylakoid membranes and interacts with the light-harvesting complex II (LHCII) of photosystem II. *Chembiochem* 16, 1060–1067. doi: 10.1002/cbic.201402669

Chan, T. Y. K. (2015). Ciguatera fish poisoning in East Asia and Southeast Asia. *Mar. Drugs* 13, 3466–3478. doi: 10.3390/md13063466

Chen, T., Xuqing, X., Jinjiao, W., Chen, J., Miu, R., Huang, L., et al. (2013). Food-borne disease outbreak of diarrhetic shellfish poisoning due to toxic mussel consumption: the first recorded outbreak in China. *PLoS ONE* 8:e65049. doi: 10.1371/journal.pone.0065049

Ching, P. K., Ramos, R. A., de los Reyes, V. C., Sucaldito, M. M., and Tayag, E. (2015). Lethal paralytic shellfish poisoning from consumption of green mussel broth, Western Samar, Philippines, August 2013. *Western Pac. Surveill. Response J.* 6, 22–26. doi: 10.5365/WPSAR.2015.6.1.004

Deeds, J. R., and Schwartz, M. D. (2010). Human risk associated with palytoxin exposure. *Toxicon* 56, 150–162. doi: 10.1016/j.toxicon.2009.05.035

EFSA (2008a). Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Okadaic acid and analogues. *EFSA J.* 589, 1–62.

EFSA (2008b). Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Azaspiracids. *EFSA J.* 723, 1–52.

EFSA (2009a). Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Saxitoxin Group. *EFSA J.* 1019, 1–76.

EFSA (2009b). 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.

EFSA (2010a). Scientific Opinion on Marine Biotoxins in Shellfish – Emerging toxins: Brevetoxin Group. *EFSA J.* 1677, 1–29.

EFSA (2010b). Scientific Opinion on Marine Biotoxins in Shellfish – Emerging toxins: Ciguatoxin Group. *EFSA J.* 1627, 1–38.

EFSA (2010c). Scientific Opinion on Marine Biotoxins in Shellfish – Cyclic Imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). *EFSA J.* 1628, 1–39.

Espiña, B., Louzao, M. C., Ares, I. R., Cagide, E., Vieytes, M. R., Vega, F. V., et al. (2008). Cytoskeletal toxicity of pectenotoxins in hepatic cells. *Br. J. Pharmacol.* 155, 934–944. doi: 10.1038/bjp.2008.323

FAO/IOC/WHO (2004). *Report of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs.* Norway: FAO/IOC/WHO, 1–31.

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

Fleming, L. E., Kirkpatrick, B., Backer, L. C., Bean, J. A., Wanner, A., Reich, A., et al. (2007). Aerosolized red-tide toxins (brevetoxins) and asthma. *Chest* 131, 187–194. doi: 10.1378/chest.06-1830

Furey, A., O'Doherty, S., O'Callaghan, K., and Lehane, M. (2010). Azaspiracid poisoning (AZP) toxins in shellfish: toxicological and health considerations. *Toxicon* 56, 173–190. doi: 10.1016/j.toxicon.2009.09.009

García, C., Bravo, M. C., Lagos, M., and Lagos, N. (2004). Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in Patagonia fjords. *Toxicon* 43, 149–158. doi: 10.1016/j.toxicon.2003.11.018

Gebhard, E., Levin, M., Bogomolni, A., and De Guise, S. (2015). Immunomodulatory effects of brevetoxin (PbTx-3) upon in vitro exposure in bottlenose dolphins (*Tursiops truncatus*). *Harmful Algae* 44, 54–62. doi: 10.1016/j.hal.2015.02.010

Harju, K., Koskela, H., Kremp, A., Suikkanen, S., de la Iglesia, P., Miles, C. O., et al. (2016). Identification of gymnodimine D and presence of gymnodimine variants in the dinoflagellate *Alexandrium ostenfeldii* from the Baltic Sea. *Toxicon* 112, 68–76. doi: 10.1016/j.toxicon.2016.01.064

Hellyer, S. D., Selwood, A. I., Rhodes, L., and Kerr, D. S. (2013). Neuromuscular blocking activity of pinnatoxins E, F and G. *Toxicon* 76, 214–220. doi: 10.1016/j.toxicon.2013.10.009

Hess, P., Abadie, E., Hervé, F., Berteaux, T., Séchet, V., Aráoz, R., et al. (2013). Pinnatoxin G is responsible for atypical toxicity in mussels (*Mytilus galloprovincialis*) and clams (*Venerupis decussata*) from Ingril, a French Mediterranean lagoon. *Toxicon* 75, 16–26. doi: 10.1016/j.toxicon.2013.05.001

Hidalgo, J., Liberona, J., Molgó, J., and Jaimovich, E. (2002). Pacific ciguatoxin-1b effect over Na+and K+currents, inositol 1,4,5-triphosphate content and intracellular Ca2+ signals in cultured rat myotubes. *Br. J. Pharmacol.* 137, 1055–1062. doi: 10.1038/sj.bjp.0704980

Hossen, V., Jourdan-da Silva, N., Guillois-Bécel, Y., Marchal, J., and Kris, S. (2011). Food poisoning outbreaks linked to mussels contaminated with okadaic acid and ester dinophysistoxin-3 in France, June 2009. *Euro Surveill.* 16, 1–7.

Hossen, V., Soliño, L., Leroy, P., David, E., Velge, P., Dragacci, S., et al. (2015). Contribution to the risk characterization of ciguatoxins: loael estimated from eight ciguatera fish poisoning events in Guadeloupe (French West Indies). *Environ. Res.* 143, 100–108. doi: 10.1016/j.envres.2015.09.014

Hu, T., Curtis, J., Oshima, Y., Quilliam, M., Walter, J., Watson-Wright, W., et al. (1995). Spirolides B and D, two novel macrocycles isolated from the digestive glands of shellfish. *J. Chem. Soc. Chem. Commun.* 20, 2159–2161. doi: 10.1039/c39950002159

Jeffery, B., Barlow, T., Moizer, K., Paul, S., and Boyle, C. (2004). Amnesic shellfish poison. *Food Chem. Toxicol.* 42, 545–557. doi: 10.1016/j.fct.2003.11.010

Lefebvre, K. A., and Robertson, A. (2010). Domoic acid and human exposure risks: a review. *Toxicon* 56, 218–230. doi: 10.1016/j.toxicon.2009.05.034

Li, A., Ma, J., Cao, J., and McCarron, P. (2012). Toxins in mussels (*Mytilus galloprovincialis*) associated with diarrhetic shellfish poisoning episodes in Cina. *Toxicon* 60, 420–425. doi: 10.1016/j.toxicon.2012.04.339

Litaker, R. W., Vandersea, M. W., Faust, M. A., Kibler, S. R., Chinain, M., Holmes, M. J. H., et al. (2009). Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). *Phycologia* 48, 344–390. doi: 10.2216/07-15.1

MacKenzie, L. (1994). More blooming problems: toxic algae and shellfish biotoxins in the South Island (January-May 1994). *Seaf. N. Z.* 2, 47–52.

Mafra, L. L., Bricelj, V. M., and Fennel, K. (2010). Domoic acid uptake and elimination kinetics in oysters and mussels in relation to body size and anatomical distribution of toxin. *Aquat. Toxicol.* 100, 17–29. doi: 10.1016/j.aquatox.2010.07.002

Marrouchi, R., Rome, G., Kharrat, R., Molgo, J., and Benoit, E. (2013). Analysis of the action of gymnodimine-A and 13-desmethyl spirolide C on the mouse neuromuscular system in vivo. *Toxicon* 75, 27–34. doi: 10.1016/j.toxicon.2013.08.050

Maso, M., and Garcés, E. (2006). Harmful microalgae blooms (HAB); problematic and conditions that induce them. *Mar. Pollut. Bull.* 53, 620–630. doi: 10.1016/j.marpolbul.2006.08.006

Mattei, C., Vetter, I., Eisenblätter, A., Krock, B., Ebbecke, M., Desel, H., et al. (2014). Ciguatera fish poisoning: a first epidemic in Germany highlights an increasing risk for European countries. *Toxicon* 91, 76–83. doi: 10.1016/j.toxicon.2014.10.016

McCarthy, M., Bane, V., García-Altares, M., van Pelt, F. N. A. M., Furey, A., and O'Halloran, J. (2015). Assessment of emerging biotoxins (pinnatoxin G and spirolides) at Europe's first marine reserve: Lough Hyne. *Toxicon* 108, 202–209. doi: 10.1016/j.toxicon.2015.10.007

Nielsen, L. T., Hansen, P. J., Krock, B., and Vismann, B. (2016). Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*. *Toxicon* 117, 84–93. doi: 10.1016/j.toxicon.2016.03.021

Paz, B., Daranas, A. H., Norte, M., Riobó, P., Franco, J. M., and Fernández, J. J. (2008). Yessotoxins, a group of marine polyether toxins: an overview. *Mar. Drugs* 6, 73–102. doi: 10.3390/md20080005

Poletti, R., Milandri, A., and Pompei, M. (2003). Algal biotoxins of marine origin: new indications from the European Union. *Vet. Res. Commun.* 27, 173–182. doi: 10.1023/B:VERC.0000014136.98850.b1

Richter, I., and Fidler, A. W. (2015). Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast. *Toxicon* 95, 13–22. doi: 10.1016/j.toxicon.2014.12.011

Rundberget, T., Bunæs Aasen, J. A., Selwood, A. I., and Miles, C. O. (2011). Pinnatoxins and spirolides in Norwegian blue mussels and seawater. *Toxicon* 58, 700–711. doi: 10.1016/j.toxicon.2011.08.008

Salgado, P., Riobó, P., Rodríguez, F., Franco, J. M., and Bravo, I. (2015). Differences in the toxin profiles of *Alexandrium ostenfeldii* (Dinophyceae) strains isolated from different geographic origins: evidence of paralytic toxin, spirolide, and gymnodimine. *Toxicon* 103, 85–98. doi: 10.1016/j.toxicon.2015.06.015

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.

Schwarz, M., Jandová, K., Struk, I., Marešová, D., Pokorný, J., and Riljak, V. (2014). Low dose domoic acid influences spontaneous behavior in adult rats. *Physiol. Res.* 63, 369–376.

Selwood, A. I., Miles, C. O., Wilkins, A. L., Van Ginkel, R., Munday, R., Rise, F., et al. (2010). Isolation, structural determination and acute toxicity of pinnatoxins E, F and G. *J. Agric. Food Chem.* 50, 6532–6542. doi: 10.1021/jf100267a

Silva, M., Rodriguez, I., Barreiro, A., Kaufmann, M., Neto, A. I., Hassouani, M., et al. (2015). First report of ciguatoxins in two startfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. *Toxins* 7, 3740–3757. doi: 10.3390/toxins7093740

Smayda, T. J. (2007). Reflections on the ballast water dispersal-harmful algal bloom paradigm. *Harmful Algae* 6, 601–622. doi: 10.1016/j.hal.2007.02.003

Sun, P., Leeson, C., Zhi, X., Leng, F., Pierce, R. H., Henry, M. S., et al. (2016). Characterization of an epoxide hydrolase from the Florida red tile dinoflagellate, *Karenia brevis*. *Phytochemistry* 122, 11–21. doi: 10.1016/j.phytochem.2015.11.002

Taylor, M., McIntyre, L., Ritson, M., Stone, J., Bronson, R., Bitzikos, O., et al. (2013). Outbreak of diarrhetic shellfish poisoning associated with mussels, British Columbia, Canada. *Mar. Drugs* 11, 1669–1676. doi: 10.3390/md11051669

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

Turner, A. D., and Goya, A. B. (2015). Occurrence and profiles of lipophilic toxins in shellfish harvested from Argentina. *Toxicon* 102, 32–42. doi: 10.1016/j.toxicon.2015.05.010

Vale, P. (2012). Two simple models for accounting mussel contamination with diarrhoetic shellfish poisoning toxins at Aveiro lagoon: control by rainfall and atmospheric forcing. *Estuar. Coast. Shelf Sci.* 98, 94–100. doi: 10.1016/j.ecss.2011.12.007

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, Italy. *Chemosphere* 90, 1077–1082. doi: 10.1016/j.chemosphere.2012.09.012

Wang, Z., Broadwater, M. H., and Ramsdell, J. S. (2015). Analysis of diarrhetic shellfish poisoning toxins and pectenotoxin-2 in the bottlenose dolphin (*Tursiops truncatus*) by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1416, 22–30. doi: 10.1016/j.chroma.2015.08.066

Watkins, S. M., Reich, A., Fleming, L. E., and Hammond, R. (2008). Neurotoxin shellfish poisoning. *Mar. Drugs* 6, 431–455. doi: 10.3390/md20080021

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 June 2016
doi: 10.3389/fmicb.2016.00994

Comparison of the Effects of Environmental Parameters on the Growth Variability of *Vibrio parahaemolyticus* Coupled with Strain Sources and Genotypes Analyses

Bingxuan Liu[1†], *Haiquan Liu*[1,2,3†], *Yingjie Pan*[1,2,3], *Jing Xie*[1,2,3] *and Yong Zhao*[1,2,3*]

[1] College of Food Science and Technology, Shanghai Ocean University, Shanghai, China, [2] Laboratory of Quality and Safety Risk Assessment for Aquatic Products on Storage and Preservation, Ministry of Agriculture, Shanghai, China, [3] Shanghai Engineering Research Center of Aquatic-Product Processing and Preservation, Shanghai, China

OPEN ACCESS

Edited by:
Rosanna Tofalo,
University of Teramo, Italy

Reviewed by:
Antonio Valero,
University of Cordoba, Spain
Giorgia Perpetuini,
University of Teramo, Italy

***Correspondence:**
Yong Zhao
yzhao@shou.edu.cn

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

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

Received: 26 January 2016
Accepted: 09 June 2016
Published: 23 June 2016

Citation:
Liu B, Liu H, Pan Y, Xie J and Zhao Y (2016) Comparison of the Effects of Environmental Parameters on the Growth Variability of Vibrio parahaemolyticus Coupled with Strain Sources and Genotypes Analyses. Front. Microbiol. 7:994. doi: 10.3389/fmicb.2016.00994

Microbial growth variability plays an important role on food safety risk assessment. In this study, the growth kinetic characteristics corresponding to maximum specific growth rate (μ_{max}) of 50 *V. parahaemolyticus* isolates from different sources and genotypes were evaluated at different temperatures (10, 20, 30, and 37°C) and salinity (0.5, 3, 5, 7, and 9%) using the automated turbidimetric system Bioscreen C. The results demonstrated that strain growth variability increased as the growth conditions became more stressful both in terms of temperature and salinity. The coefficient of variation (CV) of μ_{max} for temperature was larger than that for salinity, indicating that the impact of temperature on strain growth variability was greater than that of salinity. The strains isolated from freshwater aquatic products had more conspicuous growth variations than those from seawater. Moreover, the strains with $tlh^+/tdh^+/trh^-$ exhibited higher growth variability than $tlh^+/tdh^-/trh^-$ or $tlh^+/tdh^-/trh^+$, revealing that gene heterogeneity might have possible relations with the growth variability. This research illustrates that the growth environments, strain sources as well as genotypes have impacts on strain growth variability of *V. parahaemolyticus*, which can be helpful for incorporating strain variability in predictive microbiology and microbial risk assessment.

Keywords: *Vibrio parahemolyticus,* maximum growth rate, growth variability, environmental factor, temperature, salinity, gene heterogeneity

INTRODUCTION

Vibrio parahaemolyticus is a kind of halophytic, Gram-negative bacterium that can cause headaches, diarrhea, fever, gastroenteritis, and even life-threatening sepsis (Makino et al., 2003). Since the first *V. parahaemolyticus* was isolated in Japan by Fujino Komiro in 1950 (Fujino et al., 1950), it has been considered as the major food-borne pathogen involving in bacterial seafood poisoning incidents in Asia (Fujikawa et al., 2009). According to annual statistics obtained from the detection network of microbial foodborne illness in China, *V. parahaemolyticus* has been classified

as the major foodborne pathogen (accounting for 65% of the total; Wu et al., 2014). Similarly, cases of foodborne illness caused by *V. parahaemolyticus* are common in Europe and the United States (Yang et al., 2008; Shen et al., 2009, 2010). In fact, the largest outbreak of *V. parahaemolyticus* gastroenteritis all over the world did occur in the United States in 1978 and caused up to 1133 cases (Daniels et al., 2000). As demonstrated by recent surveillance data (Ma et al., 2014; Tang et al., 2014), the control of *V. parahaemolyticus* continues to be challenging worldwide.

It was announced that the strain variability gave the importance as well as the difficulty in controlling *V. parahaemolyticus* in the previous research (Lianou and Koutsoumanis, 2011). Owing to the fact that *V. parahaemolyticus* is mainly distributed in estuaries, coastal waters, sediments, and aquatic products (such as shrimp, cod, mackerel, and shellfish), it has become the major sources of food-borne pathogen (Wu et al., 2014). Since multiple strain composites of foodborne pathogens with robust growth or inactivation characteristics are preferred in food safety researches that aimed at assessing the behavior of bacterial pathogens in food products (NACMCF (National Advisory Committee on Microbiological Criteria for Foods), 2005; Scott et al., 2005), the characterizations of a variety of strains with respect to phenotypic responses, such as the growth behavior under different environmental conditions, should be analyzed (Nishina et al., 2004). Additionally, *V. parahaemolyticus* strains in the environment exhibit a halophilic and seasonal distribution, which are directly related to the salinity and temperature (DePaola et al., 2003; Zimmerman et al., 2007; Johnson et al., 2010; Sobrinho et al., 2014; Esteves et al., 2015). For the purpose of evaluation, the quantitative microbial risk assessment (QMRA) of *V. parahaemolyticus* should be estimated by at least two factors: temperature (T) and salinity (sodium chloride) (Nauta, 2002; U.S. Food and Drug Administration, 2005). It means that the use of predictive models of growth variability is mainly associated with the *T*-value and sodium chloride (NaCl) concentration (Ratkowsky et al., 1982; Larsen et al., 2015).

In previous studies of *V. parahaemolyticus*, only a few of them investigated the relationship between growth environments and strain variability (Fujikawa et al., 2009; Larsen et al., 2015). In west countries such as the USA, aquatic animals are mostly cultured in seawater (Depaola et al., 1990); however, in China, a majority of aquatic farmers practice freshwater aquaculture (Wu et al., 2014). It has been determined that the differences of the source for *V. parahaemolyticus* strains result in a large amount of diversity in the predictive models of growth variability (McMeekin et al., 1993). Furthermore, most previous research findings of the strain variability of the growth kinetic behavior of foodborne pathogens are based on marine culture (Wong et al., 2000; Alam et al., 2002; Larsen et al., 2015), which would be discrepant from China's actual conditions. Thus, new models with the purpose of developing a safe food production process in China should be built. Moreover, further studies on the influence of gene heterogeneity on growth variability were even less (Lianou and Koutsoumanis, 2013; Lopez-Joven et al., 2015), while as mentioned in Martins and Locke (2015), gene heterogeneity could determine phenotypic heterogeneity including strain growth variability, and therefore this variability might reflect the gene heterogeneity as well.

As the growth variability can introduce the food safety risk, the quantification of the growth variability can better service to the QMRA in microbiology. Aiming at furthering the development of precautionary food safety against *V. parahaemolyticus* in China, the influences of the T value and NaCl, together with strain sources and genotypes on the growth variability were evaluated in this research. The obtained appropriate data of the growth variability for *V. parahaemolyticus* could be useful for better characterizing the kinetic behaviors of *V. parahaemolyticus* in different growth environments (Miles et al., 1997; Yang et al., 2009). In total, 9000 optical density (OD) curves with 50 isolates of *V. parahaemolyticus* from different sources were generated for four levels temperatures and five levels NaCl concentrations, which will accomplish the following: (1) determine the influences of temperature and salinity on growth variability, and discuss the comparison between this two environmental factors; (2) reveal the growth variability of strains isolated from the aquatic products in freshwater and seawater; (3) demonstrate the effects of gene heterogeneity on the growth variability; and (4) provide a reasonable environmental condition for the storage of preserved food against *V. parahaemolyticus*.

MATERIALS AND METHODS

V. parahaemolyticus Strains

Fifty strains of *V. parahaemolyticus* were isolated from the shrimps which were cultured in freshwater or seawater. The strain information was shown in **Table 1**. $tlh^+/tdh^+/trh^-$, $tlh^+/tdh^-/trh^+$, and $tlh^+/tdh^-/trh^-$ genes were used for distinguishing the genotype of the isolates (Bej et al., 1999; Okada et al., 2009), twelve *V. parahaemolyticus* strains were $tlh^+/tdh^+/trh^-$ genotype, eleven *V. parahaemolyticus* strains were $tlh^+/tdh^-/trh^+$ genotype, one strain (42) was $tlh^+/tdh^+/trh^+$ genotype and others were $tlh^+/tdh^-/trh^-$ genotype in **Table 1**. All the strains in the present study were stored frozen (−80°C) in 25% glycerol test tubes. The *V. parahaemolyticus* strains were first dispensed onto thiosulfate-citrate-bile salts-sucrose agar culture medium (TCBS; Beijing Land Bridge Technology Company Ltd., Beijing, China) plates, and cultured for 18–24 h at 37°C. The single green strain on TCBS plates was then transferred into 10 ml tryptic soy broth (TSB; Beijing Land Bridge Technology Company Ltd., Beijing, China) with pH 8.0 and 3.0% (w/w) NaCl concentration. The 18 h cultures were incubated at 37°C for the preparation of the test inocula. The initial strain concentrations of the inocula were about 10^9 CFU/ml after incubation. The automated turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd., Raisio, Finland) was used for testing the corresponding Optical density (OD) values. OD measurements were taken at regular time intervals using the wideband filter (420–580 nm) of the instrument, for a total time period such that a considerable OD change was observed.

TABLE 1 | The sources of the 50 strains of *V. parahaemolyticus* from the shrimps.

No.	Genotype			Source	No.	Genotype			Source
	tlh	*tdh*	*trh*			*tlh*	*tdh*	*trh*	
1	+	−	+	Freshwater	26	+	−	−	Freshwater
2	+	−	−	Seawater	27	+	−	−	Freshwater
3	+	−	+	Freshwater	28	+	+	−	Freshwater
4	+	−	−	Freshwater	29	+	−	+	Freshwater
5	+	−	−	Seawater	30	+	−	+	Seawater
6	+	−	−	Seawater	31	+	+	−	Freshwater
7	+	+	−	Seawater	32	+	−	−	Seawater
8	+	−	+	Freshwater	33	+	−	−	Seawater
9	+	+	−	Seawater	34	+	−	−	Seawater
10	+	+	−	Seawater	35	+	−	+	Freshwater
11	+	−	−	Seawater	36	+	−	+	Freshwater
12	+	−	+	Freshwater	37	+	+	−	Seawater
13	+	+	−	Seawater	38	+	−	−	Freshwater
14	+	−	−	Seawater	39	+	−	−	Seawater
15	+	+	−	Seawater	40	+	−	+	Freshwater
16	+	−	−	Seawater	41	+	−	−	Freshwater
17	+	+	−	Seawater	42	+	+	+	Human
18	+	−	+	Freshwater	43	+	+	−	Human
19	+	−	−	Freshwater	44	+	−	−	Freshwater
20	+	−	+	Seawater	45	+	−	−	Seawater
21	+	−	−	Freshwater	46	+	−	−	Freshwater
22	+	−	−	Seawater	47	+	−	−	Freshwater
23	+	−	−	Freshwater	48	+	−	−	Freshwater
24	+	−	−	Seawater	49	+	−	−	Seawater
25	+	−	−	Seawater	50	+	−	−	Freshwater

"+" represents positive genotypic, and "−" means negative genotypic.

Growth Experiments

To evaluate the single effect of the T value or NaCl concentration on the growth variability in terms of the two environmental factors, a total of 20 different growth conditions were assessed with 4-levels (10, 20, 30, and 37°C) of temperature and 5-levels (0.5, 3, 5, 7, and 9%) of NaCl concentrations so as to cover the most probable growth region of the *V. parahaemolyticus* strains. The maximum and minimum boundaries of the T value (37 and 10°C, respectively) and the NaCl concentration (9 and 0.5%, respectively) were set up based on the findings of preliminary experiments in which the growth environment approximately reached the minimum growth requirements (*V. parahaemolyticus* strains approached the minimum growth rate in the condition of 10°C and 9% salinity) or the maximum growth requirements (*V. parahaemolyticus* strains attained the maximum growth rate in an optimum environment with 37°C and 3% salinity condition).The prepared initial inocula of each strain were decimally diluted in the TSB with 5 levels of NaCl concentration separately for five times. With strain concentration of approximately 10^4 CFU/ml, the inoculated TSB were transferred into 100-well microtiter plates, which were then placed in the automated turbidimetric system Bioscreen C for 4 levels of temperatures, respectively. Totally three OD measurement replicates were tested in this process. Additionally, three independent experiments were conducted at each growth condition and therefore there were three samples per strain altogether for testing. In such a way, the total number of the described OD curves would amount to 9000 patterns (3 replicates × 3 independent experiments × 20 growth conditions × 50 types of *V. parahaemolyticus*). The counted data were analyzed in order to achieve an accurate approximation of the *V. parahaemolyticus* growth states in different cultured environments. Moreover, it would be more reasonable for the *V. parahaemolyticus* strain evaluation of QMRA (Vose, 1998).

Maximum Specific Growth Rate

The maximum specific growth rate ($\mu_{\max}$) (Dalgaard and Koutsoumanis, 2001) of each strain at each growth condition was estimated according to Mytilinaios et al. (2012). By using the decimal dilution approach with Bioscreen C, the novel calculation of the maximum growth rate in the unit of $OD*h^{-1}$ can be formulated in the model of Modified Gompertz (Gibson et al., 1987; Zwietering et al., 1990; Gil et al., 2006; Juneja et al., 2007; Yoon et al., 2008), with a little regulation, as the following equation:

$$y = A + C\,exp\left\{-exp\left[\frac{\mu_m}{A}(\lambda - t) + 1\right]\right\}$$

Where A means the initial amount of bacteria, C represents the difference between the initial amount and the maximum amount of bacteria, μ_m represents maximum specific growth rate and λ is the lag time of the strain growth.

To calculate the maximum growth rate, the obtained data with both OD values and cultured times were taken into the above equation in the place of y and t, respectively. The OD curves were then fitted and the matrix of function was calculated including A, C, λ, and μ_m.

Statistical Analysis

The statistical indicators were used to compare the performance of the models: correlation coefficients (R^2), the p values from the Fisher F-test, and root mean square error (RMSE), accuracy factor (A_f), and bias factor (B_f), whose mathematical expressions are as follows:

$$R^2 = \left[1 - \frac{\sum (pred - obs)^2}{\sum (obs - mean)^2}\right]$$

$$RMSE = \sqrt{\frac{\sum (obs - pred)^2}{n}}$$

$$A_f = 10\left(\frac{\sum |Log\,(pred/obs)|}{n}\right)$$

$$B_f = 10\left(\frac{\sum Log\,(pred/obs)}{n}\right)$$

where *obs* is observed values, *pred* is predicted values by models, *mean* is average values, and the n stands for the number of observations. The RMSE values approaching zero indicate a closer fit with the data for the model (Zhang et al., 2015). A_f provides the accuracy of the model, which reflects how close the predicted values are to the observed values, while B_f indicates the mean difference between observed and predicted value. Ideally, predictive models would have $A_f = B_f = 1$ (Wang et al., 2014).

The coefficient of variation (CV) of $\mu_{\max}$ in different conditions were calculated within the formula as

$$CV = \frac{standard\ deviation\ of\ \mu_{max}}{mean\ value\ of\ \mu_{max}} \times 100\%$$

Significance testing making use of p-values was applied to verify the differences of the strain growth rate in different sources. Values differences were compared using the least significant difference (LSD) method at $p = 0.05$.Statistical analysis was performed using SPSS statistical package17.0 (SPSS Inc., Chicago, IL).

RESULTS

Tendency of Maximum Growth Rates

The estimated maximum specific growth rate $\mu_{\max}$ vs. 50 strains in various growth environments were calculated are presented in Supplementary Material, and almost all of the values were fitted in the equation given above. By statistical analysis, all the correlation coefficients achieved above 93%, and all RMSE values approached zero. Both accuracy factors and bias factors got close to 1. The results showed a satisfactory goodness-of-fit in this study. A fraction of the maximal growth rate values could not yet be evaluated by Modified Gompertz model (Lammerding, 1997; Anderson and Hattis, 1999; Nauta and Dufrenne, 1999). It should be pointed out that the equation still cannot afford the actual growth state (Li et al., 2007), which requires the construction of a microbial macro growth model in multi-parameters.

Based on the $\mu_{\max}$ in Supplementary Material, the tendency chats in various growth environments are shown in **Figure 1**. As shown in **Figure 1A** for the T of 37°C, the mean value of $\mu_{\max}$ (OD∗h^{-1}) ranged from 0.03 to 0.24 in the condition of 0.5% NaCl, from 0.02 to 0.44 at 3% NaCl, from 0.01 to 0.26 at 5% NaCl, from 0 to 0.15 at 7% NaCl, and from 0 to 0.12 at 9% NaCl among the 50 strains. While with the same NaCl concentration of 3% in the TSB, the mean $\mu_{\max}$ (OD∗h^{-1}) ranged from 0.02 to 0.44 at 37°C, from 0.005 to 0.065 at 30 °C, from 0.007 to 0.031 at 20°C, and from 0.001 to 0.014 at 10°C. Obviously, the average growth rate in the condition of 37°C and 3% NaCl concentration was found to be the largest (**Figure 1B**). Therefore, 37°C and 3% NaCl were considered as the optimal growth temperature and salinity respectively, similarly 10°C and 9% NaCl were considered as the most non-optimal temperature and salinity in this research.

Evaluation of Growth Variability in Different Temperatures and Salinities

The optimal growth condition at 37°C with 3% NaCl concentration was used as the reference in **Figure 1**. In this case, the strains from No. 1 to No. 50 tended to be staged growth with an increasing maximum specific growth rate $\mu_{\max}$. While in other conditions, the strains from No. 1 to No. 50 seemed to grow randomly with no fixed growth trend as compared with that of the optimal growth condition. For example, the strains No. 50 and No. 1 at 37°C with 3% NaCl salinity had the highest growth rate and the lowest growth rate respectively, but in the condition at 20°C with 3% NaCl salinity, the No. 50 and No. 1 both located in the intermediate range of $\mu_{\max}$ in all 50 strains, nearly 0.02 OD∗h^{-1}. Similar situations also appeared in other strains like No. 2, No. 13, No. 28 strains at 37°C with 5% NaCl salinity compared with those at 10°C with 3% NaCl concentration.

The curves related to the coefficient of variation (CV) of $\mu_{\max}$ in different conditions were drawn in **Figure 2**. The CV value of maximum growth rate represented the growth variability for *V. parahaemolyticus* strains. The CV value among the tested strains at 37°C-3% NaCl concentration was 12.7%, while at 37°C-0.5% NaCl concentration and T 37°C-5% NaCl concentration, it was 13.0 and 15.1%, respectively (**Figure 2A**). The CV value among the tested strains corresponding to a mean $\mu_{\max}$ of approximately 0.16 OD∗h^{-1} was 12.7% for 37°C-3% NaCl concentration, while corresponding to a mean $\mu_{\max}$ of approximately 0.03 OD∗h^{-1}, the CV value was 16.3% for 30°C-3% NaCl concentration in **Figure 2B**. The non-optimal T and NaCl concentration led to an increase of CV values in the activation range of 0–5% NaCl and 30–37°C. On the contrary, in the inactivation range of *V. parahaemolyticus* strains, since the maximum growth rate dropped to nearly 0 OD∗h^{-1}, the CV

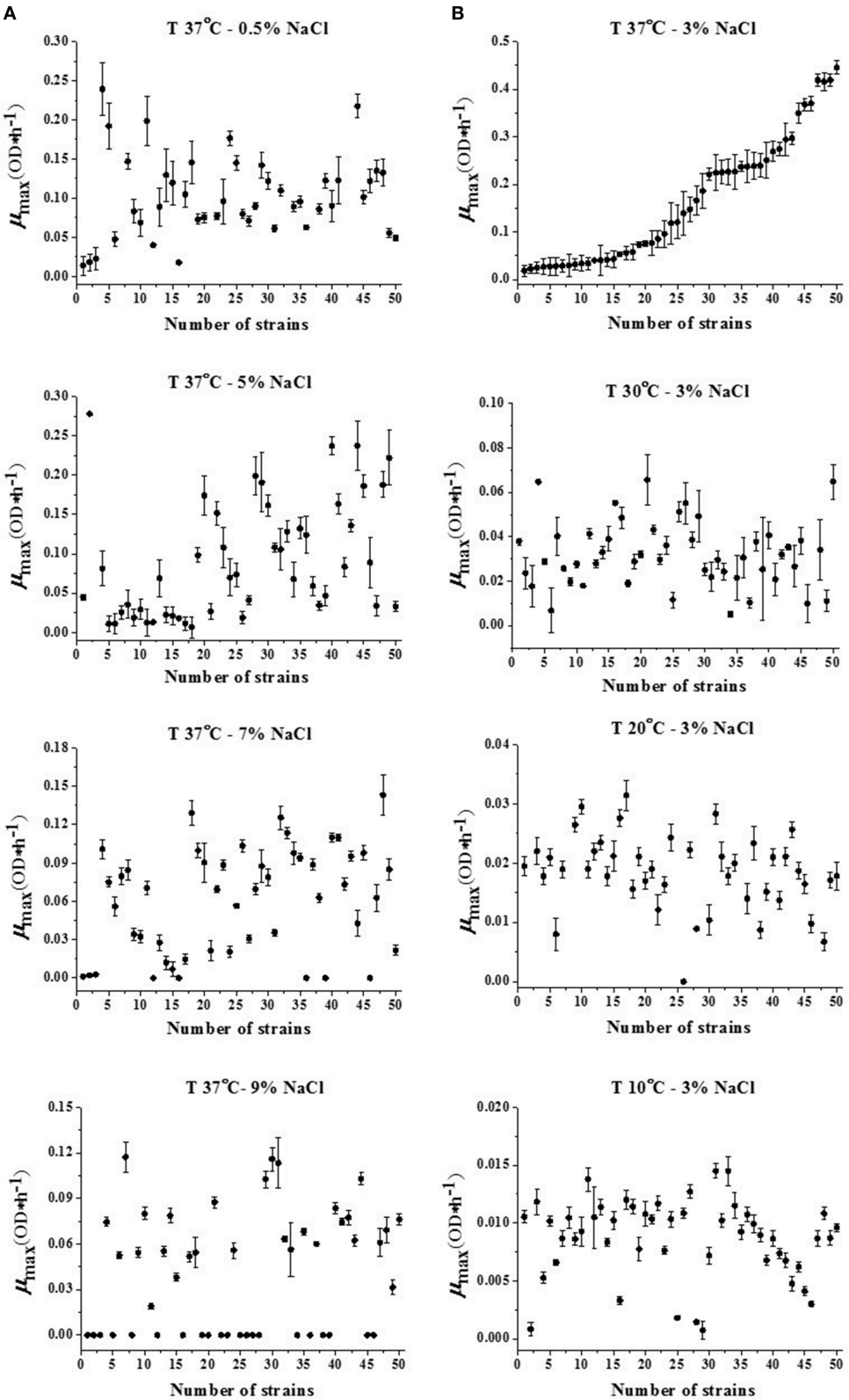


FIGURE 1 | Maximum specific growth rates (μ_{max}) of 50 *V. parahaemolyticus* strains in different (A) NaCl concentrations (37°C) and (B) T values (3% NaCl).

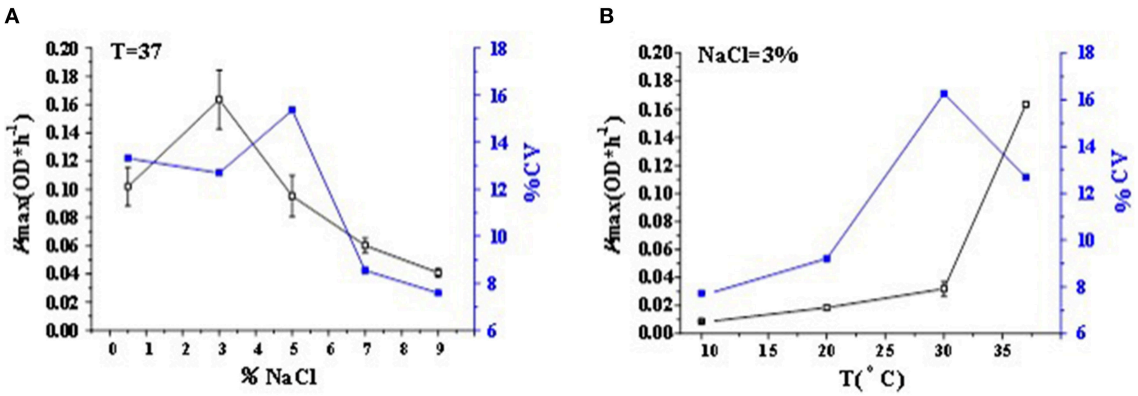


FIGURE 2 | Mean value curve of maximum specific growth rates (μ_{max}) and coefficient of variation curve of μ_{max} among strains (CV-Strain) in different (A) NaCl concentrations and (B) *T*-values of *V. parahaemolyticus*.

values of μ_{max} would similarly drop down, with less variance of growth variability in *V. parahaemolyticus* strains as shown in the points among the 7–9% NaCl concentration and 10–20°C.

Comparison of Growth Variability from Different Sources

From the different types of environmental sources in **Table 1**, the *V. parahaemolyticus* strains could be roughly divided into two categories: freshwater and seawater. The significant difference analyses between these two categories in four environmental conditions (37°C-3% NaCl, 30°C-3% NaCl, 37°C-9% NaCl, and 10°C-3% NaCl) with in the box plot method were respectively drawn in **Figure 3**. The 4 environmental conditions represented 4 typical growth kinetics of *V. parahaemolyticus* strains. These four box plots were counted by the mean of the maximum growth rate μ_{max} in freshwater and seawater accordingly. In addition, the significant differences were calculated by *p*-value, with 0, 0.063, 0.001, and 0.024 respectively, which demonstrated the growth variability in these two sources. The non-significant difference occurred in the condition at 30°C-3% NaCl, and other conditions performed as the significant difference.

Influence of Genotypes on Growth Variability

Further investigation of the growth variability of *V. parahaemolyticus* strains was studied through gene heterogeneity. For the purpose of research on the effect of genotypes on growth variability, all 50 strains were classified by growth condition and genotype. Based on each genotype with different virulence factors of *V. parahaemolyticus* strains (Letchumanan et al., 2014), four groups of virulence genes-related *V. parahaemolyticus* strains, $tlh^+/tdh^-/trh^-$, $tlh^+/tdh^+/trh^-$, $tlh^+/tdh^-/trh^+$, and $tlh^+/tdh^+/trh^+$, were introduced in this research in order to explore the internal causes of the growth variability of *V. parahaemolyticus*. Among these virulence genes, *tlh* has been expressed by all clinical and environmental strains of *V. parahaemolyticus* in previous studies (Bej et al., 1999; Okada et al., 2009); thus, the *tlh* virulence gene was contained in all four groups of isolates. The inter-specific variability of four genotype factors in the environmental factors of T and NaCl concentration is given in **Figure 4**. In **Figure 4A**, the temperature was fixed at the optimal condition of 37°C, and the genotype $tlh^+/tdh^+/trh^-$ (colored in red) embodied the largest strain growth variability. The associated CV values were set at a high level compared with 3 other genotypes. In contrast, the $tlh^+/tdh^+/trh^+$ genotype had the lowest CV values overall. Similar circumstances appeared in **Figure 4B** with the NaCl concentration set at 3% as well. The growth variability of $tlh^+/tdh^-/trh^+$ (colored in green) and $tlh^+/tdh^-/trh^-$ (colored in blue) performed moderate, overtopping the CV values only in the condition at 30°C and 3% NaCl concentration.

DISCUSSION

Effects of Temperature and NaCl Concentration on μ_{max}

It has been reported that the *V. parahaemolyticus* strains cannot grow at low temperature in nature since there is growth inhibition for *V. parahaemolyticus* strains with *T*-values below 10°C in freshwater or seawater (Cook and Ruple, 1989; Burnham et al., 2009). Regarding growth rate, the conditions at the lowest temperature (i.e., 10°C) and the highest NaCl concentration (i.e., 9%) almost approached the minimum growth requirements of this pathogen (Fujikawa et al., 2009). In contrast among all of the conditions, it is obvious that the growth condition of 37°C and 3% NaCl concentration is considered to be the optimal growth condition with the widest range and maximum mean value of specific growth rate compared to the other combinations (**Figure 1**), which has already been verified the similar completion in accordance with its own growth habits and laboratory experiments (Miles et al., 1997; Liu et al., 2008; Yang et al., 2009; Baker-Austin et al., 2010; Fernandez-Piquer et al., 2011). In this study, however, a much wider range of growth conditions in discussing both temperature and salinity simultaneously will give more comprehensive testimony for understanding the variability of the *V.*

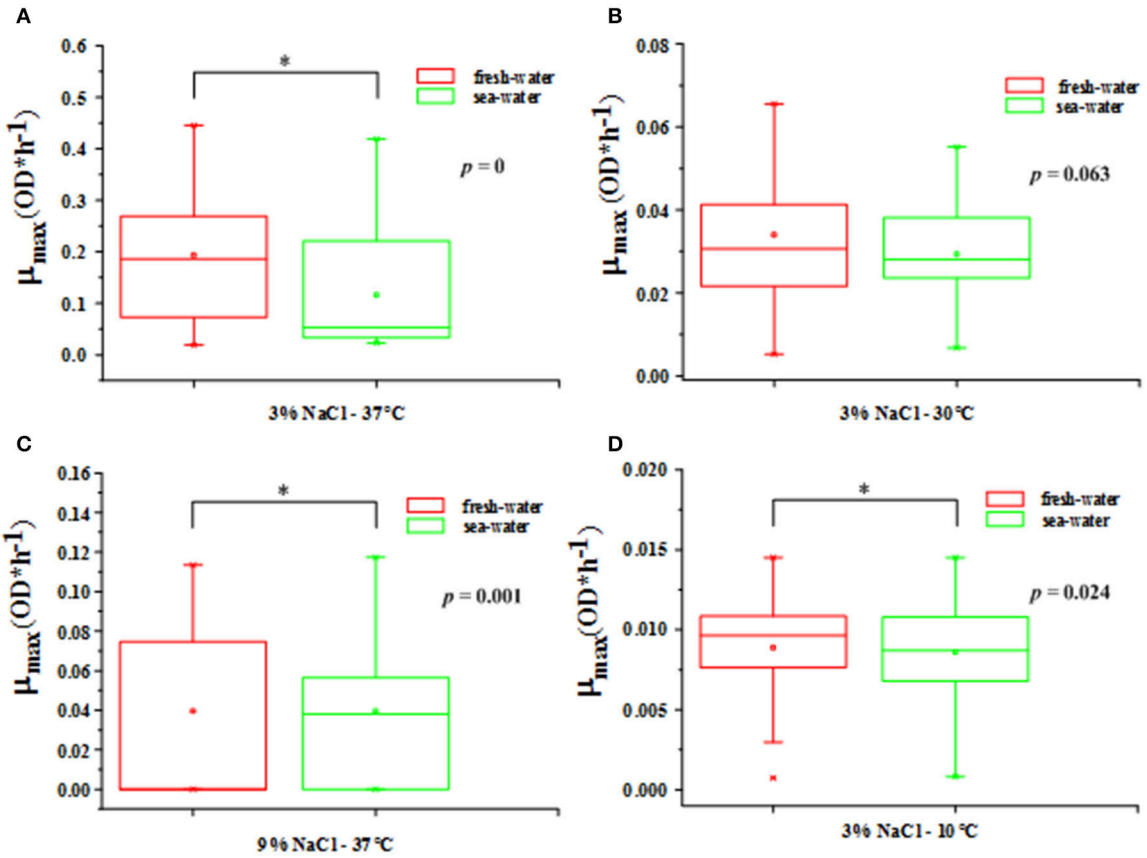


FIGURE 3 | The box plot between freshwater and seawater for *V. parahaemolyticus* strains in the shrimps in the following growth conditions. (A) 37°C-3% NaCl concentration with $p = 0$ (optimal growth temperature and salinity), **(B)** 30°C-3% NaCl concentration with $p = 0.063$ (μ_{max} most consistent), **(C)** 37°C-9% NaCl concentration with $p = 0.001$ (optimal growth temperature and most non-optimal growth salinity), and **(D)** 10°C-3% NaCl concentration with $p = 0.024$ (most non-optimal growth temperature and optimal growth salinity). Statistical significance ($p < 0.05$) is shown by *.

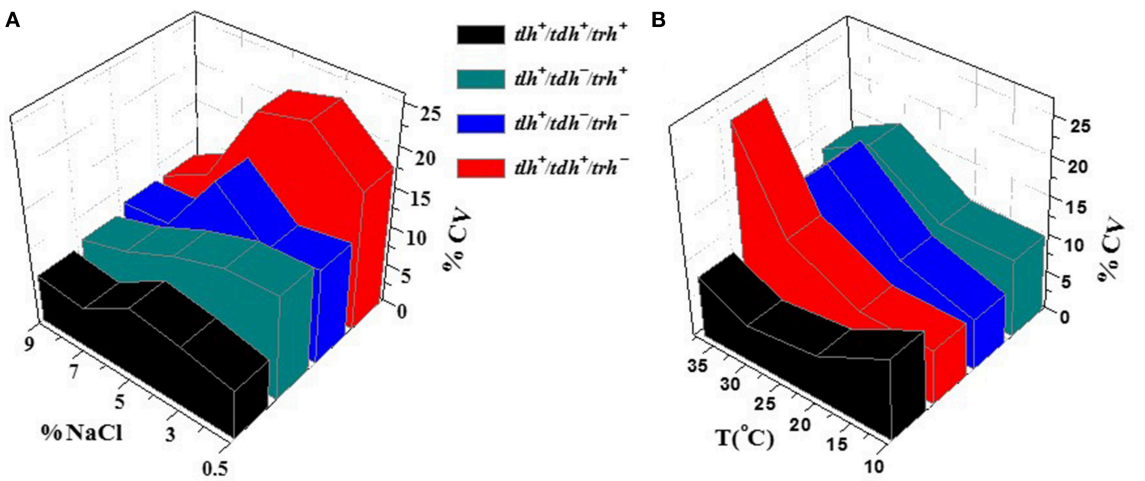


FIGURE 4 | The influence of the genotype on the growth variability of *V. parahaemolyticus* strains in various conditions. (A) various NaCl concentrations with a fixed T value of 37°C and **(B)** various temperatures with a fixed NaCl concentration of 3%.

parahaemolyticus kinetic behavior within the scope of the growth environments.

It seemed that the inter-species growth variability of the 50 strains occurred at different environmental conditions. The trend of maximum growth rate in various conditions indicates that the external environment, such as the temperature and salinity, can affect the growth variability among *V. parahaemolyticus* strains, and such inter-species growth variability performs randomly (Whiting and Golden, 2002).

Moreover, because of incomplete knowledge of the effects of environmental conditions on model parameters in current microbiological studies (Nauta, 2002), the quantitative information based on a single impact parameter, like only the *T*-value or the NaCl concentration, needs to be evaluated separately in order to analyze the strain variability of *V. parahaemolyticus* (Fujikawa et al., 2009). Since there are no methods for separating two impact parameters absolutely, the mutual comparison between the one most non-optimal factor (*T*-value or NaCl concentration in this study) and the other factor in the optimal condition that emerged by the quantitative data is used here for approaching the actual microbial growth model (Lindqvist, 2006). As can be seen in the growth condition of 37°C-9% NaCl concentration, nearly half of the strains were inactive in such an inappropriate growth environment for *V. parahaemolyticus*, and it seemed that the rest of the strains still "struggled" in a random range of growth rate, mostly from 0.12 to 0, with a mean μ_{max} value of about 0.06; In another extreme condition with the most non-optimal temperature case while the optimal salinity: 10°C-3% NaCl concentration, although there were few inactive strains, the mean value of the growth rate μ_{max} could just achieve 0.01 or below, and μ_{max} ranged at a smaller scale from 0.015 to 0. The difference indicates that although the temperature and salinity have the same net effect on strain variability, meaning that the μ_{max} variability among the strains increases as the *T*-values or NaCl concentrations become more unfavorable for *V. parahaemolyticus* growth, the extent for this growth variability appears to be different for these two environmental parameters (Nauta, 2000). It is illustrated that as one of the two main impact parameters, the influence of low temperature on the decrease of μ_{max} appears to be greater than that of high NaCl concentration in **Figure 1**.

The analysis in **Figure 1** could provide the formulations of temperature and salt which do not allow growth of *V. parahaemolyticus*. Normally, up to 9% NaCl concentration leads to inactivation for the majority of microorganisms (Francois et al., 2006); however, for halophilic bacteria like *V. parahaemolyticus*, such NaCl concentration cannot entirely prevent growth of bacteria (Anon, 1988; David et al., 1997). With a peak μ_{max} value of 0.12 for strain No. 7, 30, and 31, it seemed that the strain growth rate was not suppressed. To avoid the growth of most pathogens, the other impact parameter, the temperature, plays an important role in the suppression of μ_{max}. As it was revealed above in the data from 20°C or even 10°C conditions, the *V. parahaemolyticus* strains in a low temperature could be more easily inactivated with weaker growth behavior. This suggests that preserved foods in a salty environment should be stored in low temperatures below 10°C, which can aid in avoiding *V. parahaemolyticus* growth. The present study gives a convincing data basis for the instruction of manufacturing preserved foods.

Effects of Temperature and NaCl Concentration on Growth Variability

As reviewed by Nauta (2002), the assumption is often made by food microbiologists that strain-to-strain variation is equal to or smaller than experimental variation, thus it is not necessary to determine strain-to-strain variation. The data presented here demonstrated that the strain variability of the estimated μ_{max} values increased as the growth conditions became more stressful both in terms of NaCl (**Figure 2A**) and T (**Figure 2B**). The phenomenon that the non-optimal growth condition has a greater strain variability of growth kinetics than the optimal condition has been pointed in previous studies (Barbosa et al., 1994; Begot et al., 1997; Lianou et al., 2006).

In **Figure 2** with both two cases, the maximum CV value occurred at the environmental condition of 30°C and 3% NaCl concentration. It meant that the growth variability of 50 strains was larger than that in the optimal growth condition and any other conditions. Thus, the condition might introduce much difficulties for the control of food safety risk. Actually, this condition comes closest to the natural environment, leading to a big challenge for food safety control (Pouillot et al., 2003). Nevertheless, the *V. parahaemolyticus* strains in this condition had a relative high consistency with the medium-to-high maximum growth rate, it might therefore maintain a large variety of serotypes as much as possible, which would achieve a diversity of *V. parahaemolyticus* strains with a similar growth rate when incubated in the same TSB. It exerted favorable effect on strain selection in the growth environment of 30°C and 3% NaCl concentration. Besides, with the similar maximum growth rate of 0.04 in the condition of T 37°C-9% NaCl in **Figure 2A** and the condition of T 30°C-3% NaCl in **Figure 2B**, the CV values in the two cases were quite different, corresponding to 3 and 16.27%, respectively, which meant that the growth variability was larger for decreasing temperature than for increasing salinity. It again proves the fact that temperature variation always leads to a more gradual increase in the growth variability in *V. parahaemolyticus* strains than NaCl variation.

Impact of Different Sources on the Growth Variability

In **Figure 3**, the largest *p*-value for the difference between the freshwater and seawater occurred in the condition of 30°C and 3% NaCl, meaning this difference of μ_{max} was not significant. Obviously, this environment condition is the common state found in nature, especially in the subtropical and temperate coastal areas, which means that the *V. parahaemolyticus* strains that are grown in freshwater and in seawater result in a similar growth rate with relatively consistent growth variability in the normal state found in nature (Larsen et al., 2015). Moreover, the 30°C-3% NaCl condition could aid in strain selection since the consistency of the growth variability from fresh and sea water

was optimal in all of the tested conditions, which offered the largest variety of growth variability. In other words, the natural environment found in the coastal areas will lead to a large growth variability in the *V. parahaemolyticus* strains, which results in difficulties for the QMRA and food safety control (Pouillot et al., 2003), as discussed in **Figure 2**.

Another interesting point is that there was an extremely significant difference in the growth variability of the freshwater and seawater *V. parahaemolyticus* strains in the environmental condition of 37°C and 3% NaCl concentration. It has been evaluated that the *V. parahaemolyticus* strains reached the largest maximum growth rate in such an optimal growth condition in **Figure 3**. On the contrary, the difference in the NaCl concentration caused the freshwater strains (living in 0.1% salinity in nature) having a larger growth variability than the seawater strains (living in 3% salinity in nature), which resulted in a higher mean value of maximum growth rate with a larger distribution according to an evaluation of its standard deviation. It has been stated that strains in a non-optimal environment condition will have growth variability at a considerably higher level than those in the optimal growth condition, and larger environmental pressure will lead to larger growth variability. In addition, based on the comparison between the condition of 37°C-9% NaCl concentration and that of 10°C-3% NaCl concentration, it is confirmed that a decreasing temperature leads to a somewhat more gradual decrease of μ_{max} than an increasing NaCl concentration does.

Influence of Different Genotypes on the Growth Variability

According to collected data from **Table 1**, since there was only one strain comprising $tlh^+/tdh^+/trh^+$, it should have no typical representativeness for the properties of this genotype, while the curve in red gave some reference for the tendency of different genotypes in **Figure 4**. From the results, it was concluded that the genotype of the $tlh^+/tdh^+/trh^-$ resulted in the largest variation degree in the growth variability of the *V. parahaemolyticus* strains in all four groups, whereas the genotype with $tlh^+/tdh^+/trh^+$ illustrated the least obvious variation degree from among those cultured in the environment condition with temperature and NaCl concentration, which verified that gene heterogeneity also affected the growth inter-specific variability for *V. parahaemolyticus*. Furthermore, as one of the major virulence genotypes in *V. parahaemolyticus*, $tlh^+/tdh^+/trh^-$ modeled the most non-optimal case in evaluating QMRA, due to there being a large risk of growth variability in reality. In addition, it is suggested that in the food safety control of clinical *V. parahaemolyticus* strains, more attention should be paid to the genotype of $tlh^+/tdh^+/trh^-$, which is associated with serious virulence (Miyamoto et al., 1969; Honda and Iida, 1993; Baffone et al., 2005) and large growth variability in most of the environmental conditions. The research exploring the effect of gene heterogeneity on the grow variability in *V. parahaemolyticus* provides a useful reference for the prevention of pathogenic *V. parahaemolyticus* in nature.

CONCLUSION

In the present study, the growth kinetics characteristics of 50 *V. parahaemolyticus* isolates with different sources and genotypes were assessed at different temperatures (10, 20, 30, and 37°C) and salinity (0.5, 3, 7, and 9%). From the experimental results, it was concluded that the strain variability increased as the growth conditions became more stressful both in terms of temperature and salinity in the activation range, and temperature has larger impacts than salinity on strain growth variability. Therefore, the preserved foods in a salty environment were suggested to be stored in a low temperature below 10°C, which could promise the inactivation of *V. parahaemolyticus* strains. Moreover, the results showed the fact that the strains isolated from freshwater aquatic product had more conspicuous variations than those from seawater. And it was interpreted that gene heterogeneity also affected strain growth variability of *V. parahaemolyticus*. The findings of this study should be useful in incorporating strain variability in predictive microbiology and microbial risk assessment, and could provide scientific guidance for *V. parahaemolyticus* verification and prevention in nature as well as strain selection in experiments.

AUTHOR CONTRIBUTIONS

BL performed the data analyses and wrote the manuscript; contributed significantly to analysis and manuscript preparation; HL helped perform the analysis with constructive discussions; Substantial contributions to the design of the work and analysis the results. YP, JX drafted the work or revising it critically for important intellectual content. 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. YZ contributed to the conception of the study. Drafting the work or revising it critically for important intellectual content. Final approval of the version to be published.

FUNDING

This research was supported by the National Natural Science Foundation of China (31271870, 31571917), the project of Science and Technology Commission of Shanghai Municipality (14DZ1205100, 14320502100), Key Project of Shanghai Agriculture Prosperity through Science and Technology (2014, 3-5 and 2015, 4-8), Shanghai Engineering Research Center of Aquatic-Product Processing & Preservation (11DZ2280300), and the "Dawn" Program of Shanghai Education Commission (15SG48).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00994

REFERENCES

Alam, M. J., Tomochika, K. I., Miyoshi, S. I., and Shinoda, S. (2002). Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Inland Sea, Japan. *FEMS Microbiol. Lett.* 208, 83–87. doi: 10.1111/j.1574-6968.2002.tb11064.x

Anderson, E. L., and Hattis, D. (1999). Uncertainty and variability. *Risk Anal.* 19, 47–49. doi: 10.1111/j.1539-6924.1999.tb00386.x

Anon (1988). "Dried and smoked fishery products: preparation and composition," in *Fish Smoking and Drying. The Effect of Smoking and Drying on the Nutritional Properties of Fish,* ed J. R. Burt (London: Elsevier Applied Science), 121–159.

Baffone, W., Casaroli, A., Campana, R., Citterio, B., Vittoria, E., Pierfelici, L., et al. (2005). '*In vivo*' studies on the pathophysiological mechanism of *Vibrio parahaemolyticus* TDH^+—induced secretion. *Microb. Pathog.* 38, 133–137. doi: 10.1016/j.micpath.2004.11.001

Baker-Austin, C., Stockley, L., Rangdale, R., and Martinez-Urtaza, J. (2010). Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. *Environ. Microbiol. Rep.* 2, 7–18. doi: 10.1111/j.1758-2229.2009.00096.x

Barbosa, W. B., Cabedo, L., Wederquist, H. J., Sofos, J. N., and Schmidt, G. R. (1994). Growth variation among species and strains of *Listeria* in culture broth. *J. Food Prot.* 57, 765–769.

Begot, C., Lebert, I., and Lebert, A. (1997). Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. *Food Microbiol.* 14, 403–412. doi: 10.1006/fmic.1997.0097

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C., 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

Burnham, V. E., Janes, M. E., Jakus, L. A., Supan, J., DePaola, A., and Bell, J. (2009). Growth and survival differences of *Vibrio vulnificus* and *Vibrio parahaemolyticus* strains during cold storage. *J. Food Sci.* 74, M314–M318. doi: 10.1111/j.1750-3841.2009.01227.x

Cook, D. W., and Ruple, A. (1989). Indicator bacteria and *Vibrionaceae* multiplication in postharvest shellstock oysters. *J. Food Prot.* 52, 343–349.

Dalgaard, P., and Koutsoumanis, K. (2001). Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. *J. Microbiol. Methods* 43, 183–196. doi: 10.1016/S0167-7012(00)00219-0

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., et al. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973-1998. *J. Infect. Dis.* 181, 1661–1666. doi: 10.1086/315459

David, W. M., Ross, T., Olley, J., and McMeekin, A. T. (1997). Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.* 38, 133–142. doi: 10.1016/S0168-1605(97)00100-1

Depaola, A., Hopkins, L. H., Peeler, J. T., Wentz, B., and Mcphearson, R. M. (1990). Incidence of *Vibrio parahaemolyticus* in United States coastal waters and oysters. *Appl. Environ. Microbiol.* 56, 2299–2302.

DePaola, A., Nordstrom, J. L., Bowers, J. C.,Wells, J. G., and Cook, D. W. (2003). Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Appl. Environ. Microbiol.* 69, 1521–1526. doi: 10.1128/AEM.69.3.1521-1526.2003

Esteves, K., Mosser, T., Aujoulat, F., Hervio-Heath, D., Monfort, P., and Jumas-Bilak, E. (2015). Highly diverse recombining populations of *Vibrio cholerae* and *Vibrio parahaemolyticus* in French Mediterranean coastal lagoons. *Front. Microbiol.* 6:708. doi: 10.3389/fmicb.2015.00708

Fernandez-Piquer, J., Bowman, J. P., Ross, T., and Tamplin, M. L. (2011). Predictive models for the effect of storage temperature on *Vibrio parahaemolyticus* viability and counts of total viable bacteria in Pacific oysters (Crassostrea gigas). *Appl. Environ. Microbiol.* 77, 8687–8695. doi: 10.1128/AEM.05568-11

Francois, K., Devlieghere, F., Standaert, A. R., Geeraerd, A. H., Van Impe, J. F., and Debevere, J. (2006). Effect of environmental parameters (temperature, pH and a_w) on the individual cell lag phase and generation time of *Listeria monocytogenes*. *Int. J. Food Microbiol.* 108, 326–335. doi: 10.1016/j.ijfoodmicro.2005.11.017

Fujikawa, H., Kimura, B., and Fujii, T. (2009). Development of a predictive program for *Vibrio parahaemolyticus* growth under various environmental conditions. *Biocontrol Sci.* 14, 127–131. doi: 10.4265/bio.14.127

Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Mukai, K., and Uebo, T. (1950). On the bacteria examination of Shirasu food poisoning. *Med. J. Osaka Univ.* 4, 299–304.

Gibson, A. M., Bratchell, N., and Roberts, T. A. (1987). The effect of sodium chloride and temperature on the rate and extent of growth of Clostridium botulinum type A in pasteurized pork slurry. *J. Appl. Bacteriol.* 62, 479–490. doi: 10.1111/j.1365-2672.1987.tb02680.x

Gil, M. M., Brandao, T. R., and Silva, C. L. (2006). A modified Gompertz model to predict microbial inactivation under time-varying temperature conditions. *J. Food Eng.* 76, 89–94. doi: 10.1016/j.jfoodeng.2005.05.017

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

Johnson, C. N., Flowers, A. R., Noriea, N. F., Zimmerman, A. M., Bowers, J. C., and DePaola, A., et al. (2010). Relationships between environmental factors and pathogenic *Vibrios* in the northern Gulf of Mexico. *Appl. Environ. Microbiol.* 76, 7076–7084. doi: 10.1128/AEM.00697-10

Juneja, V. K., Melendres, M. V., Huang, L., Gumudavelli, V., Subbiah, J., and Thippareddi, H. (2007). Modeling the effect of temperature on growth of *Salmonella* in chicken. *Food Microbiol.* 24, 328–335. doi: 10.1016/j.fm.2006.08.004

Lammerding, A. M. (1997). An overview of microbial food safety risk assessment. *J. Food Prot.* 60, 1420–1425.

Larsen, A. M., Rikard, F. S., Walton, W. C., and Arias, C. R. (2015). Temperature effect on high salinity depuration of *Vibrio vulnificus* and *V. parahaemolyticus* from the Eastern oyster (*Crassostrea virginica*). *Int. J. Food Microbiol.* 192, 66–71. doi: 10.1016/j.ijfoodmicro.2014.09.025

Letchumanan, V., Chan, K. G., and Lee, L. H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front. Microbiol.* 5:705. doi: 10.3389/fmicb.2014.00705

Li, H., Xie, G., and Edmondson, A. (2007). Evolution and limitations of primary mathematical models in predictive microbiology. *Br. Food J.* 109, 608–626. doi: 10.1108/00070700710772408

Lianou, A., and Koutsoumanis, K. P. (2011). Effect of the growth environment on the strain variability of *Salmonella* enterica kinetic behavior. *Food Microbiol.* 28, 828–837. doi: 10.1016/j.fm.2010.04.006

Lianou, A., and Koutsoumanis, K. P. (2013). Strain variability of the behavior of foodborne bacterial pathogens: a review. *Int. J. Food Microbiol.* 167, 310–321. doi: 10.1016/j.ijfoodmicro.2013.09.016

Lianou, A., Stopforth, J. D., Yoon, Y., Wiedmann, M., and Sofos, J. N. (2006). Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. *J. Food Prot.* 69, 2640–2647.

Lindqvist, R. (2006). Estimation of Staphylococcus aureus growth parameters from turbidity data: characterization of strain variation and comparison of methods. *Appl. Environ. Microbiol.* 72, 4862–4870. doi: 10.1128/AEM.00251-06

Liu, D., Ning, X., and Zhang, J. (2008). Optimization of growth condition of *Vibrio Parahaemolyticus* via response surface methodology. *Microbiology* 35, 306–310. doi: 10.3969/j.issn.0253-2654.2008.02.029

Lopez-Joven, C., Ignacio de Blas, M., and Roque, A. (2015). Prevalences of pathogenic and non-pathogenic *Vibrio parahaemolyticus* in mollusks from the Spanish Mediterranean Coast. *Front. Microbiol.* 6:736. doi: 10.3389/fmicb.2015.00736

Ma, C., Deng, X., Ke, C., He, D., and Liang, Z. (2014). Epidemiology and etiology characteristics of foodborne outbreaks caused by *Vibrio parahaemolyticus* during 2008-2010 in Guangdong province, China. *Foodborne Pathog. Dis.* 11, 21–29. doi: 10.1089/fpd.2013.1522

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., et al. (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* 361, 743–749. doi: 10.1016/S0140-6736(03)12659-1

Martins, B. M., and Locke, J. C. (2015). Microbial individuality: how single-cell heterogeneity enables population level strategies. *Curr. Opin. Microbiol.* 24, 104–112. doi: 10.1016/j.mib.2015.01.003

McMeekin, T. A., Olley, J. N., Ross, T., and Ratkowsky, D. A. (1993). *Predictive Microbiology: Theory and Application*. (Taunton, MA: John Wiley and Sons Ltd).

Miles, D. W., Ross, T., Olley, J., and McMeekin, T. A. (1997). Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.* 38, 133–142. doi: 10.1016/S0168-1605(97)00100-1

Miyamoto, Y., Kato, T., Obra, Y., Akiyama, S., Takizawa, K., and Yamai, S. (1969). *In vitro* characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J. Bacteriol.* 100, 1147.

Mytilinaios, I., Salih, M., Schofield, H. K., and Lambert, R. J. (2012). Growth curve prediction from optical density data. *Int. J. Food Microbiol.* 154, 169–176. doi: 10.1016/j.ijfoodmicro.2011.12.035

NACMCF (National Advisory Committee on Microbiological Criteria for Foods) (2005). Considerations for establishing safety-based consume-by date labels for refrigerated ready-to-eat foods. *J. Food Prot.* 68, 1761–1775.

Nauta, M. J. (2000). Separation of uncertainty and variability in quantitative microbial risk assessment models. *Int. J. Food Microbiol.* 57, 9–18. doi: 10.1016/S0168-1605(00)00225-7

Nauta, M. J. (2002). Modelling bacterial growth in quantitative microbiological risk assessment: is it possible? *Int. J. Food Microbiol.* 73, 297–304. doi: 10.1016/S0168-1605(01)00664-X

Nauta, M. J., and Dufrenne, J. B. (1999). Variability in growth characteristics of different *Escherichia coli* O157:H7 isolates and its implications for predictive microbiology. *Quant. Microbiol.* 1, 137–155. doi: 10.1023/A:1010087808314

Nishina, T., Wada, M., Ozawa, H., Hara-Kudo, Y., Konuma, H., Hasegawa, J., et al. (2004). Growth kinetics of *Vibrio parahaemolyticus* O3:K6 under varying conditions of pH, NaCl concentration and temperature. *Shokuhin Eiseigaku Zasshi.* 45, 35–37. doi: 10.3358/shokueishi.45.35

Okada, N., Iida, T., Park, K. S., Goto, N., Yasunaga, T., Hiyoshi, H., et al. (2009). Identification and characterization of a novel type III secretion system in trh-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity of pathogenic machinery beyond the species level. *Infect. Immun.* 77, 904–913. doi: 10.1128/IAI.01184-08

Pouillot, R., Albert, I., Cornu, M., and Denis, J. B. (2003). Estimation of uncertainty and variability in bacterial growth using Bayesian inference. Application to *Listeria monocytogenes*. *Int. J. Food Microbiol.* 81, 87–104. doi: 10.1016/S0168-1605(02)00192-7

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.

Scott, V. N., Swanson, K. M. J., Freier, T. A., Pruett, W. P. Jr., Sveum, W. H., Hall, P. A., et al. (2005). Guidelines for conducting Listeria monocytogenes challenge testing of foods. *Food Prot. Trends* 25, 818–825.

Shen, X., Cai, Y., and Liu, C. (2009). Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (*Crassostrea plicatula*). *Int. J. Food Microbiol.* 136, 129–132. doi: 10.1016/j.ijfoodmicro.2009.09.012

Shen, X. S., Liu, W. W., and Liu, C. C. (2010). "Effects of cold storage and thermal treatment on growth and survival of pathogenic *Vibrio Parahaemolyticus*," in *International Conference on Bioinformatics and Biomedical Technology* (Chengdu), 371–373.

Sobrinho, P. C., Destro, M. T., Franco, B. D., and Landgraf, M. (2014). A quantitative risk assessment model for *Vibrio parahaemolyticus* in raw oysters in Sao Paulo State, Brazil. *Int. J. Food Microbiol.* 180, 69–77. doi: 10.1016/j.ijfoodmicro.2014.04.008

Tang, J. Y. H., Mohd-Noor, N. H., Mazlan, N., Yeo, C. C., and Che, B. B. (2014). Survival of *Vibrio cholerae* O1 and *Vibrio parahaemolyticus* in fried and boiled Malaysian fish sausage. *Food Control* 41, 102–105. doi: 10.1016/j.foodcont.2014.01.004

U.S. Food and Drug Administration (2005). *Quantitative Risk Assessment on the Public Health Impact of Pathogenic Vibrio parahaemolyticus in Raw Oysters*. Center for Food Safety and Applied Nutrition, Food and Drug Administration, U.S. Department of Health and Human Services. Available online at: http://www.fda.gov/Food/FoodScienceResearch/RiskSafetyAssessment/ucm050421.htm

Vose, D. J. (1998). The application of quantitative risk assessment to microbial food safety. *J. Food Prot.* 61, 640–648.

Wang, J. J., Sun, W. S., Jin, M. T., Liu, H. Q., Zhang, W. J., Sun, X. H., et al. (2014). Fate of *Vibrio parahaemolyticus* on shrimp after acidic electrolyzed water treatment. *Int. J. Food Microbiol.* 179, 50–56. doi: 10.1016/j.ijfoodmicro.2014.03.016

Whiting, R. C., and Golden, M. H. (2002). Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. *J. Food Microbiol.* 75, 127–133. doi: 10.1016/S0168-1605(02)00003-X

Wong, H. C., Liu, S. H., Ku, L. W., Lee, I. Y., Wang, T. K., Lee, Y. S., et al. (2000). Characterization of *Vibrio parahaemolyticus* isolates obtained from foodborne illness outbreaks during 1992 through 1995 in Taiwan. *J. Food Prot.* 63, 900–906.

Wu, Y., Wen, J., Ma, Y., Ma, X., and Chen, Y. (2014). Epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus*, China, 2003-2008. *Food Control* 46, 197–202. doi: 10.1016/j.foodcont.2014.05.023

Yang, Z. Q., Jiao, X. A., Li, P., Pan, Z. M., Huang, J. L., Gu, R. X.,et al. (2009). Predictive model of *Vibrio parahaemolyticus* growth and survival on salmon meat as a function of temperature. *Food Microbiol.* 26, 606–614. doi: 10.1016/j.fm.2009.04.004

Yang, Z. Q., Jiao, X. A., Zhou, X. H., Cao, G. X., Fang, W. M., and Gu, R. X. (2008). Isolation and molecular characterization of *Vibrio parahaemolyticus* from fresh, low-temperature preserved, dried, and salted seafood products in two coastal areas of eastern China. *Int. J. Food Microbiol.* 125, 279–285. doi: 10.1016/j.ijfoodmicro.2008.04.007

Yoon, K. S., Min, K. J., Jung, Y. J., Kwon, K. Y., Lee, J. K., and Oh, S. W. (2008). A model of the effect of temperature on the growth of pathogenic and nonpathogenic *Vibrio parahaemolyticus* isolated from oysters in Korea. *Food Microbiol.* 25, 635–641. doi: 10.1016/j.fm.2008.04.007

Zhang, Z., Liu, H., Lou, Y., Xiao, L., Liao, C., Malakar, P. K., et al. (2015). Quantifying viable *Vibrio parahaemolyticus* and *Listeria monocytogenes* simultaneously in raw shrimp. *Appl. Microbiol. Biotechnol.* 99, 6451–6462. doi: 10.1007/s00253-015-6715-x

Zimmerman, A. M., DePaola, A., Bowers, J. C., Krantz, J. A., Nordstrom, J. L., and Johnson, C. N. (2007). Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. *Appl. Environ. Microbiol.* 73, 7589–7596. doi: 10.1128/AEM.01700-07

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., and VantRiet, K. (1990). Modelling of the bacterial growth curve. *Appl. Environ. Microbiol.* 56, 1875–1881.

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 June 2016
doi: 10.3389/fmicb.2016.00942

Rapid Detection and Isolation of *Escherichia coli* O104:H4 from Milk Using Monoclonal Antibody-coated Magnetic Beads

Mirella Luciani[1], Tiziana Di Febo[1], Katiuscia Zilli[1], Elisabetta Di Giannatale[1], Gisella Armillotta[1], Laura Manna[1], Fabio Minelli[2], Manuela Tittarelli[1] and Alfredo Caprioli[2]*

[1] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy,* [2] *EU Reference Laboratory for E. coli, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Rome, Italy*

Monoclonal antibodies (MAbs) specific for the lipopolysaccharide (LPS) of *Escherichia coli* O104:H4 were produced by fusion of Sp2/O-Ag-14 mouse myeloma cells with spleen cells of Balb/c mice, immunized with heat-inactivated and sonicated *E. coli* O104:H4 bacterial cells. Four MAbs specific for the *E. coli* O104:H4 LPS (1E6G6, 1F4C9, 3G6G7, and 4G10D2) were characterized and evaluated for the use in a method for the detection of *E. coli* O104:H4 in milk samples that involves antibody conjugation to magnetic microbeads to reduce time and increase the efficiency of isolation. MAb 1E6G6 was selected and coupled to microbeads, then used for immuno-magnetic separation (IMS); the efficiency of the IMS method for *E. coli* O104:H4 isolation from milk was evaluated and compared to that of the EU RL VTEC conventional culture-based isolation procedure. Milk suspensions also containing other pathogenic bacteria that could potentially be found in milk (*Campylobacter jejuni, Listeria monocytogenes,* and *Staphylococcus aureus*) were also tested to evaluate the specificity of MAb-coated beads. Beads coated with MAb 1E6G6 showed a good ability to capture the *E. coli* O104:H4, even in milk samples contaminated with other bacteria, with a higher number of *E. coli* O104:H4 CFU reisolated in comparison with the official method (121 and 41 CFU, respectively, at 10^3 *E. coli* O104:H4 initial load; 19 and 6 CFU, respectively, at 10^2 *E. coli* O104:H4 initial load; 1 and 0 CFU, respectively, at 10^1 *E. coli* O104:H4 initial load). The specificity was 100%.

Keywords: detection, *Escherichia coli* O104:H4, immuno-magnetic separation, milk, monoclonal antibodies

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Pendru Raghunath, Texila American University, Guyana
Giovanni Normanno, University of Foggia, Italy

****Correspondence:***
Mirella Luciani
m.luciani@izs.it

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

Received: *18 February 2016*
Accepted: *01 June 2016*
Published: *15 June 2016*

Citation:
Luciani M, Di Febo T, Zilli K, Di Giannatale E, Armillotta G, Manna L, Minelli F, Tittarelli M and Caprioli A (2016) Rapid Detection and Isolation of Escherichia coli O104:H4 from Milk Using Monoclonal Antibody-coated Magnetic Beads. Front. Microbiol. 7:942. doi: 10.3389/fmicb.2016.00942

INTRODUCTION

Escherichia coli (Enterobacteriaceae) is a Gram-negative, facultative anaerobic bacterium that is commonly found in the lower intestinal tract of healthy animals and humans. However, several *E. coli* strains have acquired virulence traits that allow them to cause disease in humans and animals. At least six categories of pathogenic *E. coli* able to affect the human gut have been described: Shiga-toxin-producing *E. coli*, also called verocytotoxin-producing *E. coli* (STEC or VTEC), of which enterohaemorrhagic *E. coli* (EHEC) are a highly pathogenic sub-group causing

bloody diarrhea and the hemolytic uremic syndrome (HUS), characterized by severe acute renal failure, thrombocytopenia and micro-angiopathic haemolytic anemia (European Centre for Disease Prevention and Control [ECDC] and European Food Safety Authority [EFSA], 2011); enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAggEC); enteroinvasive *E. coli* (EIEC), and attaching and effacing *E. coli* (A/EEC) (European Centre for Disease Prevention and Control [ECDC] and European Food Safety Authority [EFSA], 2011; Farrokh et al., 2013).

Prior to 2011, STEC serogroup O104 was not considered as a major STEC serogroup, although it had been associated with an outbreak of diarrhea in the US and with sporadic cases in European countries and Korea (European Centre for Disease Prevention and Control [ECDC] and European Food Safety Authority [EFSA], 2011; Baranzoni et al., 2014).

The concern about this serogroup increased in May-July 2011, with the occurrence of two outbreaks of bloody diarrhea and HUS in Europe: one in Germany (around 4000 cases of bloody diarrhea, 850 cases of HUS and 50 deaths), and a much smaller outbreak in southwest France (15 cases of bloody diarrhea, 9 of which progressed to HUS). Both outbreaks were caused by a STEC strain belonging to serotype O104:H4 and linked to the consumption of contaminated sprouts from fenugreek seeds (Grad et al., 2012; Baranzoni et al., 2014). The genetic analysis of the outbreak strain revealed that it carried virulence genes associated with both STEC and EaggEC (Bielaszewska et al., 2011; Scheutz et al., 2011; Baranzoni et al., 2014); in addition, all isolates also expressed the phenotypes that define STEC and EaggEC, specifically production of Shiga-toxin 2 (Stx2) and the aggregative adherence pattern on intestinal epithelial cells, and were resistant to all penicillins and cephalosporins and to co-trimoxazole (trimethoprim-sulfamethoxazole). The specific combination of the higher adherence to intestinal cells, physical survival, Stx2 production and antibiotic resistance, shows the high genomic plasticity of *E. coli* O104:H4 and could explain the high virulence of the epidemic strain (Bielaszewska et al., 2011; Scheutz et al., 2011).

The severity of the oubreaks caused by this foodborne pathogen highlights the need for sensitive screening methods allowing its rapid identification and isolation from food matrices, as sprouts, milk and meat.

Raw cow's and goat's milk provides a potential growth medium for bacteria and its consumption has been frequently associated with STEC infections in Europe, USA and Canada. Most of these cases were associated with STEC O157, although other serotypes or serogroups, including O22:H8, O110:H$^-$, O80:H$^-$, and O145 have been identified as causative agents. Consumption of contaminated soft and semi-soft cheeses has also been implicated in outbreaks: *E. coli* O157:H7 was linked to the majority of cases, but O27:H20, O103, O26, O145, O119:B14, O27:H20, and O104:H21 have also been implicated (Centers for Diseases Control and Prevention [CDC], (1995); Farrokh et al., 2013). Generally, there are two suggested routes by which potentially pathogenic STEC can contaminate raw milk: rare sub-clinical mastitis causing STEC excretion from the udder and contamination during the milking process, when teats are soiled with feces. STEC could also potentially persist if milking equipment is not adequately cleaned. Contamination of dairy products (cheeses, cream, ice-cream, yogurt and butter) is commonly due to the use of raw/unpasteurized milk, to defective pasteurization of milk and/or post processing contamination (Farrokh et al., 2013).

The aim of this work was the development of an immunomagnetic separation (IMS) method based on the use of beads coated with monoclonal antibodies (MAbs) specific for the lipopolysaccharide (LPS) of *E. coli* O104:H4 for the rapid and efficient isolation of *E. coli* O104:H4 from milk samples.

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* O104:H4 strain used for the production and the screening of MAbs and for the immunomagnetic capture was isolated from an Italian child with HUS in 2009 (Scavia et al., 2011). Other five enteroaggregative *E. coli* O104 strains were used to test the MAb 1E6G6. These included two VT2-positive strains and one VT-negative strain kindly provided by the Statens Seruminstitut (Copenhagen, Denmark), a VT2-positive strain kindly provided by the Robert Koch Institute (Berlin, Germany), and another VT2-positive strain kindly provided by the Hopital Robert Debre (Paris, France). The *E. coli* O157, *E. coli* O26, *E. coli* O103, *E. coli* O111, and *E. coli* O145 strains used for MAbs characterization, were part of the culture collection of the European Union Reference Laboratory for *E. coli* (EU-RL-ISS). *Campylobacter jejuni* ATCC 33291, *Listeria monocytogenes* ATCC 7644, and *Staphylococcus aureus* ATCC 6538 used to evaluate the specificity of MAbs, were obtained from ATCC Bacterial Collection LGC.

Preparation of *E. coli* Antigens for MAbs Production and Characterization

Escherichia coli O104:H4, *E. coli* O157, *E. coli* O26, *E. coli* O103, *E. coli* O111, and *E. coli* O145 were grown in Brain Heart Infusion Broth (BHI) (Oxoid, Basingstoke, Hampshire, UK) at 37°C for 14–16 h, inactivated at 100°C for 1 h and centrifuged at 5,500 *g* for 30 min. The pellet was washed for three times with 0.01 M phosphate buffered saline, pH 7.2 (PBS), resuspended in PBS and stored at –20°C. One aliquot of the *E. coli* O104:H4 suspension was sonicated in ice bath for two cycles of 2.5 min each with an interval of 5 min and immediately used for mice immunization.

The protein concentration of antigen preparations was determined using the BCA Protein Assay Kit (Pierce Rockford, IL, USA).

Preparation of the *E. coli* O104 Lipopolysaccharide

The *E. coli* O104 LPS was extracted by the hot phenol–water method (Westphal and Jann, 1965). The LPS was precipitated with 3 M sodium-acetate and absolute ethanol, ultracentrifuged, resuspended in deionized water and stored at –80°C until

use. The quantity of LPS was determined by the 2-keto-3-deoxyoctonate (KDO) assay (Karkharis et al., 1978).

Immunization of Mice

For MAbs production, 6/8 week-old Balb/c mice were inoculated with heat-inactivated and sonicated *E. coli* O104:H4 preparations. Animal experimentation was carried out in compliance with Italian national law (Decreto legislativo 27 Gennaio, 1992, n. 116) implementing Directive 86/609/EEC of the Council of the European Communities on the protection of animals used for experimental and other scientific purposes (European Commission [EC], 1986). The protocol was approved by the Italian Ministry of Health with number 5146 of 26.04.2012. The whole antigen, diluted to a protein concentration of 100 μg/ml, was emulsified with complete Freund adjuvant (Sigma, St. Louis, MO, USA) and administered intraperitoneally; 14 days later a second immunization was performed using the same concentration of antigen emulsified with incomplete Freund adjuvant (Sigma). Subsequently, on days 28 and 56, 100 μg/ml of antigen diluted in PBS was given (intravenous booster). Three days later, the mice were sacrificed, the spleen collected and splenocytes subjected to cell fusion with murine myeloma cells Sp2/O-Ag-14 (ATCC CRL-1581^{TM}).

Characterization of Monoclonal Antibodies vs. *E. coli* O104:H4

The antibody-secreting hybridomas, cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% bovine fetal serum, 2 mM L-glutamine, 100X Penicillin–Streptomycin–Amphotericin, 50 mg/ml gentamicin and 50X HAT, were screened by i-ELISA. Briefly, 96-well microplates (PolySorp, Nunc Brand Products, Roskilde, DK) were coated with 10 μg/ml of *E. coli* O104 LPS and of the bacterial whole antigens (*E. coli* O104:H4, *E. coli* O157, *E. coli* O26, *E. coli* O103, *E. coli* O111, *E. coli* O145) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. After washing and blocking with 1% yeast extract in PBS containing 0.05% Tween 20 (PBST) at 37°C for 1 h, 100 μl/well of MAbs supernatants were added and incubated for 1 h at 37°C. As secondary antibody, ECL anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used; the 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) was adopted as chromogenic substrate. Microplate reading was performed with a biophotometer (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm. Clones that showed optical densities (OD_{450}) greater than or equal to 3 times the OD_{450} of the negative control (serum of a naive Balb/c mouse) were considered as positives and were cloned by the limiting dilution method (Luciani et al., 2006). MAbs were produced *in vitro* on a large scale by means of serial cultures of hybridomas and collection of the supernatants and were isotyped using the Mouse-Typer Isotyping Panel (Bio-Rad).

For immunoblotting analysis, MAbs with IgG and IgM isotype were purified on affinity chromatography using, respectively, a HiTrap rProtein A FF column (GE Healthcare, Uppsala, SW) and a HiTrap IgM Purification HP column (GE Healthcare), according to the manufacturer's instructions.

Purified MAbs were concentrated with 100 kDa cut-off centrifugal filters (Millipore, Billerica, MA, USA) and resuspended in PBS. The protein concentration of purified MAbs was determined by spectrophotometry (Absorbance at 280 nm/IgG molar extinction coefficient).

The heat-treated *E. coli* O104:H4 suspensions (2.5 μg/well) were subjected to SDS-PAGE separation at 200 V with NuPAGE 4–12% Bis-Tris Gels Mini (Novex, Life Technologies, Carlsbad, CA, USA) and transferred onto nitrocellulose membrane with iBlot Dry Blotting System (Life Technologies). After blocking with 5% skimmed milk (Fluka Analytical, Sigma-Aldrich) in PBST for 2 h at room temperature (RT), membrane strips were incubated overnight at 4°C with the purified MAbs. The detection of immune complexes was performed using the ECL anti-mouse IgG HRP-conjugated (GE Healthcare) and a chemiluminescent substrate (ECL Select Western Blotting Detection Reagent, GE Healthcare). The analysis of the results was performed using the Chemidoc MP (Bio-Rad) and the Quantity One Quantitation Software version 4.3 (Bio-Rad).

Magnetic Beads Coupling Procedure

Purified MAbs were coupled to Dynabeads M-450 Tosylactivated (Life Technologies). Twenty-five micro litre of dynabeads were first washed with 0.1 M sodium phosphate buffer, pH 7.4, and then incubated with 5 μg of each purified MAb at 37°C for 24 h using the Dynabeads MX4 Mixer (Life Technologies). Supernatant was removed and dynabeads were resuspended in 0.1 M Tris containing 0.1% BSA (pH 8.5) and incubated for 4 h at 37°C with gentle and continuous agitation. Supernatant was discarded and activated dynabeads were resuspended in PBS containing 0.1% BSA, 2 mM EDTA (pH 7.4), and stored at 4°C until use.

Non-coated dynabeads were also prepared using the same protocol, but without the incubation with the MAbs (non-coated-beads control as blank).

Preparation of Bacterial Strains for Immuno-Magnetic Separation

Escherichia coli O104:H4 was grown in Tryptic Soy Broth (TSB) (Becton Dickinson, Franklin Lakes, NJ, USA) for 24 h at 37°C, and then the cultures were streaked onto Tryptic Soy Agar (TSA) (Becton Dickinson) plates and incubated for 24 h in aerobic atmosphere at 37°C. A well separated colony from the plate was transferred into 10 ml of TSB and incubated at 37°C in order to obtain a bacterial suspension with a turbidity between 2 and 3 McFarland standards. The number of cells in the bacterial suspension was also estimated by reading the absorbance at 600 nm (OD_{600}). The final concentration of bacterial suspension was 10^{11} CFU. The culture was decimally diluted in sterile PBS (until 10^{-11}); the number of bacteria in each diluted suspension was confirmed by plating onto MacConkey Agar at 37°C for 24 h.

Campylobacter jejuni, L. monocytogenes, and *S. aureus* (pool of contaminating bacteria) were grown, respectively, on Karmali Agar, Aloe Agar, and TSA Agar. For each strain, a bacterial suspension in PBS with a concentration of 0.8 McFarland (about 10^8 CFU) was prepared.

Immuno-Magnetic Separation and Evaluation of MAb-Coated Beads Specificity

The specificity of MAb-coated beads was first evaluated using PBS suspensions, prepared as described above, containing decreasing concentration of *E. coli* O104:H4 from 10^6 CFU to 10^1 CFU. Tests were repeated using *E. coli* O104:H4 PBS suspensions containing *L. monocytogenes*, *C. jejuni* and *S. aureus* at the concentration of 10^3 CFU each.

One ml of each PBS-diluted bacterial suspension was added with 25 µl of MAb-activated dynabeads and incubated at 4°C for 30 min with gentle and continuous agitation. Then, dynabeads were washed four times with PBS containing 0.1% BSA and 2 mM EDTA, (pH 7.4), resuspended in 100 µl of PBS, plated onto MacConkey Agar plates and incubated at 37°C for 24 h. The same procedure was repeated using non-coated beads to ensure the absence of aspecific interactions between the non-coated beads and bacteria.

TABLE 1 | Monoclonal antibodies isotypes.

Clone	Isotype
1E6G6	IgG1 anti κ
1F4C9	IgG3 anti κ
3G6G7	IgG1 anti κ
4G10D2	IgG3 anti κ

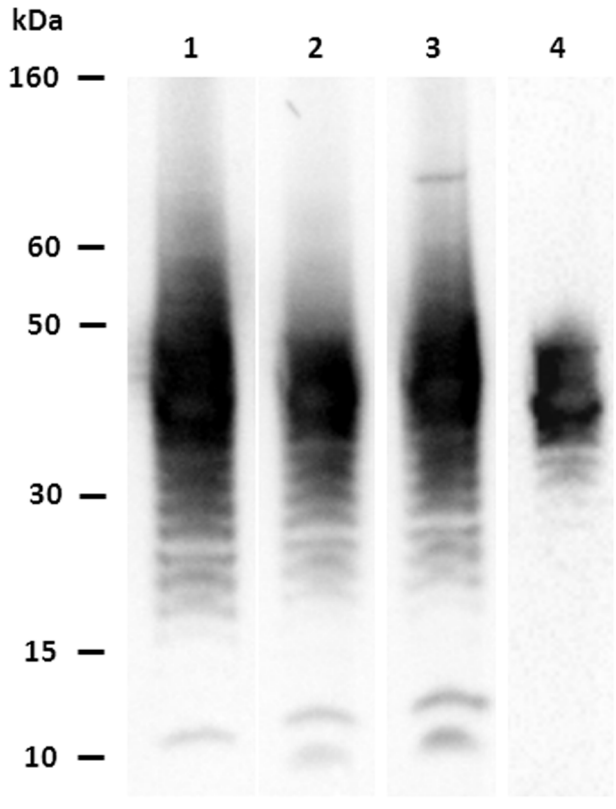


FIGURE 1 | Characterization of MAbs vs. *Escherichia coli* O104:H4: western blotting results. Lane 1: MAb 1E6G6; lane 2: MAb 1F4C9; lane 3: MAb 3G6G7; lane 4: MAb 4G10D2.

Inoculation and Enrichment of Milk and *E. coli* O104:H4 Detection from Artificially Contaminated Milk Samples

Six aliquots (9 ml each) of bovine raw milk were artificially contaminated with 1 ml of each one of the six bacterial suspensions previously prepared (*E. coli* O104:H4 concentration from 10^6 CFU to 10^1 CFU). One ml of each *E. coli* O104:H4 contaminated milk sample was incubated with MAb-coated and non-coated beads as described above. In the same time, one ml aliquot of each milk sample was analyzed using the EU RL VTEC official method, which include an overnight enrichment step. Aliquots (100 µl) of the IMS-treated samples and of the enrichment broth cultures prepared according to the EU RL VTEC method were plated onto MacConkey Agar plates. After incubation for 24 h at 37°C in aerobic condition, the colonies obtained with the EU RL VTEC method and with MAb-coated beads were enumerated.

To verify the absence of cross-reaction between MAbs and bacteria other than *E. coli* O104:H4, the same tests described above were repeated using four aliquots for each of the six cream milk samples artificially contaminated with the six bacterial suspensions at the concentration of *E. coli* O104:H4 from 10^6 CFU to 10^1 CFU and with each species of contaminating bacteria at the concentration of 10^3 CFU. Each of four aliquots of the six milk suspensions were, respectively, plated onto MacConkey Agar, onto Blood Agar for 24 h at 37°C in aerobic condition for *L. monocytogenes* and *S. aureus* isolation, and Karmali Agar for 48 h at 42°C in microaerobic conditions for *C. jejuni* isolation.

DNA Extraction and PCR for Strains Typing

Escherichia coli O104:H4 DNA extraction was performed using the InstaGene Matrix (Bio-Rad) according to manufacturer's instructions. Primers used for the PCR assay were specific for the rfb_{O104} gene, encoding the O-antigen specific for *E. coli* O104 (O104*rfb*O-f 5′ TGAACTGATTTTTAGGATGG 3′; O104*rfb*O-r 5′ AGAACCTCACTCAAATTATG 3′, amplicon size 351 bp) (Bielaszewska et al., 2011). A 50 µl PCR mixture contained: 25 µl 2× TopTaq Master Mix (Qiagen, Venlo, NL) (1.25 units of TopTaq DNA Polymerase, 1× PCR buffer, dNTPs concentration 0.2 mM each, 1.5 mM $MgCl_2$), 1 µl of each primer at the concentration of 0.2 µM, 18 µl of Nuclease-Free Water and 5 µl of template DNA. Amplifications were performed using the GeneAmp® PCR System 9700 (Applied Biosystems, Life Technologies) and the following temperature cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis on a 1,5% agarose gel.

TABLE 2 | Evaluation of MAb-coated beads specificity using *Escherichia coli* O104:H4 PBS pure suspensions.

E. coli O104:H4 in the initial suspension (CFU number)	*E. coli* O104:H4 re-isolated after immuno-magnetic separation (IMS) (CFU number)
10^6	>200
10^5	>200
10^4	>200
10^3	76
10^2	3
10^1	0

Statistical Analysis

In order to determine differences between the EU RL VTEC conventional culture-based isolation procedure and the IMS method described in this paper, the Wilcoxon test for dependent samples was applied (confidence interval = 95%).

RESULTS

Characterization of Monoclonal Antibodies vs. the LPS of *E. coli* O104:H4

A total of 28 hybridomas were positive against the *E. coli* O104:H4 whole heat-treated antigen in indirect ELISA. Four MAbs (1E6G6, 1F4C9, 3G6G7, and 4G10D2) were found positive for both the *E. coli* O104:H4 LPS and whole antigen, and negative against the other O serogroups tested. MAbs isotypes are shown in **Table 1**. Characterization by western blotting revealed that the four selected MAbs reacted with the LPS of *E. coli* O104:H4; **Figure 1** shows the ladder-like pattern of LPS bands with molecular weights between 60 and 15 kDa recognized by the MAbs. One of the four selected MAbs, MAb 1E6G6, was tested by slide agglutination against five additional *E. coli* O104:H4 strains with positive results and subsequently conjugated to magnetic microbeads, to be used for a specific IMS of *E. coli* O104:H4 from milk samples.

Immuno-Magnetic Separation and Evaluation of MAb-Coated Beads Specificity

The results obtained from *E. coli* O104:H4 re-isolation from PBS pure bacterial suspensions are shown in **Table 2**. At *E. coli* concentrations higher than or equal to 10^4, the number of CFU re-isolated was higher than 200. At the concentrations of 10^3 and 10^2 CFU, 76 and 3 CFU, respectively, were re-isolated following the IMS.

When the *E. coli* O104:H4 PBS suspensions containing other bacteria (*L. monocytogenes*, *C. jejuni* and *S. aureus*) were used, more than 200 *E. coli* O104:H4 CFU were re-isolated from suspensions with *E. coli* concentrations higher than or equal to 10^3; 19 and 2 CFU were re-isolated from the 10^2 and 10^1 CFU *E. coli* O104:H4 suspensions, respectively, (**Table 3**). None of the contaminating bacteria were captured by the MAb-coated beads. No aspecific binding between the non-coated beads and the four bacterial suspensions used in the experiments was observed.

E. coli O104:H4 Detection in Artificially Contaminated Milk Samples

Table 4 shows the results of the *E. coli* O104:H4 isolation from milk samples contaminated with scalar dilutions (from 10^6 to 10^1 CFU) of *E. coli* O104:H4. With the Dynabeads method, a higher number of *E. coli* O104:H4 CFU was isolated with respect to the EU RL method. Similar results were obtained with milk samples contaminated with *E. coli* O104:H4 and with other bacteria (*L. monocytogenes*, *C. jejuni* and *S. aureus*) (**Table 5**). The p-value obtained using the Wilcoxon test for dependent samples was 0.0517 with a confidence interval of 95%.

Moreover, none of the other contaminating bacteria were captured by MAb-coated beads, as shown by the absence of growth in the specific media. MAb-coated beads isolates were further confirmed by PCR: all the bacteria captured by coated beads were *E. coli* O104:H4 (**Figure 2**).

DISCUSSION AND CONCLUSION

Shiga toxin–producing *Escherichia coli* strains are important foodborne pathogens that have been responsible for numerous

TABLE 3 | Evaluation of MAb-coated beads specificity using *E. coli* O104:H4 PBS suspensions containing contaminating bacteria (*Listeria monocytogenes*, *Campylobacter jejuni,* and *Staphylococcus aureus*).

E. coli O104:H4 in the initial suspension (CFU number)	Contaminating bacteria in the initial suspension (CFU number)	*E. coli* O104:H4 re-isolated after IMS (CFU number)	Contaminating bacteria re-isolated after IMS (CFU number)
10^6	10^3	>200	0
10^5	10^3	>200	0
10^4	10^3	>200	0
10^3	10^3	>200	0
10^2	10^3	19	0
10^1	10^3	2	0

TABLE 4 | *Escherichia coli* O104:H4 isolation from artificially contaminated milk samples in the absence of contaminating bacteria: comparison between the EU RL VTEC official method and the *E. coli* O104 Dynabeads method.

E. coli O104:H4 in the initial suspension (CFU number)	*E. coli* O104:H4 isolated with the EU RL method (CFU number)	*E. coli* O104:H4 isolated with the O104 Dynabeads method (CFU number)
10^6	>200	>200
10^5	159	>200
10^4	117	>200
10^3	43	>200
10^2	8	27
10^1	0	4

TABLE 5 | *Escherichia coli* O104:H4 detection from artificially contaminated milk samples in the presence of contaminating bacteria (*L. monocytogenes*, *C. jejuni,* and *S. aureus*): comparison between the EU RL VTEC official method and the *E. coli* O104 Dynabeads method.

E. coli O104:H4 in the initial suspension (CFU number)	Contaminating bacteria in the initial suspension (CFU number)	*E. coli* O104:H4 isolated with EU RL method (CFU number)	*E. coli* O104:H4 isolated with the *E. coli* O104 Dynabeads method (CFU number)	*S. aureus* isolated with the *E. coli* O104 Dynabeads method (CFU number on Blood Agar plate)	*L. monocytogenes* isolated with the *E. coli* O104 Dynabeads method (CFU number on Blood Agar plate)	*C. jejuni* isolated with the *E. coli* O104 Dynabeads method (CFU number on Karmali Agar plate)
10^6	10^3	>200	>200	0	0	0
10^5	10^3	>200	>200	0	0	0
10^4	10^3	137	>200	0	0	0
10^3	10^3	41	121	0	0	0
10^2	10^3	6	19	0	0	0
10^1	10^3	0	1	0	0	0

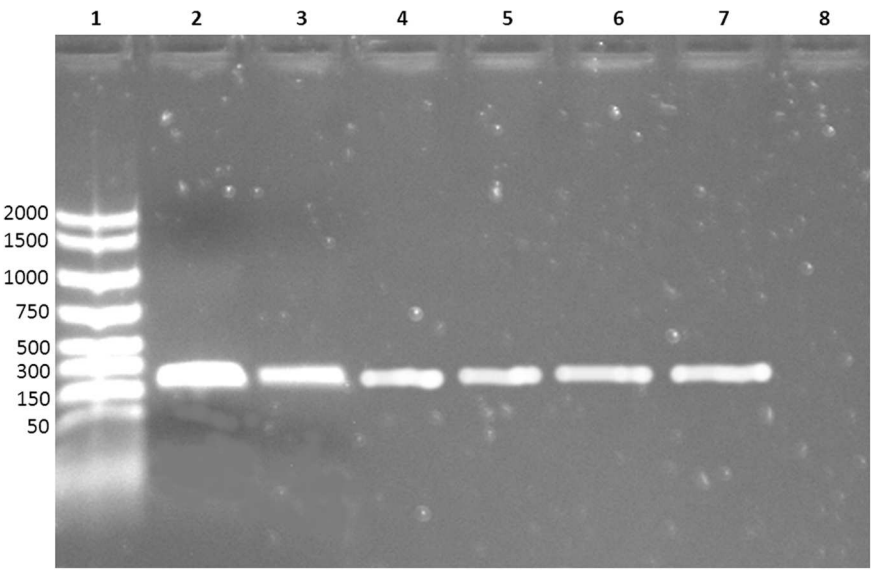


FIGURE 2 | Electrophoresis of the PCR products. Lane 1: molecular weight marker; lane 2–6: *E. coli* O104:H4 captured by MAb-coated magnetic beads; lane 7: positive control (DNA extracted from *E. coli* O104:H4 ISS-certified); lane 8: negative control (nuclease-free water).

outbreaks of hemorrhagic colitis and HUS worldwide. *E. coli* O157:H7 is the most frequently implicated in human disease, although infections can be caused by other serotypes or serogroups, as *E. coli* O104:H4. Consequently it is necessary to have the availability of rapid, sensitive and specific methods for food control.

In this study, MAbs vs. *E. coli* O104:H4 were produced andcharacterized. The efficiency of the *E. coli* O104:H4 isolation from milk samples, using Dynabeads activated with a selected MAb (1E6G6) specific for *E. coli* O104:H4 LPS, was evaluated and compared with that of the EU RL VTEC isolation procedure (European Reference Laboratory for *E.coli* [EU-RL VTEC], 2011). The specificity of the adsorbed antibodies was also evaluated by preparing *E. coli* O104:H4 suspensions containing other pathogenic bacteria that could potentially be found in milk, such as *Campylobacter jejuni, Listeria monocytogenes,* and *Staphylococcus aureus* (Farrokh et al., 2013).

The use of MAbs, instead of polyclonal ones (PAbs), could be a valid alternative to improve the specificity of pathogen detection. PAbs may have a lack of homogeneity between lots, due to the variability of the animal immune response. They may also show cross-reactions with related pathogens. Conversely, MAbs are highly specific reagents, easily purified and with batch to batch homogeneity.

Immuno-magnetic separation is a rapid and sensitive method that permit the revelation of low concentrations of pathogens in contaminated matrices. The magnetic beads method has already been applied for the detection of *E. coli* O157:H7 (Weagant et al., 2011) and *E. coli* O104 (Baranzoni et al., 2014) from sprouts with good results. In particular, Baranzoni et al. (2014) used commercially available *E. coli* IMS beads in association with optimized enrichment conditions of sprouts samples. The results indicated that the IMS enrichment enhanced the ability to detect *E. coli* O104 and reduced the interference

of background microflora. In our work, we used O104-specific magnetic beads prepared in-house to detect this pathogen in artificially contaminated milk samples, skipping the use of a previous enrichment step. The procedure showed an analytical sensitivity higher than that of the isolation procedure included in the official EURL method for *E. coli* O104. Even if the statistic analysis gave a value slightly higher than the expected one, numeric data show that the immunomagnetic method is more sensitive, particularly at low concentrations of bacteria.

The MAb-coated beads showed a good capability to capture *E. coli* O104:H4 in artificially contaminated milk samples, even in the presence of other contaminating bacteria, such as *C. jejuni*, *L. monocytogenes,* and *S. aureus*, with an increase in the number of CFU re-isolated with respect to the current official method (>200 and 137 CFU, respectively, at 10^4 *E. coli* O104:H4 initial load; 121 and 41 CFU, respectively, at 10^3 *E. coli* O104:H4 initial load; 19 and 6 CFU, respectively, at 10^2 *E. coli* O104:H4 initial load and 1 and 0 CFU, respectively, at 10^1 *E. coli* O104:H4 initial load). The Dynabeads method also showed a good analytical specificity, since no contaminating bacteria were captured by the MAb-coated beads in both PBS bacterial suspensions and contaminated milk samples and no aspecific reactions were obtained with non-coated beads. MAb 1E6G6 was able to retain its antigen-binding activity even in the presence of a fat matrix such as full cream milk. Moreover, the direct application of the O104 IMS to a liquid matrix such as milk, skipping the broth enrichment step that requires an overnight incubation period, allowed a more rapid isolation of *E. coli* O104, with respect to the current official method.

Thus, magnetic beads linked with the MAb 1E6G6 against *E. coli* O104:H4 could be a useful tool for improving the detection of this *E. coli* serogroup in milk samples. Further studies are needed to evaluate the performances of MAb 1E6G6 coated-beads when matrices other than from milk are tested.

AUTHOR CONTRIBUTIONS

ML, TD, and AC designed the study; ML, TD, GA, LM, FM, and MT produced, characterized and purified monoclonal antibodies; AC, FM, KZ, and ED performed microbiological analysis; ML coordinated animal management; ML, TD, and KZ drafted the manuscript; AC, ED, and MT revised the paper critically. All authors read and approved the final version of the manuscript.

REFERENCES

Baranzoni, G. M., Fratamico, P. M., Rubio, F., Glaze, T., Bagi, L. K., and Albonetti, S. (2014). Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) O104 from sprouts. *Int. J. Food Microbiol.* 173, 99–104. doi: 10.1016/j.ijfoodmicro.2013.12.020

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., et al. (2011). Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect. Dis.* 11, 671–676. doi: 10.1016/S1473-3099(11)70165-7

Centers for Diseases Control and Prevention [CDC] (1995). Outbreak of acute gastroenteritis attributable to *Escherichia coli* serotype O104:H21 – Helena, Montana, 1994. *MMWR Morb. Mortal. Wkly. Rep.* 44, 501–503.

Decreto legislativo 27 Gennaio (1992). n. 116. Attuazione della direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. *Official J.* 40, 1–32.

European Centre for Disease Prevention and Control [ECDC] and European Food Safety Authority [EFSA] (2011). *Shiga Toxin/Verotoxin-Producing Escherichia coli in Humans, Food and Animals in the EU/EEA, with Special Reference to the German Outbreak Strain STEC O104.* ECDC/EFSA Joint Technical Report (Stockolm: ECDC), 1–18.

European Commission [EC] (1986). Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). *Official J.* L358, 1–28.

European Reference Laboratory for *E.coli* [EU-RL VTEC] (2011). *Detection and Identification of Verocytotoxin-Producing Escherichia coli (VTEC) O104:H4 in food by Real Time PCR*. Rome: Istituto Superiore di Sanità.

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

Grad, Y. H., Lipsitch, M., Feldgarden, M., Arachchi, H. M., Cerqueira, G. C., Fitzgerald, M., et al. (2012). Genomic epidemiology of the *Escherichia coli* O104:H4 outbreaks in Europe, 2011. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3065–3070. doi: 10.1073/pnas.1121491109

Karkharis, Y. D., Zeltner, J. Y., Jackson, J. J., and Carlo, D. J. (1978). A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. *Anal. Biochem.* 85, 595–601. doi: 10.1016/0003-2697(78)90260-9

Luciani, M., Armillotta, G., Magliulo, M., Portanti, O., Di Febo, T., Di Giannatale, E., et al. (2006). Production and characterization of monoclonal antibodies specific for *Escherichia coli* O157:H7. *Vet. Ital.* 42, 173–182.

Scavia, G., Morabito, S., Tozzoli, R., Michelacci, V., Marziano, M. L., Minelli, F., et al. (2011). Similarity of Shiga toxin-producing *Escherichia coli* O104:H4 strains from Italy and Germany. *Emerg. Infect. Dis.* 17, 1957–1958. doi: 10.3201/eid1710.111072

Scheutz, F., Nielsen, E. M., Frimodt-Møller, J., Boisen, N., Morabito, S., Tozzoli, R., et al. (2011). Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. *Euro Surveill.* 16, 5–10.

Weagant, S. D., Jinneman, K. C., Yoshitomi, K. J., Zapata, R., and Fedio, W. M. (2011). Optimization and evaluation of a modified enrichment procedure combined with immunomagnetic separation for detection of *E. coli* O157:H7 from artificially contaminated alfalfa sprouts. *Int. J. Food Microbiol.* 149, 209–217. doi: 10.1016/j.ijfoodmicro.2011.06.008

Westphal, O., and Jann, K. (1965). Bacterial lipopolysaccharides, extraction with phenol–water and further application of the procedure. *Methods Carbohydr. Chem.* 5, 83–91.

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 June 2016
doi: 10.3389/fmicb.2016.00887

Tracing Back Clinical *Campylobacter jejuni* in the Northwest of Italy and Assessing Their Potential Source

*Elisabetta Di Giannatale[1], Giuliano Garofolo[1], Alessandra Alessiani[1], Guido Di Donato[1], Luca Candeloro[2], Walter Vencia[3], Lucia Decastelli[3] and Francesca Marotta[1]**

[1] National Reference Laboratory for Campylobacter, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Teramo, Italy, [2] Department of Statistics and GIS, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G.Caporale", Teramo, Italy, [3] Food Hygiene and Safety Department, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy

Food-borne campylobacteriosis is caused mainly by the handling or consumption of undercooked chicken meat or by the ingestion of contaminated raw milk. Knowledge about the contributions of different food sources to gastrointestinal disease is fundamental to prioritize food safety interventions and to establish proper control strategies. Assessing the genetic diversity among *Campylobacter* species is essential to our understanding of their epidemiology and population structure. We molecularly characterized 56 *Campylobacter jejuni* isolates (31 from patients hospitalized with gastroenteritis, 17 from raw milk samples, and 8 from chicken samples) using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) in order to trace the source of the disease. We also used a population genetic approach to investigate the source of the human cases from six different reservoirs of infection. MLST identified 25 different sequence types and 11 clonal complexes (CCs) (21, 658, 206, 353, 443, 48, 61, 257, 1332, 354, 574) and these included several alleles not cited previously in the PubMLST international database. The most prevalent CCs were 21, 206, and 354. PFGE showed 34 pulsotypes divided between 28 different clusters. At the fine scale, by means of PFGE and MLST, only two human cases were linked to raw milk, while one case was linked to chicken meat. The investigation revealed the presence of several genotypes among the human isolates, which probably suggests multiple foci for the infections. Finally, the source attribution model we used revealed that most cases were attributed to chicken (69.75%) as the main reservoir in Italy, followed to a lesser extent by the following sources: cattle (8.25%); environment (6.28%); wild bird (7.37%); small ruminant (5.35%), and pork (2.98%). This study confirms the importance of correlating epidemiological investigations with molecular epidemiological data to better understand the dynamics of infection.

Keywords: *Campylobacter*, PFGE, population genetic approach, epidemiological investigations, MLST genotyping

OPEN ACCESS

Edited by:
Maria Schirone,
University of Teramo, Italy

Reviewed by:
Odile Tresse,
French National Institute for Agricultural Research, France
Issmat Kassem,
The Ohio State University, USA

****Correspondence:***
Francesca Marotta
f.marotta@izs.it

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

Received: *17 February 2016*
Accepted: *25 May 2016*
Published: *13 June 2016*

Citation:
Di Giannatale E, Garofolo G, Alessiani A, Di Donato G, Candeloro L, Vencia W, Decastelli L and Marotta F (2016) Tracing Back Clinical Campylobacter jejuni in the Northwest of Italy and Assessing Their Potential Source. Front. Microbiol. 7:887. doi: 10.3389/fmicb.2016.00887

INTRODUCTION

Human campylobacteriosis in the European Union (EFSA, 2014) continues to be the most commonly reported zoonosis, and with 214,268 confirmed cases, this disease has considerable socio-economic impact. The *Campylobacter* species most commonly associated with human infections are *Campylobacter jejuni* followed by *C. coli* and *C. lari*, but other species, including

the non-thermophilic *C. fetus*, also occasionally cause human diseases (EFSA, 2014; Rodrigues et al., 2015). In Italy, 1252 cases of human campylobacteriosis were reported in 2014 (EFSA and ECDC, 2015). However, the data are likely to grossly underestimate the real number of cases (EFSA and ECDC, 2015) because the Italian reporting system for human infectious illnesses does not differentiate between gastroenteritis caused by *Campylobacter* and gastroenteritis caused by the other agents listed in the National Legislation in Italy (Calistri and Giovannini, 2008). Therefore, campylobacteriosis is not a statutory notification illness and the only data available on these infections are those reported voluntarily by Enter-Net, the international network for the surveillance of human gastrointestinal infections.

Generally, campylobacteriosis infections are self-limiting and only last for a few days. However, post-infection complications or extra-intestinal infections such as reactive arthritis and neurological manifestations can also arise (Haddad et al., 2010). Outbreaks caused by thermophilic *Campylobacter* species are most commonly connected with dairy and poultry products, as well as *Campylobacter*-contaminated food and untreated water (Meldrum et al., 2005; EFSA, 2008, 2014; Taylor et al., 2013). Unpasteurized or inadequately pasteurized cow's milk has been implicated as the sources of infection in some outbreaks (Schildt et al., 2006; Heuvelink et al., 2009), and recently, the European Union summary report on food-borne disease outbreaks confirmed the importance of milk as a source of human campylobacteriosis (EFSA, 2013).

In a recent survey conducted in Northern Italy, *C. jejuni* was detected in 12% of the bulk tank milk samples that were examined (Bianchini et al., 2014), and some disease outbreaks have been reported in the same Italian regions (two in Emilia Romagna, one in Veneto, and one in Marche) following the consumption of raw milk from self-service automatic vending machines (Amato et al., 2007; Arrigoni et al., 2009; Petruzzelli et al., 2011). Despite no official data existing for the incidence of campylobacteriosis associated with raw milk consumption, quantitative risk assessment modeling has estimated that the worst possible scenario for human infections with *C. jejuni* linked to the consumption of raw milk in Italy would exceed 300,000 cases per year (Giacometti et al., 2015).

The high genome diversity and plasticity within the *Campylobacter* genus hampers the surveillance and the outbreak detection. Nevertheless, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) are important and well-established tools that can be used to elucidate the epidemiology of *Campylobacter* cases. MLST is the most common genotyping method successfully applied in population genetic models for clarify the reservoirs of infection, while PFGE is a well standardized technique very useful in localized outbreak investigation (Dingle et al., 2001; Fitzgerald et al., 2001; Sheppard et al., 2009).

The objective of this study was to undertake an epidemiological investigation of *Campylobacter* infections in 31 patients hospitalized in the summer of 2012 from the Piemonte region in Northern west Italy. Source investigation using MLST and PFGE comprised *C. jejuni* isolates from raw bovine milk and chicken meat. A polymorphism in the *hip0* gene was also identified in some *Campylobacter* strains from raw bovine milk, and new PCR primer set for PCR identification were designed and successfully implemented in this study.

MATERIALS AND METHODS

C. jejuni Isolates

A total of 31 cases of campylobacteriosis were registered in August, 2012 in the local health district of Turin, Northern Italy. A temporal and spatial proximity was suspected because of a cluster of infections; consequently, active surveillance was carried on suspicious retail food items. The monitoring found 17 raw milk samples from vending machines and 8 chicken meat samples from a retail market were positive for *Campylobacter*. Here, the 56 *C. jejuni* isolates we investigated were cultured on Columbia blood agar and incubated at 42°C for 48 h in a microaerobic atmosphere.

DNA Isolation

Genomic DNA was extracted using an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, CA, USA) according to the manufacturer's instructions. The DNA concentration was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Italy).

Strain Identification

The isolates were typed using a PCR method described previously (Marshall et al., 1999; Di Giannatale et al., 2014).

Six strains isolated from raw milk were non-typable using the PCR method described above. Nevertheless, the strains were determined to be *C. jejuni* by 16S-rRNA gene sequencing (Zhou et al., 1997). Furthermore, a new PCR protocol targeting the *hip0* gene was developed to check for miss-typed isolates. The sequences of the primers, which were designed against the regions upstream and downstream of the original amplification sites, were as follows: P3Fs: 5′-GGAAAAACAGGCGTTGTGGGG G-3′ and P3Rs: 5′-CCGAAGAAGCCATCATCGCACC-3′; P3Fi: 5′-CCTGCTTGAAGAGGGTTTGGGTGG-3′ and P3Ri: 5′-TG CAACCTCACTAGCAAAATCCACA-3′. The PCR was performed using Master Mix 2X (Promega, Italy), the final concentration of the primers was 1 μM, the annealing temperature was 55°C, and the procedure involved 35 cycles in a thermal cycler 9700 Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Sequencing of the amplified fragment was performed using an ABI PRISM BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and the sequences were analyzed with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

PFGE

Pulsed-field gel electrophoresis was conducted according to the instructions from the 2009 USA PulseNet protocol for *Campylobacter* (CDC, 2009). The isolates previously identified by PCR were grown on Columbia agar (48 h at 42°C) in a

microaerophilic atmosphere and embedded in agarose blocks (Seakem Gold agarose, Lonza, Rockland, MD, USA). Following DNA purification, 1 mm of agar plugs slices was digested 18 h with SmaI restriction enzyme (Promega, Milan, Italy) and the DNA fragments were separated by PFGE (Chef Mapper II, Biorad Laboratories, Hercules, CA, USA) in 1% agarose gel (Seakem Gold agarose, Lonza).

Salmonella Braenderup H9812, digested with *Xba*I enzyme (Promega, Milan, Italy), was used as the standard molecular weight marker. The gel was stained with SYBR Safe DNA gel stain (Invitrogen, Cergy Pontoise, France) and photographed on a UV transilluminator (Alpha Innotech Corporation, San Leandro, CA, USA). The image analysis was performed using Bionumerics program v. 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity analysis was carried out using the Dice coefficient (position tolerance, 1%). The unweighted pair group mathematical average was used to cluster patterns. Isolates with <90% similarity were clustered as separate pulsotypes.

MLST Analysis

Multilocus sequence typing was performed as reported by Dingle et al. (2001) for all *C. jejuni* isolates. MLST amplified a segment of seven housekeeping genes: *aspA* (aspartase, 477 bp), *glnA* (glutamine synthase, 477 bp), *gltA* (citrate synthase, 402 bp), *glyA* (serine hydroxyl methyl transferase, 507 bp), *pgm* (phosphor glucomutase, 498 bp), *tkt* (transketolase, 459 bp), and *uncA* (ATP synthase, alpha subunit, 489 bp) to yield a total composite sequence length (all seven loci) of 3309 bp. PCRs and sequencing reactions were carried out according to the guidelines on the *Campylobacter* MLST website. Briefly, purified PCR products were sequenced using an ABI PRISM BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and then analyzed by the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The alleles, STs, and CCs were identified using the MLST database[1]. Novel alleles were submitted to the PubMLST *C. jejuni/C. coli* database curators for number assignment.

Source Attribution Analysis

According to a previous study conducted in the Netherlands (Mughini Gras et al., 2012), *Campylobacter* isolates from humans were found to share significant similar ST frequency distribution with those from Europe (the Netherlands, the UK, and Switzerland), but were genetically dissimilar from abroad isolates (from the USA and New Zealand). We have assumed this situation is also true for Italy and the details in our reference database included 558 *C. jejuni* strains from previously published Italian data; Piccirillo et al., 2014) and from our previous survey (Marotta et al., 2015), as well as the 6854 *C. jejuni* from European countries accessible in PubMLST[2].

The reservoir data we identified were pooled and arranged in six groups: (i) chicken, (ii) cattle, (iii) environment, (iv) wild bird, (v) small ruminant, and (vi) pork. Environmental strains comprised those from water, sand, and soil. The population genetics approach used for attributing human campylobacteriosis isolates to the six putative reservoirs was the asymmetric island model described by Wilson et al. (2008). This model estimates the recombination rates within the reservoirs, between the reservoirs and from each reservoir in the human population to estimate the posterior distribution used to infer the fraction of human cases attributable to each source.

Diversity Index and Evaluation of the Combined Typing Methods

The discriminatory ability of the two typing systems (PFGE and MLST) was measured according to Simpson's diversity index. The concordance of the methods was determined by calculating the adjusted Rand and Wallace coefficients. The first coefficient is used to evaluate the extent of agreement between two typing methods, and the second method is an estimate of how much new information is obtained from a typing method in comparison with another one (Behringer et al., 2011). The adjusted Rand and Wallace coefficients were used to calculate the index from the Online Tool for Quantitative Assessment of Classification Agreement[3].

RESULTS

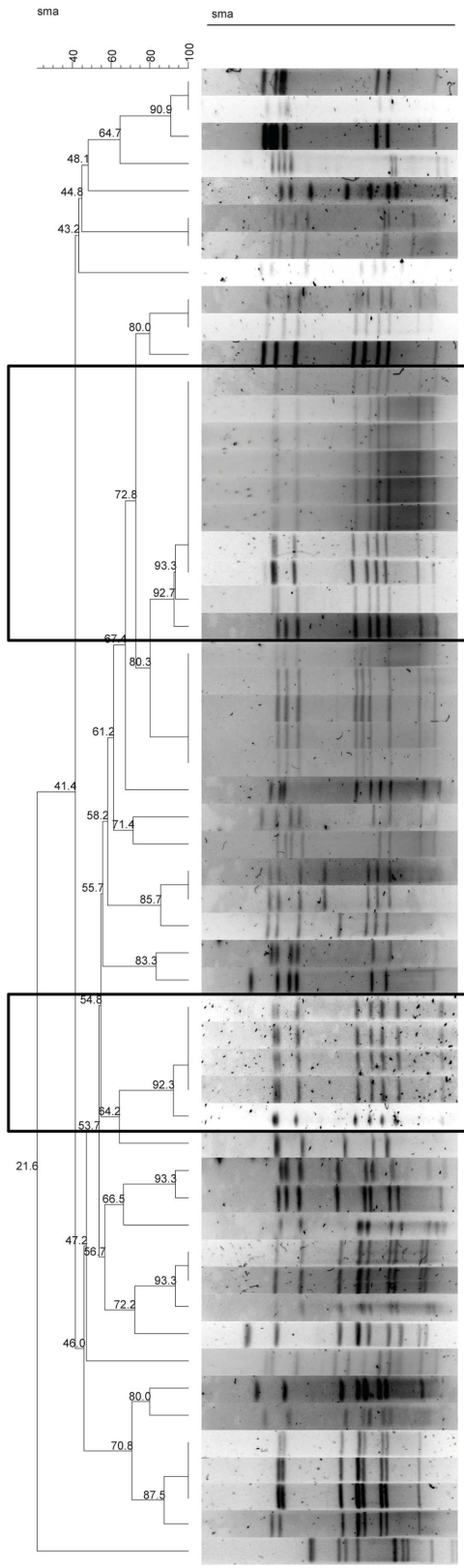
A total of 55 *C. jejuni* isolates were typed with PFGE using *Sma*I restriction enzyme. One chicken isolate could not be typed. Thirty-four different pulsotypes were obtained from the isolates typed (**Figure 1**). The cluster analysis distinguished the isolates according to 28 main clusters, with a similarity of 90% (**Figure 1**). Among these, only two clusters included non-human sources and three clinical isolates. Cluster VIII contained two human isolates, six raw milk isolates and two chicken meat isolates, and cluster XVII, contained one human and four raw milk isolates (**Figure 1**). MLST typing for the 56 isolates identified 25 STs featured in 11 clonal CCs (**Figure 2**). The most prevalent CCs were 21, 206, and 354. CC21 was the largest one shared by humans, cattle, and chickens, while CC206 and CC354 included human and cattle isolates or human and chicken isolates, respectively. At the fine scale, in three cases human isolates were linked to chicken sources sharing the STs 19 and 2863, and in six cases the human isolates were linked to raw milk sources sharing STs 122 and 21. MLST revealed the presence of 18 STs that were not attributable to any of the sources we investigated, including three new STs (STs 7407, 7418, and 6788), probably suggesting multiple foci of infections (**Figure 2**, **Table 1**).

Overall, the 31 human samples were assigned to 22 STs belonging to 11 CCs; among them, two clonal complexes have not been assigned to the international database yet. By means of the two techniques employed here and as shown in **Figure 1**, only 3 out of 31 cases were truly traceable back to the two cases linked to raw milk from vending machines, to chicken meat and one human case linked to raw milk.

[1]http://pubmlst.org/campylobacter/

[2]http://pubmlst.org/

[3]http://darwin.phyloviz.net/ComparingPartitionsv2/index.php?link=Tool



Isolate	Source	Pulsotype	MLST	Cluster	
18978	Human stool	R	1	122_ST-206 complex	I
24011	Human stool	S	1	122_ST-206 complex	I
24008	Human stool	S	2	122_ST-206 complex	I
19570	Human stool	S	3	572_ST-206 complex	II
18976	Human stool	R, NI	4	7407_ST	III
18987	Human stool	S	5	6788_ST-1332 complex	IV
19571	Human stool	S	5	6788_ST-1332 complex	IV
24004	Human stool	R	6	5_ST-353 complex	V
20164	Human stool	S	7	1811_ST-21 complex	VI
24009	Human stool	S	7	50_ST-21 complex	VI
21445	Human stool	S	8	122_ST-206 complex	VII
19567	Human stool	R	9	19_ST-21 complex	VIII
17327/1	cattle	S	9	21_ST-21complex	VIII
17327/2	cattle	S	9	21_ST-21complex	VIII
17327/3	cattle	S	9	21_ST-21complex	VIII
17327/4	cattle	S	9	21_ST-21complex	VIII
17327/5	cattle	S	9	21_ST-21complex	VIII
20206/1	chicken	R	9	19_ST-21 complex	VIII
20206/2	chicken	S	9	19_ST-21 complex	VIII
10419/2	cattle	S	10	21_ST-21complex	VIII
18980	Human stool	S	11	21_ST-21 complex	VIII
17344	cattle	S	12	61_ST-61 complex	IX
17348/1	cattle	R	12	21_ST-21complex	IX
17348/2	cattle	R	12	21_ST-21complex	IX
18102	cattle	R	12	21_ST-21complex	IX
18103/2	cattle	R	12	21_ST-21complex	IX
18984	Human stool	S	13	2789_ST-21 complex	X
20162	Human stool	S	14	48_ST-48 complex	XI
18100	cattle	R	15	61_ST-61 complex	XII
18982	Human stool	S	16	2361_ST-443 complex	XIII
20165	Human stool	S	16	443_ST-443 complex	XIII
21443	Human stool	S	17	51_ST-443 complex	XIV
20157	Human stool	S	18	572_ST-206 complex	XV
21448	Human stool	S	19	572_ST-206 complex	XVI
10419/1	cattle	S	20	21_ST-21complex	XVII
10419/3	cattle	S	20	21_ST-21complex	XVII
10419/4	cattle	S, R	20	21_ST-21complex	XVII
10419/5	cattle	S	20	21_ST-21complex	XVII
24006	Human stool	S	21	21_ST-21 complex	XVII
19566	Human stool	S	22	257_ST-257 complex	XVIII
18989	Human stool	S	23	760_ST-21 complex	XIX
19562	Human stool	S	24	21_ST-21 complex	XIX
19565	Human stool	S	25	3102_ST-21 complex	XX
18988	Human stool	S	26	1044_ST-658 complex	XXI
21439	Human stool	S	26	1044_ST-658 complex	XXI
19564	Human stool	S	27	658_ST-658 complex	XXI
20205/2	chicken	S	28	824_ST-257 complex	XXII
17347	cattle	S	29	122_ST-206 complex	XXIII
18981	Human stool	S	30	2116_ST-353 complex	XXIV
18985	Human stool	S	31	7418_ST	XXV
20204/1	chicken	S	32	2863_ST-354 complex	XXVI
20204/2	chicken	S	32	2863_ST-354 complex	XXVI
20204/3	chicken	R	32	2863_ST-354 complex	XXVI
23994	Human stool	S	33	2863_ST-354 complex	XXVII
22501/3	chicken	R	34	305_ST-574 complex	XXVIII

FIGURE 1 | Comparison of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) among *Campylobacter jejuni* isolates from different sources.

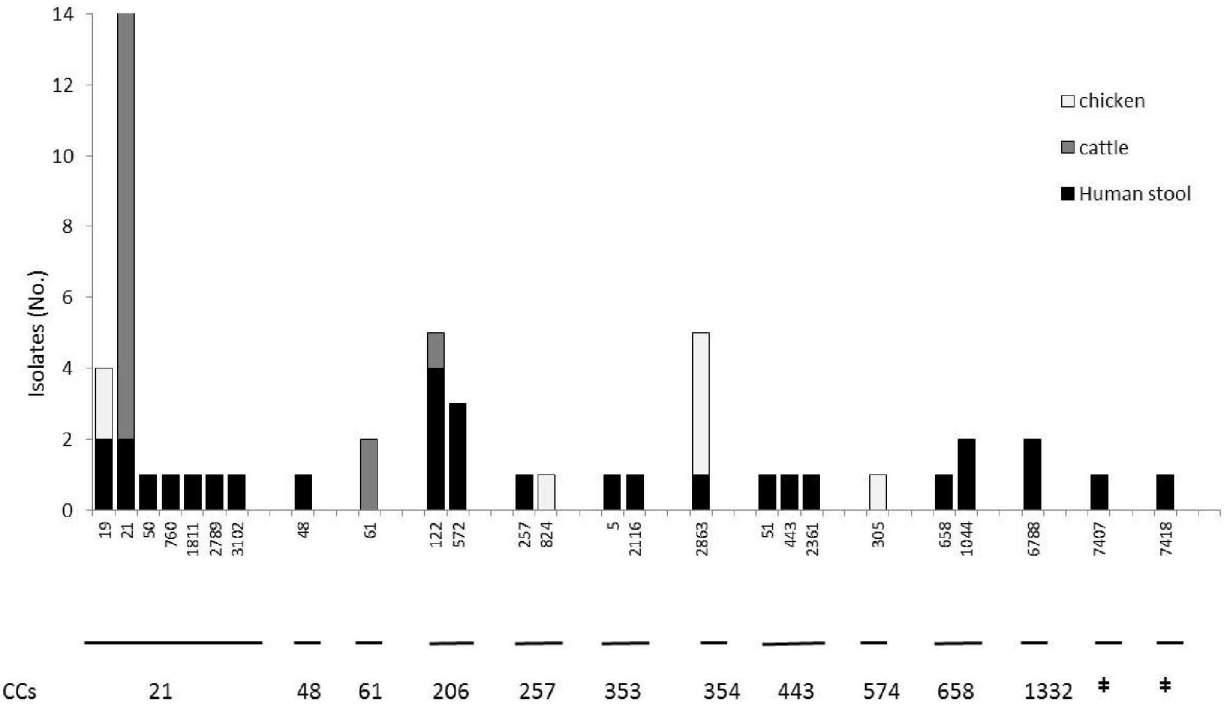


FIGURE 2 | Frequency distribution of the *C. jejuni* clonal complexes (CCs) isolated from chickens, human stool and cattle samples ($n = 56$) in the Piemonte region of Italy. STs belonging to the CCs listed below are reported on the *x* axis, while the *y* axis represents the number of isolates. ‡No clonal complex applicable.

The Simpson's index and the confidence intervals for PFGE (0.949, 95% CI = 0.920–0.979) were higher than for MLST (0.897, 95% CI = 0.834–0.962), $p = 0.044$. PFGE is a more discriminatory typing method than MLST; however, when comparing the 95% confidence intervals, it is noteworthy that they overlap. Therefore, we cannot exclude the hypothesis that both methods have similar discriminatory power (at the 95% confidence level).

The concordance of the methods based on the calculation of the adjusted Rand coefficient and 95% CI shows a concordance between MLST and PFGE of 0.32 (95% CI = 0.209–0.437). The concordance based on the Wallace coefficient was also higher for the PFGE–MLST combination (Wallace = 0.505, 95% CI = 0.290–0.714), with respect to the MLST–PFGE combination (Wallace = 0.236, 95% CI = 0.120–0.343), $p = 0.019$.

The attribution analysis revealed that 69.75% of the human cases were predominantly related to chicken (**Figure 3**). Interestingly, only two human cases belonging to CC1332 were predominantly related to wild bird (62.42%, **Figure 3**). In the asymmetric island model, most human cases (69.7%) were attributed to the chicken reservoir (95% CI 47.6–87.5%). Cattle were the second most probable source reservoir (8.2%, 95% CI 0–25.6%) followed by wild birds (7.3% CI 0–19.9%), the environment (6.2% CI 0–22%), small ruminants (5.3% CI 0–17.8%) and pork (2.9% CI 0–10.7%). Although there was a large credibility interval for the chicken source, the model gave the most probable source at a probability of 99.8%.

For each patient, the model restituted the posterior probability for the source of infection as shown in **Figure 4**. One dominant color confirmed that chicken is the likely source for the cases under consideration. It is worth noting that, for the generalist CC21 and CC48 regularly isolated from multiple reservoirs, the model showed a minor probability of chicken in favor of ruminants (cattle and small ruminants). CC1332 featured two clinical isolates and resulted with wild birds as the most probable source of infection. Overall, the 31 human cases investigated were probably infected in 29 instances by chicken and in the last two cases by wild birds. A minor probable source was confirmed for pork.

DISCUSSION

The percentage of human cases with campylobacteriosis ascribed to eating or handling raw poultry differs between countries and studies. Estimates of cases of a food-borne origin range from 30 to 58% (Mullner et al., 2009), and up to 80% can be attributed to the chicken sources as a whole (EFSA, 2011; Mughini Gras et al., 2012). Furthermore, the sources of the notified cases have not been able to be determined, indicating that the real number of food-borne cases is unknown (Meinersmann et al., 2005; EFSA, 2013). It is well known that the presence of *C. jejuni* in bovine raw milk samples and fresh dairy products is increasing (Fernandes et al., 2015). Recent data show that the consumption of undercooked chicken meat and raw milk are the most important sources of human campylobacteriosis in Europe (EFSA, 2014), accounting for hundreds of cases.

TABLE 1 | Genetic diversity and frequency distribution of 56 *Campylobacter jejuni* strain isolates from human stool, chicken, and cattle sources.

Clonal complex	ST	Multilocus sequence typing (MLST) allelic profile							Pulsed-field gel electrophoresis (PFGE) profile[a]	Source and number of isolates
		aspA	glnA	gltA	glyA	pgm	tkt	uncA	SmaI cluster	
21	21	2	1	1	3	2	1	5	**VIII–XVII**	Human stool (3)
										Cattle (14)
	19	2	1	5	3	2	1	5	**VIII**	Human stool (1)
										Chicken (2)
	1811	2	4	12	3	2	1	5	VI	Human stool (1)
	2789	2	1	12	3	2	3	5	X	Human stool (1)
	3102	2	84	12	3	11	1	5	XX	Human stool (1)
	50	2	1	12	3	2	1	5	VI	Human stool (1)
	760	2	1	52	3	2	100	5	XIX	Human stool (1)
658	658	2	4	2	4	19	3	6	XXI	Human stool (1)
	1044	2	10	2	4	19	3	6	XXI	Human stool (2)
206	122	6	4	5	2	2	1	5	XXIII	Cattle (1)
									I–II	Human stool (4)
	572	62	4	5	2	2	1	5	II–XV–XVI	Human stool (3)
353	2116	7	17	52	10	89	3	6	XXIV	Human stool (1)
	5	7	2	5	2	10	3	6	V	Human stool (1)
443	443	24	17	2	15	23	3	12	XIII	Human stool (1)
	2361	7	254	2	15	23	3	12	XIII	Human stool (1)
	51	7	17	2	15	23	3	12	XIV	Human stool (1)
48	48	2	4	1	2	7	1	5	XI	Human stool (1)
61	61	1	1	4	2	2	6	3	XII–IX	Cattle (2)
257	257	9	2	4	62	4	5	6	XVIII	Human stool (1)
	824	9	2	2	2	11	5	6	XXII	Chicken (1)
‡	7407[§]	24	28	2	28	10	1	125	III	Human stool (1)
‡	7418[§]	24	17	52	10	89	418	6	XXV	Human stool (1)
1332[§]	6788[§]	2	1	4	28	58	29	58	IV	Human stool (2)
354	2863	198	2	2	2	11	61	6	XXVI	Chicken (4)
									XXVII	Human stool (1)
574	305	9	53	2	10	11	3	3	XXVIII	Chicken (1)

[a]*PFGE profiles for the restriction enzyme SmaI. Bold font: the two identified clusters shared among humans and cattle or humans and chicken.*

[§] *New allelic combination.*

[‡]*No clonal complex applicable.*

Outbreaks associated with the consumption of raw or pasteurized milk contaminated with *Campylobacter* were recently recorded in Italy (Giacometti et al., 2012). A quantitative risk assessment focused on one region of Northern Italy evaluated that 1–2% of the population were consumers of raw milk from vending machines and only 57% of users boiled the raw milk before consumption; therefore, the estimated proportion of the people consuming unboiled raw milk is 0.5–0.9% (4548–9096 people; Giacometti et al., 2012).

The present study aimed to investigate an outbreak of campylobacteriosis among humans and to define the infection sources. An active surveillance was undertaken that sampled raw milk from vending machines and chicken meat from a retail market. The PFGE and MLST results suggested that the human cases we studied were not caused by a single-point source, but were likely triggered by multiple foci of infection instead. The evidence supporting this conclusion was the presence of 26 different pulsotypes discovered by PFGE and 22 STs for MLST. The MLST results found nine clinical cases with similar profiles for the raw milk and chicken samples. In particular, ST21 and ST122 were shared among humans and cattle, while ST19 and ST 354 were shared between humans and chickens.

Calculation of the two coefficients (adjusted Rand and Wallace coefficients) and their confidence intervals permits quantification of the congruence between the two different method results (Wallace, 1983; Pinto et al., 2008). In our study, the adjusted Rand coefficient gave low congruence between the different methods. The PFGE analysis showed that isolates with the same MLST profile were partially congruent and sometimes grouped inside different clusters, as was also revealed by the adjusted Rand coefficient between MLST and PFGE of 0.32 (95% CI = 0.209–0.437). However, the Wallace coefficient for PFGE and MLST gave a value of 0.5, which is higher than the 0.2 value from the MLST–PFGE combination, suggesting that PFGE performs better in an outbreak scenario.

Nevertheless, only three cases featured in VIII and XVII groups clustered together with chicken and raw milk samples, a finding in agreement with the MLST results. In contrast, ST

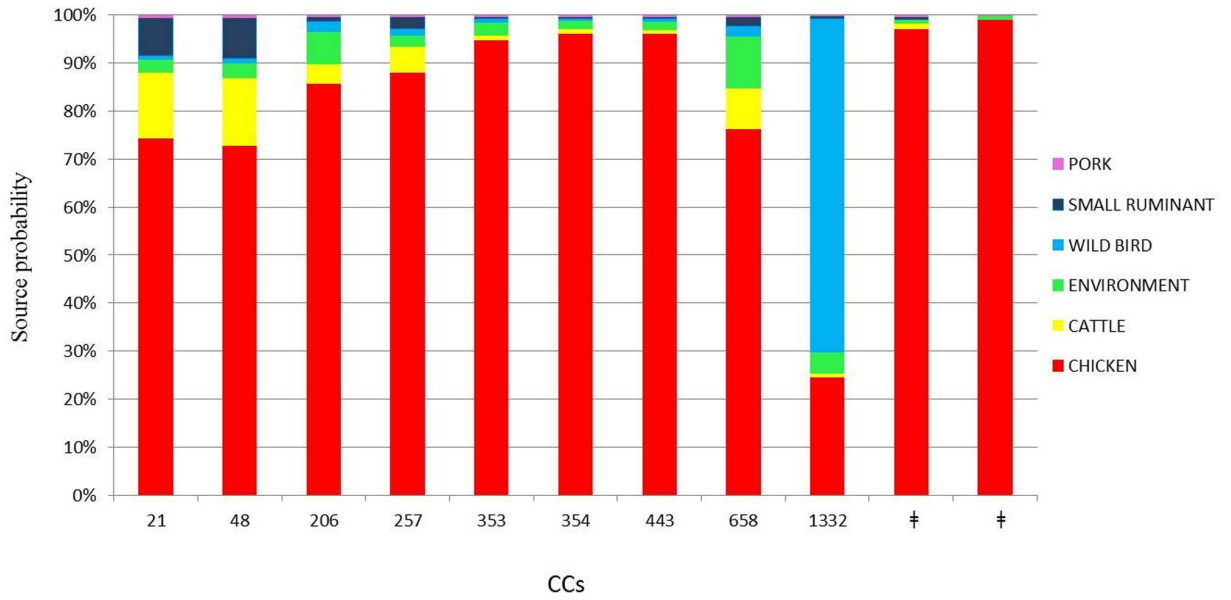


FIGURE 3 | Attributed probability (%) for the six most represented clonal complexes originating from chicken, cattle, environment, wild bird, small ruminant, and pork sources. ‡No clonal complex applicable.

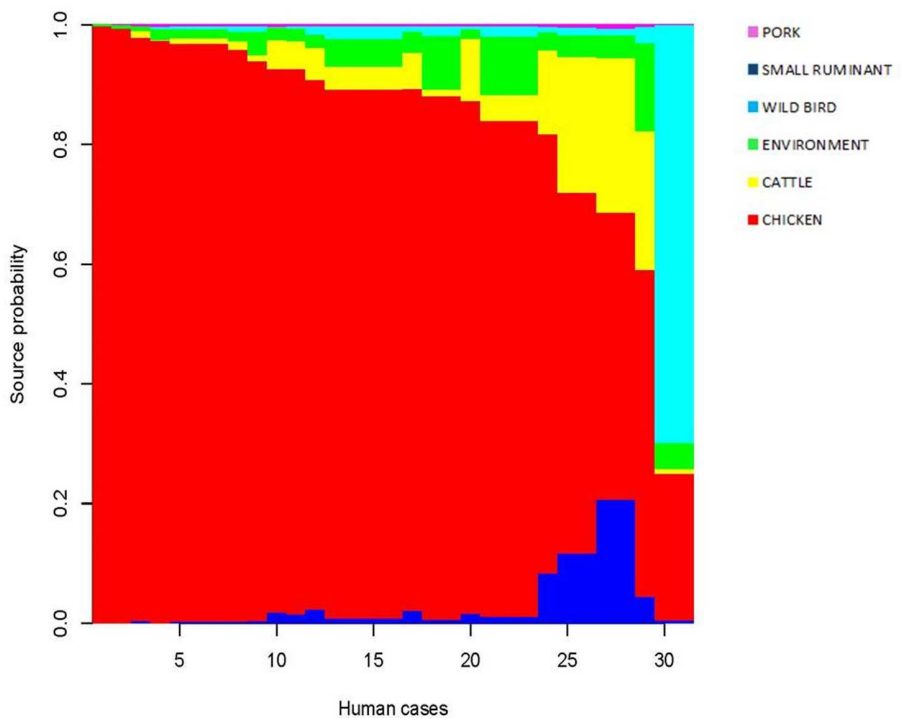


FIGURE 4 | Posterior probability of the source for each isolate.

122 and ST 354 did not cluster together in the PFGE profiles, suggesting a possible diverse origin for them. In this study, the clustering concordance of the two molecular techniques is not similar, but we propose that complementation of the results could lead to a higher level of isolate discrimination.

The data suggest that genomic-based techniques like MLST and PFGE can provide useful insight for outbreak investigations. PFGE has been standardized for *Campylobacter* spp., and has been used in localized outbreak investigations (Fitzgerald et al., 2001), while MLST is useful for investigating the DNA sequence

diversity via the index of variations in housekeeping genes (Dingle et al., 2001).

Recently, MLST has commonly been employed to increase the analysis resolution of outbreak-associated isolates, leading to quicker and much more accurate source identification, as well as investigating different epidemiological hypotheses.

Our findings revealed three human cases that had similar genetic profiles to chicken and cattle isolates. However, in this study, there were no clues for tracing the remaining isolates investigated and we did not retrieve similar profiles from the source strains isolated during the active surveillance. Therefore, to try to understand the source of infection, we applied a genetic population approach using MLST data from our database and the data from other studies conducted in Europe.

From our analysis, chicken was estimated to be the most dominant source of infection for the human isolates we studied. Using asymmetric island model, the MLST profiles associated with human disease were most similar to those from a chicken source in 69.75% of the cases of *C. jejuni* infection. Ruminants (cattle and small ruminants) contributed far fewer cases of *C. jejuni* infection (13.6%) and the contribution from environment, wild bird, and pig sources was very low (6.28, 7.37, and 2.98%, respectively). This finding is in line with many other studies performed in industrialized countries, even if divergence in the percentage of cases attributable to the chicken source varied among these studies (Wilson et al., 2008; Mullner et al., 2009; Sheppard et al., 2009). Our results should indicate the importance of chicken as the main reservoir of human campylobacteriosis and lend to the suggestion that this disease could be greatly reduced by focusing interventions on chickens. The cases studied are not representative of the Italian epidemiological context but seem to be congruent with other European scenarios.

Notably, inspecting the three cases that were traced back to a potential source revealed that the model estimated chicken as the most probable source of infection for all the cases as well. Contrastingly, our investigation found that one case featured in PFGE cluster VIII and ST 21 was attributable to raw milk instead of chicken. In fact, CC21 is a multi-host lineage shared among different sources, and this can lead to an incorrect assignment in the genetic model. The model calculates the likely source of the infection but cannot rule out completely the minor reservoir as the actual source. In this case, the back-tracing analysis using PFGE and MLST is likely to be the best approach for drawing the correct reconstruction of the food-borne illness. In this scenario, whole-genome sequencing (WGS) would probably provide the best fit for the epidemiological analysis, but this approach is not yet ready to be applied routinely because of the lack of standardization of the analysis of the WGS data in the context of the molecular epidemiology of food-borne pathogens.

CONCLUSION

The number of clinical isolates, albeit limited to only 56 samples from a restricted area and in a short time window, confirms the high genetic diversity present in *Campylobacter*. Genetic assessment of *Campylobacter* spp. is fundamental to our understanding of its epidemiology. Additional analyses of isolates from various sources will consent major progresses in the knowledge of the epidemiology and population structure of *C. jejuni* in Italy. This study confirms the interest of molecular epidemiology as a powerful support for epidemiological investigations.

AUTHOR CONTRIBUTIONS

EG has contributed to the conception and design of the work and wrote the paper. AA performed experiments. FM performed experiments, analyzed data and wrote the paper. GG performed the source attribution analysis, analyzed data and wrote the paper. LC performed the source attribution analysis. GD helped draft the manuscript. WV and LD contributed strains.

REFERENCES

Amato, S., Maragno, M., Mosele, P., Sforzi, M., Mioni, R., Barco, L., et al. (2007). "An outbreak of *Campylobacter jejuni* linked to the consumption of raw milk in Italy," in *Proceedings of the Zoonoses and Public Health Conference* (Oxford: Blackwell Publishing), 23–23.

Arrigoni, N., Scavia, G., and Tamba, M. (2009). "Raw milk: experiences and hygiene-sanitary problems in Emilia Romagna Region," in *Proceedings of the Conference on Large Animal Review: SIVAR-Societa Italiana Veterinari per Animali da Reddito*, Cremona, 215–219.

Behringer, M., Miller, W. G., and Oyarzabal, O. A. (2011). Typing of *Campylobacter jejuni* and *Campylobacter coli* isolated from live broilers and retail broiler meat by flaA-RFLP, MLST, PFGE and REP-PCR. *J. Microbiol. Methods* 84, 194–201. doi: 10.1016/j.mimet.2010.11.016

Bianchini, V., Luini, M., Borella, L., Parisi, A., Jonas, R., Kittl, S., et al. (2014). Genotypes and antibiotic resistances of *Campylobacter jejuni* isolates from cattle and pigeons in dairy farms. *Int. J. Environ. Res. Public Health* 11, 7154–7162. doi: 10.3390/ijerph110707154

Calistri, P., and Giovannini, A. (2008). Quantitative risk assessment of human *campylobacter*iosis related to the consumption of chicken meat in two Italian regions. *Int. J. Food Microbiol.* 128, 274–287. doi: 10.1016/j.ijfoodmicro.2008.08.021

CDC (2009). *Standard Operating Procedure for PulseNet PFGE of Campylobacter jejuni*. Available at: http://www.cdc.gov/pulsenet/pdf/campylobacter-pfge-protocol-508c.pdf

Di Giannatale, E., Di Serafino, G., Zilli, K., Alessiani, A., Sacchini, L., Garofolo, G., et al. (2014). Characterization of antimicrobial resistance patterns and detection of virulence genes in *Campylobacter* isolates in Italy. *Sensors* 14, 3308–3322. doi: 10.3390/s140203308

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

EFSA (2008). 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.

EFSA and ECDC (2015). The european union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J.* 13, 3991.

EFSA (2011). The european union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. *EFSA J.* 9, 2090.

EFSA (2013). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA J.* 11, 3129.

EFSA (2014). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. *EFSA J.* 12, 3547.

Fernandes, A. M., Balasegaram, S., Willis, C., Wimalarathna, H. M., Maiden, M. C., and McCarthy, N. D. (2015). Partial failure of milk pasteurization as a risk for the transmission of *campylobacter* from cattle to humans. *Clin. Infect. Dis.* 61, 903–909. doi: 10.1093/cid/civ431

Fitzgerald, C., Helsel, L. O., Nicholson, M. A., Olsen, S. J., Swerdlow, D. L., Flahart, R., et al. (2001). Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. *J. Clin. Microbiol.* 39, 2386–2390. doi: 10.1128/JCM.39.7.2386-2390.2001

Giacometti, F., Bonilauri, P., Amatiste, S., Arrigoni, N., Bianchi, M., Losio, M. N., et al. (2015). Human campylobacteriosis related to the consumption of raw milk sold by vending machines in Italy: quantitative risk assessment based on official controls over four years. *Prev. Vet. Med.* 121, 151–158. doi: 10.1016/j.prevetmed.2015.06.009

Giacometti, F., Serraino, A., Bonilauri, P., Ostanello, F., Daminelli, P., Finazzi, G., et al. (2012). Quantitative risk assessment of verocytotoxin-producing *Escherichia coli* O157 and *Campylobacter jejuni* related to consumption of raw milk in a province in Northern Italy. *J. Food Prot.* 75, 2031–2038. doi: 10.4315/0362-028X.JFP-12-163

Haddad, N., Marce, C., Magras, C., and Cappelier, J.-M. (2010). An overview of methods used to clarify pathogenesis mechanisms of *Campylobacter jejuni*. *J. Food Prot.* 73, 786–802.

Heuvelink, A. E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J. J., Bos, M. H., Heilmann, F. G., et al. (2009). Two outbreaks of *campylobacter*iosis associated with the consumption of raw cows' milk. *Int. J. Food Microbiol.* 134, 70–74. doi: 10.1016/j.ijfoodmicro.2008.12.026

Marotta, F., Garofolo, G., Di Donato, G., Aprea, G., Platone, I., Cianciavicchia, S., et al. (2015). Population diversity of *campylobacter* jejuni in poultry and its dynamic of contamination in chicken meat. *Biomed Res. Int*. 2015, 859845. doi: 10.1155/2015/859845

Marshall, S. M., Melito, P. L., Woodward, D. L., Johnson, W. M., Rodgers, F. G., and Mulvey, M. R. (1999). Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* 37, 4158–4160.

Meinersmann, R. J., Phillips, R. W., Hiett, K. L., and Fedorka-Cray, P. (2005). Differentiation of *Campylobacter* populations as demonstrated by flagellin short variable region sequences. *Appl. Environ. Microbiol.* 71, 6368–6374. doi: 10.1128/AEM.71.10.6368-6374.2005

Meldrum, R., Griffiths, J., Smith, R., and Evans, M. R. (2005). The seasonality of human *campylobacter* infection and *Campylobacter* isolates from fresh, retail chicken in Wales. *Epidemiol. Infect.* 133, 49–52. doi: 10.1017/S0950268804003188

Mughini Gras, L., Smid, J. H., Wagenaar, J. A., de Boer, A. G., Havelaar, A. H., Friesema, I., et al. (2012). Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. *PLoS One* 7:e42599. doi: 10.1371/journal.pone.0042599

Mullner, P., Spencer, S. E., Wilson, D. J., Jones, G., Noble, A. D., Midwinter, A. C., et al. (2009). Assigning the source of human campylobacteriosis in New Zealand: a comparative genetic and epidemiological approach. *Infect. Genet. Evol.* 9, 1311–1319. doi: 10.1016/j.meegid.2009.09.003

Petruzzelli, A. A. G., Foglini, M., Orazietti, N., Mancini, P., Omiccioli, E., Brandi, G., et al. (2011). "Identificazione di patogeni in un allevamento di bovine da latte mediante metodo in multiplex real-time pcr," in *Proceedings of the Convegno: XIII Congresso Nazionale S.I.Di.L.V.*, Trani, 322–323.

Piccirillo, A., Giacomelli, M., Salata, C., Bettanello, S., De Canale, E., and Palù, G. (2014). Multilocus sequence typing of *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in North-Eastern Italy. *New Microbiol.* 37, 557–562.

Pinto, F. R., Melo-Cristino, J., and Ramirez, M. r. (2008). A confidence interval for the Wallace coefficient of concordance and its application to microbial typing methods. *PLoS ONE* 3:e3696. doi: 10.1371/journal.pone.0003696

Rodrigues, R. C., Bronnec, V., Tresse, O., Cappelier, J.-M., and Haddad, N. (2015). "A review of *Campylobacter jejuni* pathogenesis: main virulence factors and their use as biomarkers," in *Campylobacter Infections: Epidemiology, Clinical Management and Prevention*, ed. B. A. Bertucci (New York, NY: Nova Science Publishers Inc.).

Schildt, M., Savolainen, S., and Hänninen, M. L. (2006). Long-lasting *Campylobacter jejuni* contamination of milk associated with gastrointestinal illness in a farming family. *Epidemiol. Infect.* 134, 401–405. doi: 10.1017/S0950268805005029

Sheppard, S. K., Dallas, J. F., Strachan, N. J., MacRae, M., McCarthy, N. D., Wilson, D. J., et al. (2009). *Campylobacter* genotyping to determine the source of human infection. *Clin. Infect. Dis.* 48, 1072–1078. doi: 10.1086/597402

Taylor, E. V., Herman, K. M., Ailes, E., Fitzgerald, C., Yoder, J., Mahon, B., et al. (2013). Common source outbreaks of *Campylobacter* infection in the USA, 1997-2008. *Epidemiol. Infect.* 141, 987–996. doi: 10.1017/S0950268812001744

Wallace, D. L. (1983). Comment. *J. Am. Stat. Assoc.* 78, 569–576. doi: 10.1080/01621459.1983.10478009

Wilson, D. J., Gabriel, E., Leatherbarrow, A. J., Cheesbrough, J., Gee, S. S, Bolton, E., et al. (2008). Tracing the source of campylobacteriosis. *PLoS Genet.* 4:e1000203. doi: 10.1371/journal.pgen.1000203

Zhou, J., Bowler, L. D., and Spratt, B. G. (1997). Interspecies recombination, and phylogenetic distortions, within the glutamine synthetase and shikimate dehydrogenase genes of *Neisseria meningitidis* and commensal *Neisseria* species. *Mol. Microbiol.* 23, 799–812. doi: 10.1046/j.1365-2958.1997.2681633.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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 June 2016
doi: 10.3389/fmicb.2016.00816

Prevalence of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* in Retail Ready-to-Eat Foods in China

*Xiaojuan Yang †, Jumei Zhang †, Shubo Yu †, Qingping Wu *, Weipeng Guo, Jiahui Huang and Shuzhen Cai*

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

Staphylococcus aureus, particularly methicillin-resistant *S.*aureus (MRSA), is a life-threatening pathogen in humans, and its presence in food is a public health concern. MRSA has been identified in foods in China, but little information is available regarding MRSA in ready-to-eat (RTE) foods. We aimed to investigate the prevalence of *S. aureus* and MRSA in Chinese retail RTE foods. All isolated *S. aureus* were tested for antimicrobial susceptibility, and MRSA isolates were further characterized by multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. Of the 550 RTE foods collected from 2011 to 2014, 69 (12.5%) were positive for *S. aureus*. Contamination levels were mostly in the range of 0.3–10 most probable number (MPN)/g, with five samples exceeding 10 MPN/g. Of the 69 *S. aureus* isolates, seven were identified as MRSA by cefoxitin disc diffusion test. Six isolates were *mecA*-positive, while no *mecC*-positive isolates were identified. In total, 75.8% (47/62) of the methicillin-susceptible *S. aureus* isolates and all of the MRSA isolates were resistant to three or more antibiotics. Amongst the MRSA isolates, four were identified as community-acquired strains (ST59-MRSA-IVa ($n = 2$), ST338-MRSA-V, ST1-MRSA-V), while one was a livestock-associated strain (ST9, harboring an unreported SCC*mec* type 2C2). One novel sequence type was identified (ST3239), the SCC*mec* gene of which could not be typed. Overall, our findings showed that Chinese retail RTE foods are likely vehicles for transmission of multidrug-resistant *S. aureus* and MRSA lineages. This is a serious public health risk and highlights the need to implement good hygiene practices.

Keywords: ***Staphylococcus aureus*, methicillin-resistant, ready-to-eat foods, prevalence, antibiotic resistance**

Edited by:
Pierina Visciano, University of Teramo, Italy

Reviewed by:
Jyoti Prakash Tamang, Sikkim University, India
Adrian Canizalez-Roman, Autonomous University of Sinaloa, Mexico

****Correspondence:***
Qingping Wu
wuqp203@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: *30 March 2016*
Accepted: *13 May 2016*
Published: *07 June 2016*

Citation:
Yang X, Zhang J, Yu S, Wu Q, Guo W, Huang J and Cai S (2016) Prevalence of Staphylococcus aureus and Methicillin-Resistant Staphylococcus aureus in Retail Ready-to-Eat Foods in China. Front. Microbiol. 7:816. doi: 10.3389/fmicb.2016.00816

INTRODUCTION

Staphylococcus aureus is an important cause of food poisoning worldwide. It is estimated that ∼20–25% of foodborne bacterial outbreaks are caused by *S. aureus* in China (Wang et al., 2014). In addition, it is the leading cause of infection in both healthcare facilities and communities, causing illnesses ranging from mild skin and soft tissue infections to life threatening diseases such as septicemia, necrotizing fasciitis, endocarditis, and necrotizing pneumonia

(Lowy, 1998; Chen and Huang, 2014; Rodríguez-Lázaro et al., 2015). Furthermore, the increasing antimicrobial resistance rates of this bacterium pose a serious threat to public health. Methicillin-resistant *S. aureus* (MRSA) strains exhibit resistance to all β-lactam antibiotics through acquisition of the mobile staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the antibiotic-resistant gene *mecA*. Together, healthcare-acquired MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) strains constitute a major health concern.

Epidemiological studies have revealed differences between HA-MRSA, CA-MRSA, and LA-MRSA strains, including antimicrobial resistance profiles, SCC*mec* types, and clonal complexes (CCs) identified by multilocus sequence typing (MLST). HA-MRSA isolates typically carry relatively large SCC*mec* elements (types II or III), and are resistant to many classes of antimicrobials, including β-lactams (Yamamoto et al., 2013). CA-MRSA isolates usually harbor smaller SCC*mec* elements (types IV or V) and are only resistant to β-lactam antibiotics (Rodríguez-Lázaro et al., 2015). Interestingly, specific MRSA clones have spread across different geographical regions worldwide. The New York/Japan (ST5/SCC*mec*II), Brazilian/Hungarian (ST239/SCC*mec*III), EMRSA-15 (ST22/SCC*mec*IV), and EMRSA-16 (ST36/SCC*mec*II) clones are pandemic HA-MRSA lineages (Yamamoto et al., 2013), while the Taiwan (ST59/SCC*mec*IV or V), USA300 (ST8/SCC*mec*IV), European (ST80/SCC*mec*IV), and USA400 (ST1) clones are always associated with community-acquired infections (Yamamoto et al., 2013). In China, ST239/SCC*mec*III and ST5/SCC*mec*II are predominant HA-MRSA clones, while ST59/SCC*mec*IV or V is the most prevalent CA-MRSA clone (Chuang and Huang, 2013; Chen and Huang, 2014).

The first reports of MRSA infections in animals appeared in the 1970s and, on the basis of their putative source, these MRSA strains are referred to as LA-MRSA (Petinaki and Spiliopoulou, 2012). ST398 was the first detected and most widespread LA-MRSA sequence type (ST). The isolates belonging to this clonal lineage are not typeable by pulsed field gel electrophoresis (PFGE) using *Sma*I, and often exhibit co-resistance to many non-β-lactam antimicrobials, including those commonly used in animal production.

The emergence of LA-MRSA has led to concerns about its transmission via the food chain. In recent years, LA-MRSA has frequently been detected in food-producing animals (Wagenaar et al., 2009; Petinaki and Spiliopoulou, 2012; Visciano et al., 2014), meat (Rodríguez-Lázaro et al., 2015), milk and dairy products (Song et al., 2015), fish (Hammad et al., 2012), and ready-to-eat (RTE) food products (Hammad et al., 2012; Wang et al., 2014), causing a significant public health concern.

RTE foods that are consumed without further treatment, such as cooked meat and poultry, cold vegetable dishes, cold noodles, and fried rice, are popular in China. It has been reported that these food products are associated with the introduction of microbiological hazards, including *Listeria monocytogenes* (Chen M. T. et al., 2014), *Cronobacter* (Xu et al., 2015), and *Salmonella* (Yang et al., 2016). However, fewer data are available regarding the prevalence of *S. aureus* and MRSA in these foods. The aim of this study was to determine the prevalence, antibiotic resistance, and molecular characteristics of *S. aureus* and MRSA isolated from RTE food samples collected in China.

MATERIALS AND METHODS

Sample Collection

From December 2011 to May 2014, a total of 550 samples were collected from RTE foods from retail markets. Samples included cooked pork (119), cooked chicken (153), cooked duck (127), cold vegetable dishes in sauce (53), cold noodles (52), and fried rice/sushi (46). The retail markets were located in 24 cities across most of the provincial capitals of China (**Figure 1**). Each sample was weighed, labeled, placed in a separate sterile bag, and then immediately transported to the laboratory in an ice box.

Detection and Enumeration of *S. aureus*

The prevalence and bacterial load of *S. aureus* in the samples was determined using the most probable number (MPN) method according to National Food Safety Standards of China document GB 4789.10-2010. A 25-g sample was randomly collected from each RTE food product and placed into a sterile glass flask containing 225 mL of saline solution (Huankai, Guangzhou, China). Following homogenization, trypticase soy broth (Huankai) supplemented with 10% NaCl was inoculated in triplicate with 1-ml aliquots of decimal dilutions of each sample. Broths were incubated at 37°C for 48 h. Loopfuls of the resulting cultures were streaked onto chromogenic *S. aureus* agar plates (Huankai), then incubated at 37°C for 24 h. Putative *S. aureus* isolates were tested for coagulase activity, and were further confirmed using API STAPH test strips (bioMerieux, Marcy-l'Etoile, France). The MPN value was determined on the basis of the number of positive tube(s) in each of the three sets using the MPN table.

Antimicrobial Susceptibility Testing

All isolates were evaluated for antimicrobial resistance using the Kirby–Bauer disk diffusion method according to the (Clinical Laboratory Standards Institute previously National Committee on Clinical Laboratory Standards., 2014). Susceptibility to the following 16 antimicrobial agents was tested: ampicillin (AMP), cephalothin (KF), cefoxitin (FOX), penicillin G (P), chloramphenicol (C), tetracycline (TE), ciprofloxacin (CIP), amikacin (AK), gentamicin (CN), kanamycin (K), trimethoprim-sulfamethoxazole (SXT), erythromycin (E), clindamycin (DA), rifampicin (RD), linezolid (LZD), and quinupristin/dalfopristin (QD) (Oxoid, Basingstoke, UK). The isolates were also examined using a microdilution test according to the CLSI method for vancomycin minimum inhibitory concentrations (MICs) (Clinical Laboratory Standards Institute previously National Committee on Clinical Laboratory Standards., 2014).

Detection of mecA and mecC

mecA, which has been shown to confer methicillin resistance on *S. aureus*, and *mecC*, a divergent *mecA* homolog (also called *mecALGA251*), were detected by PCR using primers

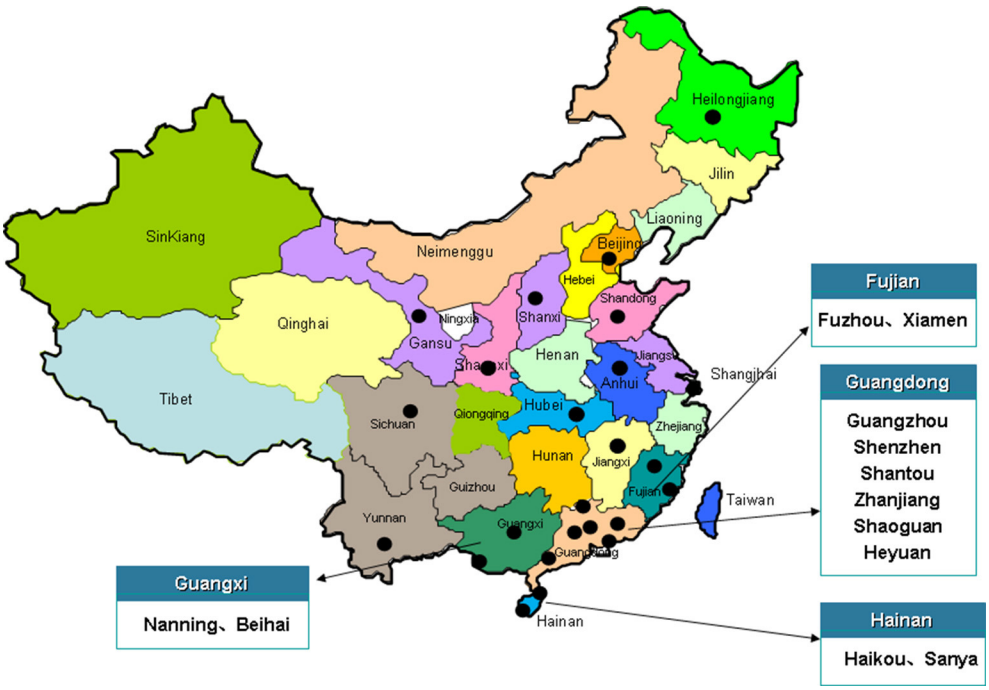


FIGURE 1 | Map of China showing the locations of the 24 cities where RTE samples were collected.

described previously (Pérez-Roth et al., 2001; Stegger et al., 2012).

MLST and SCCmec-Typing of the mecA-Positive MRSA Isolates

The *mecA*-positive MRSA isolates were characterized by MLST analysis. MLST was carried out using previously reported primers specific for seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*), and the sequence type (ST) was assigned according to the MLST database (http://www.mlst.net/).

The SCC*mec* type of *mecA*-positive strains was determined using a multiplex PCR method as previously described (Kondo et al., 2007). Strains with unanticipated fragments or completely lacking fragments were defined as non-typeable (NT).

RESULTS

Prevalence and Load of *S. aureus* in Retail RTE Food

Of the 550 retail RTE food samples, 69 (12.5%) were positive for *S. aureus* according to the MPN method. This included 15 (12.6%) of the 119 cooked pork samples, 15 (9.8%) of the 153 cooked chicken samples, 17 (13.4%) of the 127 cooked duck samples, six (11.3%) of the 53 samples from cold vegetable dishes in sauce, 10 (19.2%) of the 52 cold noodle samples, and six (13.0%) of the 46 fried rice/sushi samples. Overall, 50.7% (35/69) of positive samples had a bacterial load of <1 MPN/g, and 42.0% (29/69) reached 1 MPN/g. Five samples exceeded 10 MPN/g (**Table 1**).

Antimicrobial Susceptibility of the *S. aureus* Isolates

Of the 69 *S. aureus* isolates recovered, seven were confirmed as MRSA by cefoxitin disc diffusion test. Six of the isolates were *mecA*-positive, and none tested positive for *mecC* by PCR. All of the MRSA isolates were resistant to ampicillin and penicillin G, and 66.7% were resistant to clindamycin, erythromycin, tetracycline, and kanamycin. All MRSA isolates were susceptible to cephalothin, amikacin, linezolid, quinupristin/dalfopristin, and vancomycin (MICs <1 μg/mL). The antimicrobial resistance profiles of the MRSA isolates are shown in **Table 2**.

Among the 62 methicillin-susceptible *S. aureus* (MSSA) isolates, all were resistant to at least one antimicrobial agent, and 47 isolates (75.8%) were resistant to more than three antimicrobials. The highest levels of resistance were observed for ampicillin (98.4%), penicillin G (98.4%), and tetracycline (43.5%). The antimicrobial resistance profiles of the MSSA strains are shown in **Table 3**.

MLST and SCCmec-Typing of mecA-Positive MRSA Isolates

In the present study, seven phenotypically MRSA isolates were identified; however, only six isolates were *mecA*-positive by PCR. Among the six *mecA*-positive MRSA isolates, two were recovered from different cooked duck samples in the same city (Nanning). These were both identified as ST59 and harbored SCC*mec* type IVa (2B). Amongst the remaining *mecA*-positive isolates, one belonged to ST338 and harbored SCC*mec* type

TABLE 1 | Prevalence and load of *Staphylococcus aureus* in retail RTE foods in China.

Type of products	Samples tested no.	No. (%) Samples positive for *Staphylococcus aureus*	No. of samples			
			Staphylococcus aureus (MPN/g)			
			0.3–1	1–10	10–110	>110
Cooked pork	119	15 (12.6)	10	5		
Cooked chicken	153	15 (9.8)	6	8	1	
Cooked duck	127	17 (13.4)	7	7	3	
Cold vegetable dishes in sauce	53	6 (11.3)	4	2		
Cold noodles	52	10 (19.2)	5	4	1	
Fired rice/sushi	46	6 (13.0)	3	3		
Total	550	69 (12.5)	35	29	5	

TABLE 2 | Prevalence and characteristics of methicillin-resistant *Staphylococcus aureus* isolates.

City	Source	Sample	Isolate	Cefoxitin	*MecA*	Sequence type	*SCCmec* type	Antimicrobial resistance profile
Guagnzhou	Cold noodles	112	S8	+	+	ST338	V (5C2)	AMP-FOX-P-C-TE-K-E-DA
Sanya	Cold noodles	560	S27	+	+	ST3239*	NT (2/)	AMP-FOX-P-CN-K-SXT-E-DA-RD
Nanning	Cooked duck	678	S33	+	+	ST59	IVa (2B)	AMP-FOX-P-TE-K-E-DA
Fuzhou	Cooked chicken	2236	S51	+	+	ST9	❖ (2C2)	AMP-FOX-P-C-TE-CIP-CN-K-SXT-E-DA
Nanning	Cooked duck	2284	S53	+	+	ST59	IVa (2B)	AMP-FOX-P-TE
Nanchang	Fired rice/sushi	2512	S66	+	+	ST1	V (5C2)	AMP-FOX-P
Haikou	Cold vegetables dishes in sauce	509	S23	+	−			AMP-FOX-P-E

**Novel sequence type. NT, not typeable. /, no amplification product. ❖, undescribed SCCmec type.*
AMP, ampicillin; E, erythromycin; DA, clindamycin; FOX, cefoxitin; RD, rifampicin; P, penicillin G; C, chloramphenicol; TE, tetracycline; CIP, ciprofloxacin; CN, gentamicin; K, kanamycin; SXT, trimethoprim-sulfamethoxazole.

V (5C2), one belonged to ST1 and harbored SCC*mec* type V (5C2), and one belonged to ST9 and harbored an unreported SCC*mec* type (2C2). One novel ST (characterized as ST3239) was identified for a MRSA isolate recovered from sausage. SCC*mec* typing of this isolate was not possible as multiplex PCR-1, which provides the *ccr* gene complex type, produced a 937-bp DNA fragment, consistent with type 2, but multiplex PCR-2, which types the *mecA* gene complex class, did not amplify.

DISCUSSION

In recent years, the consumption of RTE foods in China has increased markedly. However, reports on *S. aureus* and MRSA contamination in these foods are scarce. The prevalence of *S. aureus* in food samples in the current study was 12.5% (69/550), which is lower than that observed (25.1%) in RTE foods (cooked meat, vegetable salads, boiled peanuts, cold noodles, and dried tofu) from Shaanxi Province, China (Wang et al., 2014). However, the prevalence of MRSA in our study (1.3%) was higher than that (0.6%) of the study by Wang et al. (2014). As our results were obtained from a large number of samples from most regions in China, the data are more comprehensive and systematic, and more representative of China as a whole.

The prevalence of *S. aureus* and MRSA in RTE foods in the current study differs from that reported in raw food products in China (Wang et al., 2014; Song et al., 2015), which generally have relatively higher levels of *S. aureus* (19.3–24.2%) and MRSA (1.7–5.6%). In addition, antimicrobial susceptibility testing revealed that not only all of the MRSA isolates, but a significant number of the MSSA isolates (47/62; 75.8%), were resistant to three or more antibiotics. The high prevalence of penicillin, ampicillin, tetracycline, and erythromycin resistance observed in our study is similar to results from previous studies in raw food products in China (Wang et al., 2014) and other countries (Rodríguez-Lázaro et al., 2015). However, the findings of our study are even more serious in terms of public health because RTE foods are consumed without further cooking, which would eliminate or reduce the microbial load. Consequently, the incidence of *S. aureus* and MRSA in RTE foods, along with the spread of antibiotic resistant strains, represents a potential health hazard to humans.

All MRSA isolates in the current study displayed resistance to three β-lactams, as well as showing high levels of resistance to clindamycin, erythromycin, tetracycline, and kanamycin. Fortunately, all of these isolates were susceptible to linezolid, quinupristin/dalfopristin, and vancomycin (MICs <1 μg/mL), which are the few remaining effective agents for treatment of MRSA infections.

TABLE 3 | Antimicrobial resistance susceptibility profiles of methicillin-susceptible *Staphylococcus aureus* isolates.

Antimicrobial agents	No. of isolates(%)		
	Resistant	Intermediate	Susceptible
Ampicillin (AMP, 10 μg)	61 (98.4)	0 (0.0)	1 (1.6)
Cephalothin (KF, 30 μg)	0 (0.0)	0 (0.0)	62 (100)
Cefoxitin (FOX, 30 μg)	0 (0.0)	0 (0.0)	62 (100)
Penicillin G (P, 10 μg)	61 (98.4)	0 (0.0)	1 (1.6)
Choramphenicol (C, 30 μg)	4 (6.5)	1 (1.6)	57 (91.9)
Tetracycline (TE, 30 μg)	27 (43.5)	0 (0.0)	38 (56.5)
Ciprofloxacin (CIP, 5 μg)	1 (1.6)	1 (1.6)	60 (96.8)
Amikacin (AK, 30 μg)	1 (1.6)	0 (0.0)	61 (98.4)
Gentamicin (CN, 10 μg)	6 (9.7)	0 (0.0)	56 (90.3)
Kanamycin (K, 30 μg)	10 (16.1)	8 (12.9)	44 (71.0)
Trimethoprim-Sulfamethoxazole (SXT, 25 μg)	11 (17.8)	2 (3.2)	49 (79.0)
Erythromycin (E, 15 μg)	17 (27.4)	5 (8.1)	40 (64.5)
Clindamycin (DA, 2 μg)	7 (11.3)	2 (3.2)	53 (85.5)
Rifampicin (RD, 5 μg)	4 (6.5)	0 (0.0)	58 (93.5)
Linezolid (LZD, 30 μg)	1 (1.6)	0 (0.0)	61 (98.4)
Quinupristin/dalfopristin (QD, 15 μg)	0 (0.0)	0 (0.0)	62 (100)
Vancomycin (VA, MIC)	0 (0.0)	0 (0.0)	62 (100)
Pansusceptible	0 (0.0)		
≥1 Antimicrobial	62 (100)		
≥3 Antimicrobial	47 (75.8)		
≥6 Antimicrobial	9 (12.9)		

Our study provides evidence for the existence of two different lineages of MRSA in RTE foods in China: CA-MRSA and LA-MRSA. ST59 (2/6, 33.3%) was the predominant ST in our study, and its single locus variant ST338 was also detected. ST59 and ST338 belong to CC59, which is the dominant CA-MRSA CC in Asia and is a significant cause of human infection attributable to MRSA (Li et al., 2013; Chen and Huang, 2014). CC59 MRSA isolates also prevail in China, and are also the dominant clone in healthy carriers (Du et al., 2011) and in patients with community-acquired infections (Li et al., 2013). Amongst CC59 strains, two dominant SCC*mec* types (IV and V) and different antimicrobial resistance profiles have been described. A previous study showed that ST59 and ST338 were the first and second most common STs in Chinese pediatric community-acquired pneumonia, especially the dominant ST59-MRSA-IVa and ST338-MRSA-V clones (Geng et al., 2010). ST59-MRSA-IV and ST338-MRSA-V have also been associated with cases of bacteremia in China (He et al., 2013).

ST1 isolates are generally considered to be CA-MRSA (Porrero et al., 2013), although they have also been found in animals (Porrero et al., 2013). ST1 MRSA has most commonly been reported in the United States and Canada (Chuang and Huang, 2013), and has only been found sporadically in China. However, multiple ST1-MRSA-IV clones were reported to cause community-acquired pneumonia and skin/soft-tissue infections in Chinese children (Geng et al., 2010). ST1 has also been associated with staphylococcal food poisoning in Korea (Cha et al., 2006) and China (Yan et al., 2012). Some studies have shown that rates of resistance to non-β-lactam agents amongst ST1 MRSA isolates vary between countries and clones. ST1 MRSA strains in the United States usually show resistance to several non-β-lactam agents, including erythromycin and clindamycin, while MRSA strains in Australia are often uniformly susceptible to almost all non-β-lactams (Chen J. et al., 2014). The ST1 MRSA isolate in our study was also susceptible to all non-β-lactams, indicating that it might differ from that prevailing in the United States.

One ST9 LA-MRSA isolate was also identified in the current study. ST9 is the most prevalent LA-MRSA clone in most Asian countries, including China (Cui et al., 2009; Wagenaar et al., 2009; Petinaki and Spiliopoulou, 2012), despite ST398 being the most widespread ST in the rest of the world (Weese and Van Duijkeren, 2010; Petinaki and Spiliopoulou, 2012). ST9 MRSA is the predominant clone in food animals and animal-derived products (pork, chicken, and raw milk) in China (Cui et al., 2009; Wagenaar et al., 2009; Boost et al., 2013), and has also been found in farm workers (Cui et al., 2009) and associated with human infections (Liu et al., 2009; Yu et al., 2014). A previous study described ST9-MRSA-SCC*mec*V/NT isolates from patients with severe clinical illness (Yu et al., 2014). However, these patients were not livestock handlers and did not keep close contact with livestock (Yu et al., 2014), indicating that MRSA ST9 can pose a threat to humans through the food chain. ST9 MRSA strains with SCC*mec* types II (Wang et al., 2014), III (Cui et al., 2009), IVb (Boost et al., 2013; Wang et al., 2014; Yan et al., 2014), and V (Yu et al., 2014) have also been reported in China. However, the ST9 MRSA isolate recovered in our study showed considerable heterogeneity, harboring a previously undescribed SCC*mec* type with a type 2 *ccr* (A2B2) and a class C2 *mec* gene complex (2C2). These results suggest that ST9 MRSA acquired novel genomic islands during evolution from ST9 MSSA. Notably, the ST9 MRSA isolate in the current study was multidrug resistant, with resistance to β-lactams as well as eight other antibiotics widely used in chemotherapy. This finding suggests that the isolate originated from livestock.

Human infections caused by foodborne MRSA strains have been reported (Jones et al., 2002). Therefore, the potential role of food in the dissemination of successful MRSA lineages cannot be ignored. While ST59 and ST9 MRSA strains have been detected in food in China (Wang et al., 2014; Song et al., 2015), ST338 and ST1 MRSA isolates from food have never been reported. However, the previously reported ST59 MRSA strains were ST59-MRSA-II and ST59-MRSA-NT clones, not the more virulent ST59-MRSA-IVa clone identified in our study, which is associated with severe infections. Our results suggested that the presence of MRSA in RTE foods is the result of human contamination through poor personal hygiene, or through cross-contamination of carcasses during food processing. On the other hand, the MRSA clonal complexes identified in the present study are prevalent amongst clinical isolates associated with severe infection in China, suggesting food as a potential

environmental source of *S. aureus* isolates with significant clinical relevance.

CONCLUSION

To our knowledge, this is the first comprehensive study of the prevalence of *S. aureus* and MRSA in retail RTE foods from diverse regions of China. The present study revealed a relatively high prevalence of *S. aureus* and MRSA, and high rates of antimicrobial resistance amongst the isolates. Epidemic CA-MRSA and LA-MRSA clones associated with severe infection were identified amongst the isolates. Our data confirm the potential role of RTE foods in the dissemination of multidrug-resistant *S. aureus* strains and successful MRSA lineages in China, and highlights the health risks for consumers. Effective measures should be taken to ensure the safety of our food products.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XY, QW, and JZ. Performed the experiments: XY and SY. Analyzed the data: XY and JZ. Contributed reagents/materials/analysis tools: XY, JZ, WG, JH, and SC.

FUNDING

This work was supported by the Science and Technology Projects of Guangdong (2014B050504007, 2013B020312001).

REFERENCES

Boost, M. V., Wong, A., Ho, J., and O'Donoghue, M. (2013). Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from retail meats in Hong Kong. *Foodborne Pathog. Dis.* 10, 705–710. doi: 10.1089/fpd.2012.1415

Cha, J. O., Lee, J. K., Jung, Y. H., Yoo, J. I., Park, Y. K., Kim, B. S., et al. (2006). Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal foodpoisoning in South Korea. *J. Appl. Microbiol.* 101, 864–871. doi: 10.1111/j.1365-2672.2006.02957.x

Chen, C. J., and Huang, Y. C. (2014). New epidemiology of *Staphylococcus aureus* infection in Asia. *Clin. Microbiol. Infect.* 20, 605–623. doi: 10.1111/1469-0691.12705

Chen, J., Luo, Y., Zhang, S., Liang, Z., Wang, Y., Zhang, Y., et al. (2014). Community-acquired necrotizing pneumonia caused by methicillin-resistant *Staphylococcus aureus* producing Panton-Valentine leukocidin in a Chinese teenager: case report and literature review. *Int. J. Infect. Dis.* 26, 17–21. doi: 10.1016/j.ijid.2014.02.025

Chen, M. T., Wu, Q. P., Zhang, J. M., Yan, Z. A., and Wang, J. (2014). Prevalence and characterization of *Listeria monocytogenes* isolated from retail-level ready-to-eat foods in South China. *Food Control.* 38, 1–7. doi: 10.1016/j.foodcont.2013.09.061

Chuang, Y. Y., and Huang, Y. C. (2013). Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Asia. *Lancet Infect. Dis.* 13, 698–708. doi: 10.1016/S1473-3099(13)70136-1

Clinical and Laboratory Standards Institute (previously National Committee on Clinical Laboratory Standards). (2014). *Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard-9th Edn M2-A9.* Wayne, PA: CLSI.

Cui, S., Li, J., Hu, C., Jin, S., Li, F., Guo, Y., et al. (2009). Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from swine and workers in China. *Antimicrob. Chemother.* 64, 680–683. doi: 10.1093/jac/dkp275

Du, J., Chen, C., Ding, B., Tu, J., Qin, Z., Parsons, C., et al. (2011). Molecular characterization and antimicrobial susceptibility of nasal *Staphylococcus aureus* isolates from a Chinese medical college campus. *PLoS ONE* 6:e27328. doi: 10.1371/journal.pone.0027328

Geng, W., Yang, Y., Wu, D., Huang, G., Wang, C., Deng, L., et al. (2010). Molecular characteristics of community-acquired, methicillin-resistant *Staphylococcus aureus* isolated from Chinese children. *FEMS Immunol. Med. Microbiol.* 58, 356–362. doi: 10.1111/j.1574-695X.2009.00648.x

Hammad, A. M., Watanabe, W., Fujii, T., and Shimamoto, T. (2012). Occurrence and characteristics of methicillin-resistant and susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative *staphylococci* from Japanese retail ready-to-eat raw fish. *Int. J. Food Microbiol.* 156, 286–289. doi: 10.1016/j.ijfoodmicro.2012.03.022

He, W., Chen, H., Zhao, C., Zhang, F., Li, H., Wang, Q., et al. (2013). Population structure and characterisation of *Staphylococcus aureus* from bacteraemia at multiplehospitals in China: association between antimicrobial resistance, toxin genes and genotypes. *Int. J. Antimicrob. Agents* 42, 211–219. doi: 10.1016/j.ijantimicag.2013.04.031

Jones, T. F., Kellum, M. E., Porter, S. S., Bell, M., and Schaffner, W. (2002). An Outbreak of Community-acquired foodborne illness caused by Methicillin-resistant *Staphylococcus aureus*. *Emerging Infect. Dis.* 8, 82–84. doi: 10.3201/eid0801.010174

Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J., et al. (2007). Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrob. Agents Chemother.* 51, 264–274. doi: 10.1128/AAC.00165-06

Li, J., Wang, L., Ip, M., Sun, M., Sun, J., Huang, G., et al. (2013). Molecular and clinical characteristics of clonal complex 59 methicillin-resistant *Staphylococcus aureus* infections in Mainland China. *PLoS ONE* 8:e70602. doi: 10.1371/journal.pone.0070602

Liu, Y., Wang, H., Du, N., Shen, E., Chen, H., Niu, J., et al. (2009). Molecular evidence for spread of two major methicillin-resistant *Staphylococcus aureus* clones with a unique geographic distribution in Chinese hospitals. *Antimicrob. Agents Chemother.* 53, 512–518. doi: 10.1128/AAC.00804-08

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339, 520–532. doi: 10.1056/NEJM199808203390806

Pérez-Roth, E., Claverie-Martín, F., Villar, J., and Méndez-Alvarez, S. (2001). Multiplex PCR for simultaneous identification of *Staphylococcus aureus* and detection of methicillin and mupirocin resistance. *J. Clin. Microbiol.* 39, 4037–4041. doi: 10.1128/JCM.39.11.4037-4041.2001

Petinaki, E., and Spiliopoulou, I. (2012). Methicillin-resistant *Staphylococcus aureus* among companion and food-chain animals: impact ofhuman contacts. *Clin. Microbiol. Infect.* 18, 626–634. doi: 10.1111/j.1469-0691.2012.03881.x

Porrero, M. C., Mentaberre, G., Sánchez, S., Fernández-Llario, P., Gómez-Barrero, S., Navarro-Gonzalez, N., et al. (2013). Methicillin resistant *Staphylococcus aureus* (MRSA) carriage in different free-living wild animal species in Spain. *Vet. J.* 198, 127–130. doi: 10.1016/j.tvjl.2013.06.004

Rodríguez-Lázaro, D., Ariza-Miguel, J., Diez-Valcarce, M., Fernández-Natal, I., Hernández, M., and Rovira, J. (2015). Foods confiscated from non-EU flights as a neglected route of potential methicillin-resistant *Staphylococcus aureus* transmission. *Int. J. Food Microbiol.* 209, 29–33. doi: 10.1016/j.ijfoodmicro.2014.08.016

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

Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., et al. (2012). Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either mecA or the new mecA homologue mecA (LGA251). *Clin. Microbiol. Infect.* 18, 395–400. doi: 10.1111/j.1469-0691.2011.03715.x

Visciano, P., Pomilio, F., Tofalo, R., Sacchini, L., Saletti, M. A., Tieri, E., et al. (2014). Detection of methicillin-resistant *Staphylococcus aureus* in dairy cow farms. *Food Control.* 46, 532–538. doi: 10.1016/j.foodcont.2014.06.022

Wagenaar, J. A., Yue, H., Pritchard, J., Broekhuizen-Stins, M., Huijsdens, X., Mevius, D. J., et al. (2009). Unexpected sequence types in livestock associated methicillin-resistant *Staphylococcus aureus* (MRSA): MRSA ST9 and a single locus variant of ST9 in pig farming in China. *Vet. Microbiol.* 139, 405–409. doi: 10.1016/j.vetmic.2009.06.014

Wang, X., Li, G., Xia, X., Yang, B., Xi, M., and Meng, J. (2014). Antimicrobial susceptibility and molecular typing of methicillin-resistant *staphylococcus aureus* in retail foods in Shaanxi, China. *Foodborne Pathog. Dis.* 11, 281–286. doi: 10.1089/fpd.2013.1643

Weese, J. S., and Van Duijkeren, E. (2010). Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet. Microbiol.* 140, 418–429. doi: 10.1016/j.vetmic.2009.01.039

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

Yamamoto, T., Hung, W. C., Takano, T., and Nishiyama, A. (2013). Genetic nature and virulence of communityassociated methicillin-resistant *Staphylococcus aureus*. *Biomedicine* 3, 2–18. doi: 10.1016/j.biomed.2012.12.001

Yan, X., Wang, B., Tao, X., Hu, Q., Cui, Z., Zhang, J., et al. (2012). Characterization of *Staphylococcus aureus* strains associated with food poisoning in Shenzhen, China. *Appl. Environ. Microbiol.* 78, 6637–6642. doi: 10.1128/AEM.01165-12

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

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

Yu, F., Lu, C., Liu, Y., Sun, H., Shang, Y., Ding, Y., et al. (2014). Emergence of quinupristin/dalfopristin resistance among livestock-associated *Staphylococcus aureus* ST9 clinical isolates. *Int. J. Antimicrob. Agents* 44, 416–419. doi: 10.1016/j.ijantimicag.2014.06.020

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 June 2016
doi: 10.3389/fmicb.2016.00831

Design and Performance Testing of a DNA Extraction Assay for Sensitive and Reliable Quantification of Acetic Acid Bacteria Directly in Red Wine Using Real Time PCR

*Cédric Longin *, Michèle Guilloux-Benatier and Hervé Alexandre*

Equipe VAIMiS (Vin, Aliment, Microbiologie, Stress), Institut Universitaire de la Vigne et du Vin Jules Guyot, UMR Procédés Alimentaires et Microbiologiques, AgroSup Dijon – Université de Bourgogne, Dijon, France

Although strategies exist to prevent AAB contamination, the increased interest for wines with low sulfite addition leads to greater AAB spoilage. Hence, there is a real need for a rapid, specific, sensitive, and reliable method for detecting these spoilage bacteria. All these requirements are met by real time Polymerase Chain Reaction (or quantitative PCR; qPCR). Here, we compare existing methods of isolating DNA and their adaptation to a red wine matrix. Two different protocols for isolating DNA and three PCR mix compositions were tested to select the best method. The addition of insoluble polyvinylpolypyrrolidone (PVPP) at 1% (*v/v*) during DNA extraction using a protocol succeeded in eliminating PCR inhibitors from red wine. We developed a bacterial internal control which was efficient in avoiding false negative results due to decreases in the efficiency of DNA isolation and/or amplification. The specificity, linearity, repeatability, and reproducibility of the method were evaluated. A standard curve was established for the enumeration of AAB inoculated into red wines. The limit of quantification in red wine was 3.7 log AAB/mL and about 2.8 log AAB/mL when the volume of the samples was increased from 1 to 10 mL. Thus, the DNA extraction method developed in this paper allows sensitive and reliable AAB quantification without underestimation thanks to the presence of an internal control. Moreover, monitoring of both the AAB population and the amount of acetic acid in ethanol medium and red wine highlighted that a minimum about 6.0 log cells/mL of AAB is needed to significantly increase the production of acetic acid leading to spoilage.

Keywords: acetic acid bacteria, red wine, microbiological internal control, DNA extraction, real time PCR

OPEN ACCESS

Edited by:
Rosanna Tofalo,
University of Teramo, Italy

Reviewed by:
Luca Settanni,
Università degli Studi di Palermo, Italy
Rolf Dieter Joerger,
University of Delaware, USA

****Correspondence:***
Cédric Longin
cedric.longin@u-bourgogne.fr

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

Received: *16 March 2016*
Accepted: *17 May 2016*
Published: *01 June 2016*

Citation:
Longin C, Guilloux-Benatier M and Alexandre H (2016) Design and Performance Testing of a DNA Extraction Assay for Sensitive and Reliable Quantification of Acetic Acid Bacteria Directly in Red Wine Using Real Time PCR. Front. Microbiol. 7:831. doi: 10.3389/fmicb.2016.00831

INTRODUCTION

Acetic Acid Bacteria (AAB) species typically associated with grapes and must is *Gluconobacter oxydans* (*G. oxydans*) which prefers a sugar rich environment (Joyeux et al., 1984; Bartowsky and Henschke, 2008). AAB associated with wine are *Acetobacter aceti* (*A. aceti*) and *Acetobacter pasteurianus* (*A. pasteurianus*) which prefer ethanol as a carbon source, as does *Gluconacetobacter liquefaciens* (*Ga. liquefaciens*) (Joyeux et al., 1984; Drysdale and Fleet, 1985; Yamada et al., 1997). When these AAB are present during winemaking, aging or wine storage, they metabolize

ethanol to acetaldehyde by alcohol dehydrogenase and then produce acetic acid by acetaldehyde dehydrogenase. Acetic acid is the main constituent of wine volatile acidity (Bartowsky and Henschke, 2008) and considered to be undesirable in dry wine at concentrations exceeding 0.4–0.5 g/L depending on wine type (Davis et al., 1985; Eglinton and Henschke, 1999a,b). The European regulation (CE 1308/2013) has set out limits for sale at 1.20 and 1.08 g/L acetic acid for red wines and white/rosé wines, respectively. Taking into account the ability of AAB to convert ethanol into acetic acid, these bacteria are considered as spoilage bacteria in the wine industry. Plate counting is typically used in order to quantify AAB in wine. However, culturing and enumerating AAB is challenging despite the availability of various growth media. Many studies have reported that plate counting is not appropriate for estimating AAB populations in stressful environments like wine (Sievers et al., 1992; Sokollek et al., 1998; Millet and Lonvaud-Funel, 2000; Bartowsky et al., 2003; Trcek, 2005). Indeed, this technique often underestimates AAB populations (Bartowsky and Henschke, 2008). According to Millet and Lonvaud-Funel (2000), the difficulties of isolating AAB may be due in part to the existence of a Viable But Non Culturable (VBNC) state which may result from anaerobic conditions in wine (Du Toit et al., 2005). Many techniques can be used for AAB detection like nested PCR (González et al., 2006), AAB gene (*adh*A) PCR (Trcek, 2005), PCR-Restriction Fragment Length Polymorphism (Ruiz et al., 2000), and many others, however, all these techniques are culture dependent and the VBNC state may lead to underestimating the AAB population. Independent culture quantification techniques using Denaturing Gradient Gel Electrophoresis (De Vero et al., 2006), Temperature Gradient Gel Electrophoresis (Ilabaca et al., 2008), or epifluorescence have been reported (Mesa et al., 2003; Baena-Ruano et al., 2006). These latter authors quantified AAB in vinegar fermentation using viability (i.e., measurement of cell membrane permeability) and vitality (i.e., measurement of cell enzymatic activity) dyes. This technique has a high detection limit and appreciation of fluorescence is operator dependent. Thus, qPCR techniques have been developed (Torija et al., 2010; Valera et al., 2013) to quantify AAB. The qPCR method presented by González et al. (2006) did not use any internal control and was validated with red wine inoculated with a known amount of AAB, but without any growth. However, AAB growth in wine modifies DNA extraction efficiency and qPCR reliability since polyphenols seem to be adsorbed onto the cell walls (Morata et al., 2004). In addition, problems often arise with DNA amplification due to inhibitory substances such as tannins, polysaccharides and pigments (Rossen et al., 1992; Wilson, 1997). Therefore, poor DNA isolation and/or amplification efficiency, leading to false-negative results, were observed (Tessonnière et al., 2009). The specific species quantification of AAB using TaqMan probes was also reported (Torija et al., 2010; Valera et al., 2013). However, these probes are expensive and could not be used for routine laboratory analysis to determine total AAB population. Furthermore, none of these assays used internal controls to screen for the presence of inhibitors contained in the samples, leading to unreliable quantification. The goal of the current work was to develop a qPCR technique using an internal control that provides efficient and reliable quantification of the AAB naturally present in red wine. Different DNA extraction protocols were compared in order to remove wine inhibitors and assess the sensitivity, specificity, and reproducibility of the method.

MATERIALS AND METHODS

Microorganism Strains

Four different species of AAB were used: *Acetobacter aceti* DSM 3508 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), *Acetobacter pasteurianus* CECT 7582 (Colección española de cultivos tipo, Universitat de València, Edificio de Investigación, Burjassot, Spain); *Gluconobacter oxydans* DSM 7145*; Gluconacetobacter liquefaciens* CIP 103109 (Collection of Institut Pasteur, Biological Resource Center of Institut Pasteur (CRBIP), Paris, France). *Oenococcus oeni* sabo11 (a biotechnological strain isolated from a South African wine) was also used as the majority LAB present in wine. AAB were adapted to ethanol by growing them in mannitol medium supplemented with ethanol [2.5% (w/v) mannitol; 0.5% (w/v) yeast extract; 0.3% (w/v) peptone, 5% (v/v) ethanol]. *O. oeni* was adapted in FT80 medium (Cavin et al., 1988). *Escherichia coli* K12 ER2738 (available from New England Biolabs) was cultivated in LB medium (1% (*w*/*v*) peptone, 0.5% (*w*/*v*) yeast extract, 1% (*w*/*v*) NaCl). Yeasts, namely *Zygosaccharomyces bailii* MUCL 27812 (Mycothèque, de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium), *Candida vini* MUCL 27720, *Pichia membranifaciens* PMmb2000*, P. fermentans* PFmb2005, and *Saccharomyces cerevisiae* FERMOL-PB2023, were grown in modified YPD medium (2% (*w*/*v*) glucose; 0.5% (*w*/*v*) yeasts extract, 1% (*w*/*v*) peptone) with chloramphenicol at 0.02% (*w*/*v*) added after sterilization.

Growth Conditions

For the artificial contaminations, adapted AAB were inoculated into mannitol medium supplemented with 10% (*v*/*v*) ethanol (ethanol medium), white and red wines. Growth was ensured at 28°C. Red wine made from Pinot Noir grapes was supplied by the vineyard of the University of Burgundy [pH: 3.5; 12% (*v*/*v*) alcohol] as was white wine made from Chardonnay grapes [pH: 3.5; 12% (*v*/*v*) alcohol]. The wines were filtered through a 0.2 μm sterile membrane and dispensed into sterilized Erlenmeyer flasks.

Enumeration of Microorganisms

After AAB growth in ethanol medium and white and red wines, bacteria levels were measured by flow cytometry (FCM) in BD Accuri C6 flow cytometers with a single dye $DiBAC_4(3)$ [Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol] (ThermoFisher Scientific, Molecular Probes™, B-438) (final concentration 6 μM) in PBS buffer 1X (Biosolve, 10X concentrate Molecular biology, 162323). This compound was excited by the flow cytometer laser at 488 nm and emitted fluorescence collected by the filter 530 ± 15 nm. This dye was used for counterstaining and circumventing potential culture dependent shortcomings such as the VBNC state of wine microorganisms (Millet and Lonvaud-Funel, 2000). Three enumeration repetitions (20 μL at 34 μL/min) were performed for each sample.

Internal Control for DNA Isolation and Amplification

Specific EC23S primers were selected to quantify *E. coli* K12 ER2738 (Forward: 5′-CATAAGCGTCGCTGCCG-3′; Reverse: 5′-AAAGAAAGCGTAATAGCTCACTGGTC-3′) (Ludwig and Schleifer, 2000; Chern et al., 2011). A standard curve with these primers using the 23S rRNA gene sequences as target was obtained in LB medium at 10^7 to 10^1 cells/mL. 20 μL of internal control at 5.10^5 *E. coli*/mL were added to each 1 mL sample prior to DNA isolation to obtain a concentration of 10^4 *E. coli*/mL in each sample.

DNA Isolation and Extraction

After AAB growth and the addition of the internal control, two DNA extraction methods were tested: the Ausubel (Ausubel et al., 1992) and Lipp methods (Lipp et al., 1999). The two methods use CTAB as Jara et al. (2008) recommend. The main differences are that the second method use chloroform to purify DNA and use a CTAB precipitation solution after cell lysis to eliminate the remaining polyphenols. Moreover, DNA was re-dissolved in 50 μL sterile deionized water for the methods. When indicated, cells were centrifuged and the pellets were resuspended into lysis buffer supplemented with PVPP at a final concentration of 1% (*w*/*v*). Each bacterium was tested in growth medium supplemented with 10% (*v*/*v*) ethanol, and white and red wines. All the experiments were performed in triplicate and repeated three times.

AAB Real-Time PCR Amplification (qPCR)

AAB primers used to amplify the 16S rRNA gene were selected from Valera et al. (2015). The forward primer AAB-F (5′-TGAGAGGATGATCAGCCACACT-3′) and the reverse primer AAB-R (5′-TCACACACGCGGCATTG-3′) were synthesized by Eurogentec® (France). The PCR mixture was prepared in a total volume of 25 μL with 100 nmol of each primer and 5 μL DNA extract. The amplifications were done in triplicate on a CFX90 real-time PCR system (Bio-Rad) under the following conditions: 95°C for 10 min, 40 denaturation cycles at 95°C for 15 s, and 62°C for 1 min. Then a melting curve was produced to check the presence of only one amplification fragment. To test PCR amplification quality, BSA, and PVP treatments during qPCR at 400 ng/μL and 0.5% (*w*/*v*), respectively, were performed (Tessonnière et al., 2009). A control without treatment was also performed. The PCR cycle in which fluorescence first occurred (quantification cycle: C_q) was determined automatically using Bio-Rad CFX Manager® software after setting the regression method.

Volatile Acidity According to AAB Population

The acetic acid concentration and AAB population were monitored over time in synthetic medium containing 10% (*v*/*v*) alcohol. The amount of acetic acid was measured enzymatically using a Biosentec kit (Cat. No 021) according to the manufacturer's instructions, expressed in gram per liter. The AAB population was determined throughout their growth by plating on mannitol agar (CFU/mL). The same experiment was also performed in red wine from the vineyard of the University of Burgundy but no inoculation was performed; the AAB contamination was natural. In this experiment, the AAB population was determined by both qPCR and FCM. FCM provided the total bacteria population count. LAB enumeration was performed in FT80 Petri dishes to avoid overestimating the AAB population determined by FCM. Moreover, 15 red wines were chosen randomly from different wine regions to analyze the AAB populations (qPCR and FCM). In addition, acetic acid concentration was determined.

RESULTS

qPCR Specificity

The specificity of the AAB primers was tested against a panel of microorganisms known to be naturally present in wine. *In silico* tests were performed on *O. oeni*, *Pediococcus* spp., *Lactobacillus* spp, *Zygosaccharomyces bailii*, *Candida vini*, *Pichia membranifaciens, P. fermentans,* and *S. cerevisiae*. AAB primers did not match with the main wine microorganisms. *In vitro* tests were performed in synthetic medium against *O. oeni* and a panel of yeasts (see above). The C_q-values were the same as the negative control for all populations of *O. oeni* and yeast, thus validating the specificity of the primers. The EC23S specific primers targeting the 23S rRNA gene from *E.coli* (internal) control did not amplify DNA of the wine microorganisms tested. *E. coli* has been chosen because this microorganism is not naturally present in wine.

Comparison of DNA Isolation Methods

DNA extraction for AAB quantification was performed after growth in ethanol medium, and white and red wines using either the Lipp or Ausubel methods with or without PVPP during cell lysis. In order to compare the DNA extraction methods, the C_q obtained from DNA extracted by both methods with or without PVPP for the three different media were compared for each bacterium.

As shown in **Table 1A**, for ethanol medium, the Lipp method without PVPP gave significantly better results, as shown by a lower C_q whatever the bacteria, except for *G. oxydans*, showing no significant difference in the results obtained by both methods. PVPP addition during DNA extraction using either the Lipp or Ausubel method did not improve qPCR efficiency for most of the bacteria tested. PVPP addition during extraction with the Ausubel method significantly improved C_q only for *A. aceti* and *A. pasteurianus*. Thus, the choice of DNA extraction method is essential.

For white wine (**Table 1B**), the effect of PVPP is species dependent, but the results confirmed that the Lipp method gave much better results.

Regarding red wines which are rich in qPCR inhibitor compounds, the Lipp method gave much better C_q compared to the Ausubel method, except for *G. oxydans*. When PVPP was added prior to DNA extraction, the C_q-values did not improve whatever the DNA extraction method, except in a few cases (**Table 1C**). However, when using PVPP, the Lipp method was always better than the Ausubel method, as demonstrated by the lower C_q-values. For example, in our study we obtained a C_q for

TABLE 1 | C_q results according to DNA extraction (Ausubel and Lipp methods) with (+) or without (–) PVPP for *A. aceti*, *A. pasteurianus*, *G. oxydans,* and *Ga. liquefaciens* in (A) growth medium supplemented with 10% (*v/v*) ethanol, and (B) white, and (C) red wines.

(A)

Methods	PVPP	Ethanol medium			
		A. aceti	*A. pasteurianus*	*G. oxydans*	*Ga. liquefaciens*
Ausubel	–	32.0 ± 0.0^{b}	30.5 ± 0.0^{c}	21.8 ± 1.8^{a}	23.0 ± 1.7^{b}
	+	22.7 ± 1.7^{a}	18.3 ± 0.8^{b}	22.4 ± 1.7^{a}	23.9 ± 0.4^{b}
Lipp	–	18.5 ± 0.6^{a}	14.0 ± 0.5^{a}	18.6 ± 1.7^{a}	20.3 ± 0.6^{a}
	+	20.4 ± 2.7^{a}	15.4 ± 0.6^{a}	18.9 ± 0.8^{a}	20.2 ± 0.6^{a}

(B)

Methods	PVPP	White wine			
		A. aceti	*A. pasteurianus*	*G. oxydans*	*Ga. liquefaciens*
Ausubel	–	23.9 ± 1.3^{b}	21.8 ± 0.8^{a}	22.4 ± 0.3^{c}	23.1 ± 1.3^{b}
	+	23.0 ± 2.8^{ab}	22.2 ± 0.6^{a}	22.2 ± 1.3^{c}	21.6 ± 0.0^{ab}
Lipp	–	19.8 ± 0.1^{ab}	19.8 ± 0.6^{a}	16.8 ± 0.6^{a}	21.2 ± 0.3^{a}
	+	19.6 ± 0.8^{a}	19.3 ± 2.3^{a}	19.4 ± 0.7^{b}	20.8 ± 0.4^{a}

(C)

Methods	PVPP	Red wine			
		A. aceti	*A. pasteurianus*	*G. oxydans*	*Ga. liquefaciens*
Ausubel	–	26.4 ± 1.9^{b}	28.4 ± 0.5^{b}	25.9 ± 1.7^{b}	26.2 ± 1.1^{b}
	+	27.8 ± 0.8^{b}	26.9 ± 0.9^{b}	26.3 ± 0.8^{b}	26.1 ± 0.3^{b}
Lipp	–	23.2 ± 0.5^{a}	12.7 ± 0.7^{a}	23.8 ± 1.0^{ab}	21.7 ± 0.4^{a}
	+	22.4 ± 0.4^{a}	11.2 ± 0.3^{a}	22.1 ± 0.6^{a}	21.2 ± 0.0^{a}

Values represent the C_q mean ± Standard Deviation (SD); n = 3; values followed by different letters within a column are statistically different at $p < 0.05$ (XLStat©).

A. aceti of 27.8 in red wine (Ausubel + PVPP). When using the Lipp method with PVPP, the C_q was equal to 22.4. Thus, using the suboptimal extraction method, C_q led to underestimating the real AAB population.

Another way to improve qPCR efficiency is to add either BSA or PVP in the qPCR mix (Jiang et al., 2005; Malorny and Hoorfar, 2005). These compounds are assumed to trap inhibitors in the reaction mix. **Figure 1** shows the results of PCR assays on *A. pasteurianus*. DNA was extracted using the Lipp extraction method with PVPP, and qPCR was run with or without either the addition of BSA or PVP in the qPCR mix compared to control. The addition of BSA or PVP in the qPCR mix did not lead to any improvement of the C_q obtained. No improvement of C_q-values was observed using these compounds and, as no difference was highlighted in red wine with the Lipp DNA extraction method between the four species, neither BSA nor PVP were added in the mix to quantify AAB using the Lipp method in red wine. However, BSA added to *A. pasteurianus* DNA using the Ausubel method with or without PVPP in red wine significantly improved C_q-values compared to control, but they were always significantly higher (less effective) than the Lipp method. The values from

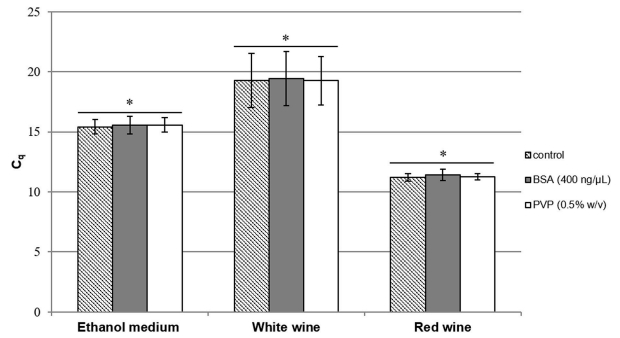


FIGURE 1 | C_q results for *A. pasteurianus* after DNA extraction by the Lipp method in mannitol supplemented with 10% (*v/v*) alcohol (containing $2.4.10^6$ cells/mL), white wine (containing $2.7.10^5$ cells/mL), and red wine (containing $9.9.10^7$ cells/mL). Cell lysis was performed in triplicate with PVPP and BSA (400 ng/μL) (■) or PVP (0.5% *w/v*) (□), added to the qPCR mix and compared to control (⊠). An Anova with Tukey's test was performed for each medium independently to analyze the C_q results with BSA and PVP compared to control (*$p > 0.05$), (XLStat©). Error bars represent standard deviations.

the Ausubel DNA extraction method with or without PVPP were higher than 11.3 and 12.8 units, respectively, compared to the values obtained with the Lipp method. These results seem to indicate the presence of PCR inhibitor compounds in DNA extract using the Ausubel method with or without PVPP.

Use of *E. coli* ER2738 as Microbiological Internal Control

An *E. coli* standard curve from DNA isolation at various levels between 10^1 and 10^7 bacteria/mL was used to evaluate qPCR efficiency (98.8%) with a correlation coefficient of 0.998. The T_m of the product had a value of 82.5 ± 0.5°C. The trend curve is: $[E.\ coli]_{concentration}(\log \text{ cells/mL}) = -0.2977 \times C_q + 11.581$. A suspension of the strain *E. coli* K12 ER2738 was added to obtain 10^4 cells/mL in ethanol medium (10% *v/v*), and white and red wines samples containing *A. aceti*, *A. pasteurianus*, *G. oxydans,* and *Ga. liquefaciens*. According to the standard curve, efficient DNA extraction and amplification with EC23S primers should give a C_q-value of 25.5 ± 0.4 for 4 log cells/mL of *E. coli*. Thus, following DNA extraction performed in triplicate of each AAB in each media with both Ausubel and Lipp methods, with or without PVPP, *E. coli* was quantified.

The values in **Table 2** are the averages of *E. coli* populations found after DNA extraction according to methods with or without PVPP. No significant differences ($p > 0.05$) can be seen between the two techniques used for DNA extraction with or without PVPP for *E. coli* enumeration in ethanol medium and white wine. No significant cell loss was highlighted for either the Ausubel or Lipp method. However, the standard deviation of the Lipp method was lower than 0.3 log *E. coli*/mL compared to the Ausubel method for both media.

In red wine, the mean values for *E. coli* concentration in the Lipp method after DNA extraction with or without PVPP were 3.7 ± 0.2 and 3.3 ± 0.2 log cells/mL, respectively, whereas Ausubel DNA extractions led to 1.8 ± 0.2 and 1.9 ± 0.5 log

TABLE 2 | Averages of *E. coli* K12 ER2738 found after initially adding 4 log cells/mL in ethanol growth medium and white and red wines containing *A. aceti*, *A. pasteurianus*, *G. oxydans,* and *Ga. liquefaciens,* and following DNA extraction.

Methods	PVPP	Ethanol medium		White wine		Red wine	
		log *E. coli*/mL	*p*-values	log *E. coli*/mL	*p*-values	log *E. coli*/mL	*p*-values
Ausubel	–	3.4 ± 0.7^{a}	$p > 0.05$	3.6 ± 0.5^{a}	$p > 0.05$	1.9 ± 0.5^{b}	$p < 0.05$
	+	3.5 ± 0.4^{a}	$p > 0.05$	3.7 ± 0.5^{a}	$p > 0.05$	1.8 ± 0.2^{b}	$p < 0.05$
Lipp	–	3.9 ± 0.3^{a}	$p > 0.05$	3.6 ± 0.1^{a}	$p > 0.05$	3.3 ± 0.2^{a}	$p > 0.05$
	+	3.6 ± 0.2^{a}	$p > 0.05$	3.6 ± 0.3^{a}	$p > 0.05$	3.7 ± 0.1^{a}	$p > 0.05$

The Lipp and Ausubel methods were used with (+) or without (–) PVPP during cell lysis. qPCR was done with EC23S primers.
An Anova representation with Tukey's test was applied to the E. coli enumeration results using as control modality of 4.0 log E. coli/mL, (XLStat©). Values followed by different letters within a column are statistically different at $p < 0.05$ (XLStat©).

cells/mL, respectively, with and without PVPP. Thus, as shown in **Table 2**, the Lipp method with or without PVPP led to the recovery of the internal control population, which was not significantly different from the added population. Although not significant, the C_q-value following Lipp DNA extraction with PVPP tended to be slightly lower in red wine compared to experiments performed without PVPP. On the basis of these results, the Lipp DNA extraction method with PVPP was used for all extractions in wine. AAB can be quantified only if *E. coli* quantification is not significantly different from the added concentration (10^4 *E. coli*/mL), since the detection of a loss of internal standard leads to an underestimation of AAB population.

AAB Quantification with the Presence of Other Wine Microorganisms

To verify the absence of interference by other microorganisms in naturally contaminated wine, the following procedure was implemented. AAB quantifications in red wine containing 10^5 *A. pasteurianus*/mL alone or supplemented with *O. oeni* and *B. bruxellensis* at 10^5 cells/ml were performed. qPCR with AAB primers was performed after validating the presence of *E. coli* at 10^4 cells/mL. The results of the samples containing *A. pasteurianus* alone or with other microorganisms (*O. oeni* and *B. bruxellensis*) were identical: 5.1 ± 0.1 and 5.1 ± 0.2 log *A. pasteurianus*/mL, respectively.

Linearity, Repeatability, and Reproducibility in Red Wine

After artificial contamination of the red wine by AAB and incubation allowing their growth, verified over time by flow cytometry, Lipp DNA extraction with PVPP was performed in triplicate with 3 repetitive independent experiments. The T_m of the product had a value of 82.5 ± 0.5°C. AAB quantification could be done after confirming the presence of 4 log *E. coli*/mL. Efficiency, r^2, and y-intercept are presented in **Table 3**. No significant differences between efficiency, r^2, and y-intercept following a Tukey's test between red wines, containing each AAB species or a mix of all AAB, were observed. Thus, all the data were compiled to determine the general standard curve allowing AAB quantification in red wine whatever the species present (**Figure 2**):

$$[AAB]_{\text{concentration}}(log\ cells/mL) = -0.2927 \times C_q + 11.135.$$

TABLE 3 | Statistical analysis of qPCR parameters obtained from independent DNA isolation experiments performed on *A. aceti, A. pasteurianus. G. oxydans,* and *Ga. liquefaciens* after growth in red wine in triplicate and with three repetitions of the experiment over time.

	Efficiency	r^2	y-intercept
A. aceti	166 ± 46^{a}	0.926 ± 0.031^{a}	35.2 ± 3.0^{a}
A. pasteurianus	176 ± 37^{a}	0.942 ± 0.069^{a}	33.8 ± 1.7^{a}
G. oxydans	167 ± 40^{a}	0.940 ± 0.018^{a}	34.4 ± 1.9^{a}
Ga. liquefaciens	178 ± 33^{a}	0.875 ± 0.098^{a}	33.5 ± 1.8^{a}
AAB mixture	125 ± 09^{a}	0.988 ± 0.012^{a}	33.6 ± 0.7^{a}
p values	$p > 0.05$	$p > 0.05$	$p > 0.05$

Three red wines containing a mix of AAB were also analyzed in triplicate. An Anova with Tukey's test were performed according to efficiency, r^2, and y-intercept, (XLStat©). Values followed by the same letter within a column are statistically identical at $p > 0.05$ (XLStat©).

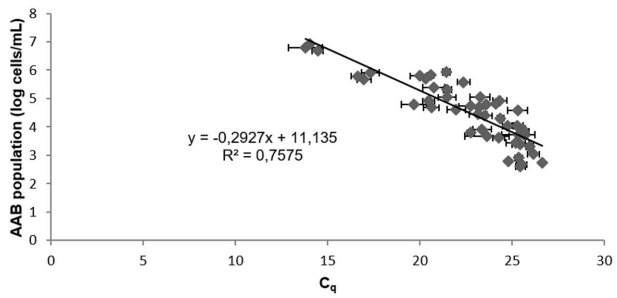


FIGURE 2 | Standard curve from 10-fold serial sample dilutions of red wines containing *A. aceti*, *A. pasteurianus*, *G. oxydans*, *Ga. liquefaciens*, and the AAB mixture. The C_q values are the average of three individual experiments performed in triplicate. Error bars represent standard deviations.

Limit of Quantification (LOQ) of AAB in Red Wine

For our study, LOQ was determined using the slope, residue standard deviation, and standard deviation of the intercept obtained from linearity validation experiments (OIV, 2005).

Using the Lipp method after AAB growth in red wine, the LOQ value of $5.2.10^3$ cells/mL was obtained. These results were the mean of the three independent experiments performed in triplicate. Moreover, the sampling volume of 10 mL instead of 1 mL led to the improvement of LOQ by 0.9 ± 0.1 log cells/mL.

Acetic Acid Monitoring According to AAB Population

Acetic acid concentration monitoring was performed after *A. pasteurianus* inoculation in ethanol medium [10% (*v*/*v*)] at 10^2 cells/mL in independent experiments performed in triplicate (**Figure 3**). The initial amount of acetic acid was 0.04 g/L. **Figure 3** shows acceptable acetic acid values of 0.04 ± 0.00 g/L for an AAB concentration from 2.2 to 5.4 log CFU/mL. The sample presented 0.52 g/L acetic acid when the *A. pasteurianus* population reached 6.9 log CFU/mL. The last analysis point has a bacterial population of 7.2 ± 0.1 log CFU/mL and shows acetic acid values from 2.39 ± 0.61 g/L. Thus, acetic acid production increased in ethanol medium and occurred when acetic acid bacteria exceeded 6 log CFU/mL. AAB growth was low between the analysis points of 10 and 14 days, indicating that culture reached a stationary phase. The high acetic acid concentration determined during this time seemed to highlight overproduction during the stationary phase of the culture, i.e., when AAB are in high concentration.

To confirm these hypotheses of acetic acid production beyond acceptable limits at a population higher than 6 log AAB/mL and during the stationary phase of AAB growth, AAB growth was monitored in red wine from the vineyard of the University of Burgundy, contaminated naturally over time (**Figure 4**). Both the AAB population and acetic acid concentration were determined over time. The enumeration of total bacteria in the samples was performed by FCM and LAB quantification in specific medium. Neither yeasts nor LAB were detected in the wine under study, consequently only AAB were present and enumerated. Therefore, it was possible to compare the AAB concentration determined by FCM/qPCR methods and acetic acid production. Acetic acid concentration as a function of AAB population is presented in **Figure 4**. For the first points of analysis containing a population ranging from 2.9 to 6 log AAB/mL, quantifications by qPCR were well-correlated with quantification by FCM. The acetic acid concentrations at these analysis points did not exceed 0.29 g/L. Then, higher acetic acid concentrations were reached for higher acetic acid bacteria concentrations. The analysis point containing an AAB population of 6.8 ± 0.2 log bacteria/mL had 0.82 ± 0.01 g/L acetic acid. The last point of AAB growth monitoring in red wine presented a high AAB concentration with acetic acid levels exceeding the European limit values and an increase in the mean difference between the qPCR and FCM methods (1.2 ± 0.6 log cells/mL). **Figure 4** validates acetic acid production by AAB during the stationary growth phase, thus when AAB exceeded 6 log bacteria/mL.

The measure of acetic acid and counting of AAB by FCM/qPCR methods were then performed on 15 red wines chosen randomly from wine regions (**Figure 5**). Twelve red wines had a mean amount of acetic acid at 0.52 ± 0.15 g/L. For these same samples, the median amount of acetic acid was 0.57 ± 0.15 g/L. These wines are considered as unspoiled. The mean relative difference between FCM vs. qPCR, allowing the determination of the AAB population, was 0.5 ± 0.3 log cells/mL. Three of the fifteen red wines analyzed presented a high amount of acetic acid and were thus spoiled. These wines had an AAB concentration higher than 6 log AAB/mL.

DISCUSSION

Cell quantification by qPCR in red wine is difficult since various qPCR inhibitors such as polyphenols and polysaccharides are abundant, thereby increasing the risk of false negative results (Demeke and Jenkins, 2009) and making the amplification of genetic material challenging. qPCR has been used to quantify AAB in wine (González et al., 2006; Andorrà et al., 2008; Torija et al., 2010; Valera et al., 2013), however, the authors of these

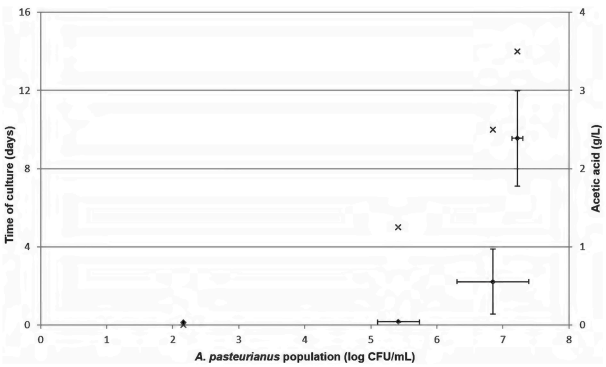


FIGURE 3 | Monitoring of *Acetobacter pasteurianus* growth in mannitol medium containing 10% (*v*/*v*) alcohol (×). Initial inoculation was 10^2 cells/mL. This experiment was performed in three independent experiments. Acetic acid concentration (♦) was determined in triplicate over time. Error bars represent standard deviations.

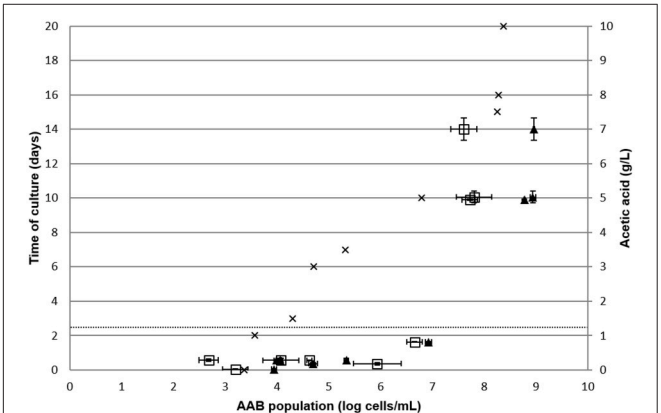


FIGURE 4 | Monitoring of natural AAB growth in red wine. The AAB population was determined by FCM (□) and qPCR (▲) in duplicate over time. Acetic acid concentration was determined in duplicated over time. The dotted line represents the limit of the European threshold. Error bars represent standard deviations.

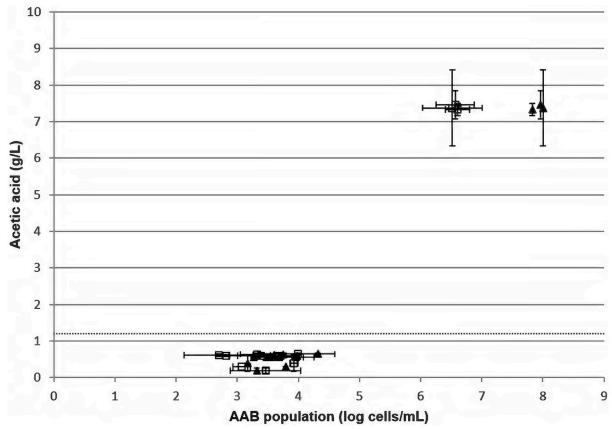


FIGURE 5 | Measure of acetic acid concentration in duplicate according to the AAB population determined by FCM (□) and qPCR (▲) in 15 red wines from different wine regions chosen randomly. The dotted line represents the limit of the European threshold.

studies did not take into account extraction efficiency or the presence of inhibitors, nor did they use any control process (cells added to the matrix). Process control can provide information on the efficiency of extraction and on the PCR procedure. The addition of a "spike" control to ensure accurate and reliable quantification is important (Fukushima et al., 2003; Stoeckel et al., 2009; van Doorn et al., 2009) and has already been reported for food analysis (Josefsen et al., 2010; Krøjgaard et al., 2011; Ishii et al., 2013), but as far as we know only once for quantifying *Brettanomyces* in wine (Tessonnière et al., 2009). In order to develop an accurate and efficient qPCR method to quantify AAB, DNA extraction procedures were first tested in order to ensure good DNA preparation. Indeed, the extraction efficiency and quality of DNA must be optimized for quantitative PCR. The selection of an appropriate DNA extraction method from those available is thus crucial (Jara et al., 2008).

DNA Extraction Method

We opted for the Lipp method (Lipp et al., 1999) since this method has proven useful for many foodstuffs, especially those rich in phenolic compounds. By comparing this method with classical CTAB extraction, we clearly demonstrated the advantage of using the former. Using our process control strain, we demonstrated that the DNA extraction method was well-suited for wine and especially red wine. Moreover, as PVPP is known to remove PCR inhibitors (Tessonnière et al., 2009) it was added in our assay and improved C_q-values. The results of our validation protocol proved the specificity of the assay. Indeed, qPCR primers showed good specificity for all the wine AAB tested and did not amplify from the other wine organisms. Moreover, our study showed that AAB population determination by qPCR with the Lipp method using PVPP was not influenced by the other microorganisms present (*O. oeni* and *B. bruxellensis*) in high concentration. Therefore, pre-amplification using a nested PCR technique (González et al., 2006) is not required to determine the AAB population in samples.

AAB qPCR Limit of Quantification

Our LOQ was 5.10^2 cells with a test sample of 10 ml. This LOQ is similar to those previously reported and was reached with AAB grown in red wines, contrary to González et al. (2006) and Torija et al. (2010). In our study, the correlation coefficient of the standard curve obtained for AAB in red wines was 0.76. This coefficient might appear low but it comes from a calibration curve used for wine. Indeed, it is essential to create calibration curves in food matrices. The creation of standard curves for the quantification of a microorganism by diluting DNA from one extraction, as has been done in most studies, should be avoided (González et al., 2006; Torija et al., 2010; Valera et al., 2013). Consequently, the influence of food matrices is not considered, resulting in an underestimation of microbial load (Cocolin and Rantsiou, 2012). Most studies have obtained a high correlation coefficient between 10-fold serial DNA dilutions, but have not performed DNA extraction from a sample containing a low bacterial population, thus biasing efficiency. DNA extraction must be performed from each dilution before running the qPCR to create a standard curve.

Evaluation of the Method with Naturally Spoiled Wines

Little is known of the spoilage caused by AAB populations and it probably depends on the matrix (sulfites, polyphenols, etc.). Also, this topic is controversial in the literature. According to Joyeux et al. (1984), a low population can activate significant volatile acidity production when exposed to air. However, Drysdale and Fleet (1985) reported that *A. pasteurianus* and *A. aceti* occur at 10^1–10^3 CFU/mL in many wines during bulk storage in wineries without causing spoilage. Moreover, Bartowsky and Henschke (2008) have shown that 2.10^4 *A. pasteurianus*/mL in a Shiraz wine leads to an acetic acid concentration of 0.6 ±0.0 g/L. This wine was not considered spoiled because the initial wine, without microorganisms, contained 0.5 ±0.0 g of acetic acid per liter. Another wine containing 9.10^4 *A. pasteurianus*/mL had 3.5 ±1.7 g/L of acetic acid, and was thus spoiled. These results may underestimate the true population due to the uncertainty in recovering all the bacteria. In the literature, there is no clear consensus regarding AAB concentration leading to spoilage risk. Therefore, we determined the level of acetic acid concentration in ethanol medium and red wine according to the AAB population over time. In the latter, the LAB concentration was determined to avoid overestimation of the AAB population by FCM bacteria enumeration, even if the LAB is not a serious issue because AAB causes wine spoilage only during aging in the cellar and after bottling (Henick-Kling, 1993), normally without the presence of LAB. In our study, neither yeasts nor LAB were detected in these red wines. Thus, the assessment of our improved qPCR assay for AAB quantification in naturally contaminated red wine proved reliable and efficient in comparison with cytometry results. The small deviations between both methods were probably derived from the matrix, which was different to that used to write the equation. In our study, during the monitoring of AAB naturally present in red wine, red wine exhibiting an AAB population lower than 6 log bacteria/mL was not altered. However, for wines

presenting an AAB population of about 6-7 log cells/mL, the acetic acid exceeded the aroma threshold (Davis et al., 1985; Eglinton and Henschke, 1999a,b; Swiegers et al., 2005) but not the European limit values for sale. For the other wines with high AAB populations and during the stationary phase, acetic acid exceeded the European limit values. Thus, the method developed in this study had an elevated LOQ without using a higher sample volume (e.g., 10 mL).

CONCLUSION

The method allowed AAB detection and quantification before spoilage occurred, which meets the needs and expectations of the wine industry when monitoring AAB populations on a regular basis. Therefore, in our study, we developed a qPCR method which allows the reliable quantification of AAB in red wine. We showed that the previously reported DNA extraction method was not efficient enough for precise quantification. The methods used in these studies probably led to underestimating the AAB population. Use of an internal control allows validating DNA extraction and qPCR efficiencies. No underestimation can be made if the initial concentration of the internal control added in the sample before DNA extraction is found. Moreover, the standard curve was established with AAB that proliferated in red wine. Finally, as far as we know, this is the first time a qPCR protocol allowing AAB quantification in red wines without bias (neither cell loss nor PCR inhibitor presence) has been validated. Moreover, the presence of other microorganisms in the sample did not alter AAB quantification.

Specific AAB species quantification is not possible with our method; however, our main goal was to quantify AAB in wines whatever the species present in order to evaluate the risk of spoilage. Furthermore, one drawback of the method is that qPCR quantifies live and dead AAB. Propidium monoazide could be used (Vendrame et al., 2013; Rizzotti et al., 2015) but cell exposure to a stress like ethanol, which is known to permeabilize membranes (Alexandre et al., 1994), can result in cells stained by propidium iodide, for example, but that are still alive (Davey and Hexley, 2011). Moreover, Shi et al. (2012) highlighted an underestimation of total yeasts, *S. cerevisiae*, total LAB, non-*O. oeni* LAB, and total AAB in wine by real-time PCR coupled with ethidium monoazide. However, they demonstrated that 40 min of incubation in recovery medium could completely cancel the underestimation of viable cell counts performed in wine, but not cell growth, which must be checked for each sample. To avoid this potential error, one way to circumvent the drawback of the method is to monitor the AAB population over time. Under these conditions, a decrease in C_q-value would reflect AAB growth and potentially the risk of spoilage.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: HA, CL, and MG. Performed the experiments: CL. Generated and analyzed the data: CL, HA, and MG. Wrote the paper: CL, HA, and MG.

FUNDING

This work was funded by the Regional Council of Burgundy and the Interprofessional Office of Burgundy Wines.

REFERENCES

Alexandre, H., Rousseaux, I., and Charpentier, C. (1994). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and *Kloeckera apiculata*. *FEMS Microbiol. Lett.* 124, 17–22. doi: 10.1111/j.1574-6968.1994.tb07255.x

Andorrà, I., Landi, S., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol.* 25, 849–856. doi: 10.1016/j.fm.2008.05.005

Ausubel, F. M., Brent, R., Robert, E., Kingston, Moore, D. D., Seidman, J. G., et al. (1992). *Short Protocols in Molecular Biology*, *2nd Edn*. London: John Wiley and Sons Inc.

Baena-Ruano, S., Jiménez-Ot, C., Santos-Dueñas, I. M., Cantero-Moreno, D., Barja, F., and García-García, I. (2006). Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process. *Process Biochem.* 41, 1160–1164. doi: 10.1016/j.procbio.2005.12.016

Bartowsky, E. J., and Henschke, P. A. (2008). Acetic acid bacteria spoilage of bottled red wine—A review. *Int. J. Food Microbiol.* 125, 60–70. doi: 10.1016/j.ijfoodmicro.2007.10.016

Bartowsky, E., J., Xia, D., Gibson, R., I., Fleet, G., H., and Henschke, P., A. (2003). Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* 36, 307–314. doi: 10.1046/j.1472-765X.2003.01314.x

Cavin, J. F., Schmitt, P., Arias, A., Lin, J., and Divies, C. (1988). Plasmid profiles in *Leuconostoc* species. *Microbiol. Aliment Nutr.* 6, 55–62.

Chern, E. C., Siefring, S., Paar, J., Doolittle, M., and Haugland, R. A. (2011). Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. *Lett. Appl. Microbiol.* 52, 298–306. doi: 10.1111/j.1472-765X.2010.03001.x

Cocolin, L., and Rantsiou, K. (2012). "Quantitative polymerase chain reaction in food microbiology," in *Quantitative Real-Time PCR in Applied Microbiology, Vol. 149*, ed M. Filion (Norfolk: Caister Academic Press), 149–160.

Davey, H. M., and Hexley, P. (2011). Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environ. Microbiol.* 13, 163–171. doi: 10.1111/j.1462-2920.2010.02317.x

Davis, C. R., Wibowo, D., Eschenbruch, R., Lee, T. H., and Fleet, G. H. (1985). Practical implications of malolactic fermentation: a review. *Am. J. Enol. Vitic.* 36, 290–301.

Demeke, T., and Jenkins, G. R. (2009). Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal. Bioanal. Chem.* 396, 1977–1990. doi: 10.1007/s00216-009-3150-9

De Vero, L., Gala, E., Gullo, M., Solieri, L., Landi, S., and Giudici, P. (2006). Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. *Food Microbiol.* 23, 809–813. doi: 10.1016/j.fm.2006.01.006

Drysdale, G. S., and Fleet, G. H. (1985). Acetic acid bacteria in some Australian wines. *Food Technol. Aust.* 37, 17–20.

Du Toit, W. J., Pretorius, I. S., and Lonvaud-Funel, A. (2005). The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *J. Appl. Microbiol.* 98, 862–871. doi: 10.1111/j.1365-2672.2004.02549.x

Eglinton, J. M., and Henschke, P. A. (1999a). Restarting incomplete fermentations: the effect of high concentrations of acetic acid. *Aust. J. Grape Wine Res.* 5, 71–78. doi: 10.1111/j.1755-0238.1999.tb00155.x

Eglinton, J. M., and Henschke, P. A. (1999b). The occurrence of volatile acidity in Australian wines. *Aust. N. Z. Grapegrow. Winemak.* 426a, 7–14.

Fukushima, H., Tsunomori, Y., and Seki, R. (2003). Duplex real-time SYBR Green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. *J. Clin. Microbiol.* 41, 5134–5146. doi: 10.1128/JCM.41.11.5134-5146.2003

González, Á., Hierro, N., Poblet, M., Mas, A., and Guillamón, J. M. (2006). Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. *FEMS Microbiol. Lett.* 254, 123–128. doi: 10.1111/j.1574-6968.2005.000011.x

Henick-Kling, T. (1993). "Malolactic fermentation," in *Wine Microbiology Biotechnology*, ed G. H. Fleet (New York, NY: Taylor & Francis Inc.), 289–326.

Ilabaca, C., Navarrete, P., Mardones, P., Romero, J., and Mas, A. (2008). Application of culture culture-independent molecular biology based methods to evaluate acetic acid bacteria diversity during vinegar processing. *Int. J. Food Microbiol.* 126, 245–249. doi: 10.1016/j.ijfoodmicro.2008.05.001

Ishii, S., Segawa, T., and Okabe, S. (2013). Simultaneous quantification of multiple food- and waterborne pathogens by use of microfluidic quantitative PCR. *Appl. Environ. Microbiol.* 79, 2891–2898. doi: 10.1128/AEM.00205-13

Jara, C., Mateo, E., Guillamón, J. M., Torija, M. J., and Mas, A. (2008). Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. *Int. J. Food Microbiol.* 128, 336–341. doi: 10.1016/j.ijfoodmicro.2008.09.008

Jiang, J., Alderisio, K. A., Singh, A., and Xiao, L. (2005). Development of procedures for direct extraction of cryptosporidium DNA from water concentrates and for relief of PCR inhibitors. *Appl. Environ. Microbiol.* 71, 1135–1141. doi: 10.1128/AEM.71.3.1135-1141.2005

Josefsen, M. H., Löfström, C., Hansen, T. B., Christensen, L. S., Olsen, J. E., and Hoorfar, J. (2010). Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. *Appl. Environ. Microbiol.* 76, 5097–5104. doi: 10.1128/AEM.00411-10

Joyeux, A., Lafon-Lafourcade, S., and Ribéreau-Gayon, P. (1984). Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol.* 48, 153–156.

Krøjgaard, L. H., Krogfelt, K. A., Albrechtsen, H.-J., and Uldum, S. A. (2011). Detection of *Legionella* by quantitative-polymerase chain reaction (qPCR) for monitoring and risk assessment. *BMC Microbiol.* 11:254. doi: 10.1186/1471-2180-11-254

Lipp, M., Brodmann, P., Pietsch, K., Pauwels, J., and Anklam, E. (1999). IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. *J. AOAC Int.* 82, 923–928.

Ludwig, W., and Schleifer, K. H. (2000). How quantitative is quantitative PCR with respect to cell counts? *Syst. Appl. Microbiol.* 23, 556–562. doi: 10.1016/S0723-2020(00)80030-2

Malorny, B., and Hoorfar, J. (2005). Toward standardization of diagnostic PCR testing of fecal samples: lessons from the detection of *Salmonellae* in pigs. *J. Clin. Microbiol.* 43, 3033–3037. doi: 10.1128/JCM.43.7.3033-3037.2005

Mesa, M. M., Macías, M., Cantero, D., and Barja, F. (2003). Use of the direct epifluorescent filter technique for the enumeration of viable and total acetic acid bacteria from vinegar fermentation. *J. Fluoresc.* 13, 261–265. doi: 10.1023/A:1025094017265

Millet, V., and Lonvaud-Funel, A. (2000). The viable but non-culturable state of wine micro-organisms during storage. *Lett. Appl. Microbiol.* 30, 136–141. doi: 10.1046/j.1472-765x.2000.00684.x

Morata, A., Gómez-Cordovés, M. C., Colomo, B., and Suárez, J. A. (2004). Cell wall anthocyanin adsorption by different *Saccharomyces* strains during the fermentation of *Vitis vinifera* L. cv Graciano grapes. *Eur. Food Res. Technol.* 220, 341–346. doi: 10.1007/s00217-004-1053-8

OIV, (2005). *Practical Guide for the Validation, Quality Control, and Uncertainty Assessment of an Alternative Oenological Analysis Method*. Int. Off. Wine OIV. Available online at: http://www.oiv.int/

Rizzotti, L., Levav, N., Fracchetti, F., Felis, G. E., and Torriani, S. (2015). Effect of UV-C treatment on the microbial population of white and red wines, as revealed by conventional plating and PMA-qPCR methods. *Food Control* 47, 407–412. doi: 10.1016/j.foodcont.2014.07.052

Rossen, L., Nørskov, P., Holmstrøm, K., and Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* 17, 37–45. doi: 10.1016/0168-1605(92)90017-W

Ruiz, A., Poblet, M., Mas, A., and Guillamón, J. M. (2000). Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Int. J. Syst. Evol. Microbiol.* 50, 1981–1987. doi: 10.1099/00207713-50-6-1981

Shi, H., Xu, W., Trinh, Q., Luo, Y., Liang, Z., Li, Y., et al. (2012). Establishment of a viable cell detection system for microorganisms in wine based on ethidium monoazide and quantitative PCR. *Food Control* 27, 81–86. doi: 10.1016/j.foodcont.2012.02.035

Sievers, M., Sellmer, S., and Teuber, M. (1992). *Acetobacter europaeus* sp. nov. a main component of industrial vinegar fermenters in central Europe. *Syst. Appl. Microbiol.* 15, 386–392. doi: 10.1016/S0723-2020(11)80212-2

Sokollek, S. J., Hertel, C., and Hammes, W. P. (1998). Cultivation and preservation of vinegar bacteria. *J. Biotechnol.* 60, 195–206. doi: 10.1016/S0168-1656(98)00014-5

Stoeckel, D. M., Stelzer, E. A., and Dick, L. K. (2009). Evaluation of two spike-and-recovery controls for assessment of extraction efficiency in microbial source tracking studies. *Water Res.* 43, 4820–4827. doi: 10.1016/j.watres.2009.06.028

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Tessonnière, H., Vidal, S., Barnavon, L., Alexandre, H., and Remize, F. (2009). Design and performance testing of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. *Int. J. Food Microbiol.* 129, 237–243. doi: 10.1016/j.ijfoodmicro.2008.11.027

Torija, M. J., Mateo, E., Guillamón, J. M., and Mas, A. (2010). Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan–MGB probes. *Food Microbiol.* 27, 257–265. doi: 10.1016/j.fm.2009.10.001

Trcek, J. (2005). Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S–23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. *Syst. Appl. Microbiol.* 28, 735–745. doi: 10.1016/j.syapm.2005.05.001

Valera, M. J., Torija, M. J., Mas, A., and Mateo, E. (2013). *Acetobacter malorum* and *Acetobacter cerevisiae* identification and quantification by Real-Time PCR with TaqMan-MGB probes. *Food Microbiol.* 36, 30–39. doi: 10.1016/j.fm.2013.03.008

Valera, M. J., Torija, M. J., Mas, A., and Mateo, E. (2015). Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. *Food Microbiol.* 46, 452–462. doi: 10.1016/j.fm.2014.09.006

van Doorn, R., Klerks, M. M., Gent-Pelzer, M. P. E., van, Speksnijder, A. G. C. L., Kowalchuk, G. A., and Schoen, C. D. (2009). Accurate quantification of microorganisms in PCR-inhibiting environmental DNA extracts by a novel internal amplification control approach using biotrove openarrays. *Appl. Environ. Microbiol.* 75, 7253–7260. doi: 10.1128/AEM.00796-09

Vendrame, M., Iacumin, L., Manzano, M., and Comi, G. (2013). Use of propidium monoazide for the enumeration of viable *Oenococcus oeni* in must and wine by quantitative PCR. *Food Microbiol.* 35, 49–57. doi: 10.1016/j.fm.2013.02.007

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63, 3741.

Yamada, Y., Hoshino, K., and Ishikawa, T. (1997). The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. *Biosci. Biotechnol. Biochem.* 61, 1244–1251. doi: 10.1271/bbb.61.1244

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 May 2016
doi: 10.3389/fmicb.2016.00787

Vibrio parahaemolyticus Strains of Pandemic Serotypes Identified from Clinical and Environmental Samples from Jiangsu, China

Jingjiao Li[1,2], Feng Xue[1], Zhenquan Yang[3], Xiaoping Zhang[4], Dexin Zeng[1], Guoxiang Chao[5], Yuan Jiang[1]* and Baoguang Li[6]*

[1] Animal Quarantine Laboratory, Jiangsu Entry-Exit Inspection and Quarantine Bureau, Nanjing, China, [2] Key Laboratory of Veterinary Biotechnology, Department of Animal Science, School of Agriculture and Biology, Shanghai JiaoTong University, Shanghai, China, [3] Jiangsu Key Laboratory of Zoonosis, School of Food Science and Engineering, Yangzhou University, Yanghzou, China, [4] Beijing Kemufeng Biopharmaceutical Company, Beijing, China, [5] Yangzhou Key Centre for Disease Control and Prevention, Yanghzou, China, [6] Division of Molecular Biology, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, Laurel, MD, USA

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università Degli Studi di Teramo, Italy

Reviewed by:
Adrian Canizalez-Roman, Autonomous University of Sinaloa, Mexico
Learn-Han Lee, Monash University Malaysia, Malaysia

***Correspondence:**
Feng Xue
fengxue1219@aliyun.com;
Yuan Jiang
jiango@yeah.net

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

Received: *15 April 2016*
Accepted: *09 May 2016*
Published: *31 May 2016*

Citation:
Li J, Xue F, Yang Z, Zhang X, Zeng D, Chao G, Jiang Y and Li B (2016) Vibrio parahaemolyticus Strains of Pandemic Serotypes Identified from Clinical and Environmental Samples from Jiangsu, China. Front. Microbiol. 7:787. doi: 10.3389/fmicb.2016.00787

Vibrio parahaemolyticus has emerged as a major foodborne pathogen in China, Japan, Thailand, and other Asian countries. In this study, 72 strains of *V. parahaemolyticus* were isolated from clinical and environmental samples between 2006 and 2014 in Jiangsu, China. The serotypes and six virulence genes including thermostable direct hemolysin (TDR) and TDR-related hemolysin (TRH) genes were assessed among the isolates. Twenty five serotypes were identified and O3:K6 was one of the dominant serotypes. The genetic diversity was assessed by multilocus sequence typing (MLST) analysis, and 48 sequence types (STs) were found, suggesting this *V. parahaemolyticus* group is widely dispersed and undergoing rapid evolution. A total of 25 strains of pandemic serotypes such as O3:K6, O5:K17, and O1:KUT were identified. It is worth noting that the pandemic serotypes were not exclusively identified from clinical samples, rather, nine strains were also isolated from environmental samples; and some of these strains harbored several virulence genes, which may render those strains pathogenicity potential. Therefore, the emergence of these "environmental" pandemic *V. parahaemolyticus* strains may poses a new threat to the public health in China. Furthermore, six novel serotypes and 34 novel STs were identified among the 72 isolates, indicating that *V. parahaemolyticus* were widely distributed and fast evolving in the environment in Jiangsu, China. The findings of this study provide new insight into the phylogenic relationship between *V. parahaemolyticus* strains of pandemic serotypes from clinical and environmental sources and enhance the MLST database; and our proposed possible O- and K- antigen evolving paths of *V. parahaemolyticus* may help understand how the serotypes of this dispersed bacterial population evolve.

Keywords: *Vibrio parahaemolyticus*, serotyping, MLST, virulence genes, TDH-related hemolysin (TRH), pandemic serotypes, epidemiology, phylogeny

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that inhabits global coastal waters and rivers, and in seafood, such as fish and shellfish (Kelly and Stroh, 1988). *V. parahaemolyticus* was initially discovered in 1950 (Parveen et al., 2008; Letchumanan et al., 2015b). A novel serotype of O3:K6 clone was discovered in India in 1996 (Okuda et al., 1997), and since then, this clone and its serovariants have disseminated worldwide and become a pandemic clonal group (Ansaruzzaman et al., 2005; Quilici et al., 2005; Nair et al., 2007; Chao et al., 2009; Velazquez-Roman et al., 2013; Li W. et al., 2014; Flores-Primo et al., 2015).

V. parahaemolyticus can cause three major clinical syndromes: gastroenteritis, wound infections, and septicemia (Daniels et al., 2000), and is also considered as the causative agent of the most prevalent food poisoning in Asia since the outbreak in 1959 (Miyamoto et al., 1962). *V. parahaemolyticus* infections usually resulted from consumption of raw or undercooked seafood, mostly causing gastroenteritis (Miyamoto et al., 1969). Recently, *V. parahaemolyticus* has been identified as a major foodborne pathogen in food poisoning incidents in China, raising public health concern (Ma et al., 2014; Zhang et al., 2015).

V. parahaemolyticus was initially discovered in 1950 (Parveen et al., 2008). Traditionally, the identification of *V. parahaemolyticus* is performed by serological and biochemical tests. *V. parahaemolyticus* is classified by serotyping and the serotypes of *V. parahaemolyticus* are determined by the combination of somatic (O) antigens and capsular (K) antigens. There are 13 O serotypes and 71 K serotypes that have been reported (Iida et al., 1997; Nair et al., 2007; Chen et al., 2012). *V. parahaemolyticus* infections are associated with pathogenic strains of numerous serotypes (clinical); whereas non-pathogenic strains comprise an even greater variety of serotypes.

Serotypes are useful distinguishing features to identify clinical isolates (Jones et al., 2012), and serotyping has been widely used in epidemiological research and etiological diagnostics for many decades. However, the increasing genetic diversity such as emerging of new serotypes and STs among *V. parahaemolyticus* strains poses a challenge to this traditional way of strain identification and differentiation due to the high cost of the antisera and the potential ambiguity encountered during serotyping (Bogdanovich et al., 2003). In the last two decades, numerous DNA-based subtyping methods such as pulsed-field gel electrophoresis (PFGE; Wong et al., 1996), multilocus sequence typing (MLST; Gonzalez-Escalona et al., 2008), repetitive element PCR (Rep PCR; Wong and Lin, 2001), multilocus variable-number tandem-repeat analysis (MLVA; Kimura et al., 2008), clustered regularly interspaced short palindromic repeats (CRISPR; Sun et al., 2015), and microarray analysis (Li et al., 2015) have been developed to investigate the genetic diversity of outbreaks caused by *V. parahaemolyticus* and other foodborne pathogens (Li B. et al., 2014). Each of these subtyping methods has its advantages and disadvantages with respect to sensitivity, specificity, cost, and speed (Li et al., 2015). In general, MLST is the most commonly used method (Maiden, 2006; Nair et al., 2007; Gonzalez-Escalona et al., 2008), due to its high specificity, repeatability, and portability (Nair et al., 2007).

In this study, in an effort to assess the homogeneity and heterogeneity between the clinical and environmental *V. parahaemolyticus* isolates from Jiangsu Provence, an east coast area in China, where seafood is widely consumed, we used the traditional classification method, serotyping, to identify 72 *V. parahaemolyticus* strains from the food poisoning case samples and the environmental samples. The genetic diversity among *V. parahaemolyticus* strains were further assessed by MLST analysis and the presence of the virulence factors such as *tdh*, *trh*, *orf8, GS-PCR, PGS-PCR,* and *HU-α*. Furthermore, we analyzed the *V. parahaemolyticus* isolates by using eBURST and START (http://eburst.mlst.net) to investigate the relationship between clinical and environmental *V. parahaemolyticus* strains based on MLST databases. Additional information on genetic variation and the distribution of virulence genes among *V. parahaemolyticus* strains from various cities in Jiangsu Province would enrich the MLST database and epidemiological archive and be beneficial for the development of an efficient risk assessment of this common foodborne pathogen.

MATERIALS AND METHODS

V. parahaemolyticus Strain Identification

Seventy-two presumed *V. parahaemolyticus* isolates were collected from nine different cities in Jiangsu province of China between 2006 and 2014, including 21 clinical isolates from patients with food poisoning and 51 isolates from food samples. All strains were characterized according to GB 4789.7–2013 Chinese Food Safety Standards (http://www.foodmate.net). The *V. parahaemolyticus* strains were inoculated onto Vibrio culture plates (CHROMagar, Paris, France) and Thiosulphate Citrate Bile salt Sucrose [(TCBS) Beijing Land Bridge, China] culture plates and incubated at 37°C for 16–24 h. The colonies with typical contour were selected and characterized by VITEK automatic biochemical analyzer (Biomerieux, France).

Serotyping

The serotype of *V. parahaemolyticus* strains was determined using two diagnostic kits; 11 antisera targeting the O1–O11 antigens and 71 antisera for the K antigens (Denka Seiken, Tokyo, Japan) and 11 antisera for O1–O13 antigens (Tianjin Biochip Corporation, Tianjin, China). Serotyping was carried out in accordance with the GB4789.7–2013 Chinese Food Safety Standard (http://www.foodmate.net). Single colonies were selected and plated onto the surface of 3% sodium chloride peptone agar plates, incubated at 37°C for 18 h. Bacterial suspension was obtained by washing the surface of agar with solution containing 3% NaCl and 5% glycerol.

O-antigen identification: The bacterial suspension was autoclaved at 121°C for 1.5 h followed by centrifugation at 12,000 g for 15 min. The pellets were washed two or three times with normal saline solution and centrifuged at 12,000 g for 15 min. The final suspension was used for O antiserum agglutination and normal saline solution was used as a negative control. If the result

was negative, all the above steps were repeated; thereafter, the negative was considered as unknown antigen O.

K-antigen identification: Multi-serum against the K-antigen was added to the bacterial suspension. Positive colonies were selected for further analysis using individual K-antigen antiserum. Solution of NaCl (3%) was used as negative control.

Identification of the *tdh, trh, orf8, GS-PCR, PGS-PCR,* and *HU-α* Genes

Single colonies of *V. parahaemolyticus* strains were picked and inoculated into liquid culture medium containing 3% sodium chloride peptone. Cultures were incubated at 37°C for 16 h, followed by centrifugation at 12,000 g for 10 min. The genomic DNA was isolated using E.Z.N.A.TM kit (OMEGA, Beijing, China), and the concentrations of DNA were determined by spectrophotometry to ensure the OD260/OD280 was between 1.8 and 2.0.

The primers for the *tdh*, *trh, orf8, GS-PCR, PGS-PCR,* and *HU-α* genes were synthesized (Chao et al., 2009; Li W. et al., 2014; **Table 1**) by Sango Biotech Co., Ltd. (Shanghai, China). PCR reactions were performed in a volume of 25 μl containing 1 μl DNA (50 ng/μl), 12.5 μl 10 × PCR mix (Takara, Dalian, China), 1 μl of the forward and reverse primers of the virulence genes, and 9.5 μl of sterile distilled water. Strains ATCC33847 (tdh^+trh^-, isolated in US in 1973) and ATCC17802 (tdh^-trh^+, isolated in Japan in 1965) were used as positive controls. PCR reactions were performed under the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 1 min, and ending with elongation at 72°C for 5 min. PCR products (1 μl) were analyzed using Agilient 2100 analyzer (Waldbronn, Germany) to determine the PCR amplicon size.

TABLE 1 | Primers used for the detection of *V. parahaemolyticus* virulence genes and pandemic marker genes by PCR.

Gene	Sequence (5′—3′)	Amplicon (*bp*)	References
tdh-F[a]	ATATCCATGTTGGC TGCATTC	531	Chao et al., 2009
tdh-R[b]	TTATTGTTGATGTTTA CATTCAAAA		
trh-F	ATGAAACTAAAACT CTACTTTGC	553	Chao et al., 2009
trh-R	TTAATTTTGTGACATAC ATTCAT		
orf8-F	GTTCGCATACAGTTGAGG	700	Nasu et al., 2000
orf8-R	AAGTACACAGGAGTGAG		
GS-PCR-F	TAATGAGGTAGAAACA	651	Matsumoto et al., 2000
GS-PCR-R	ACGTAACGGGCCTACA		
PGS-PCR-F	TTCGTTTCGCGCC ACAACT	235	Okura et al., 2004
PGS-PCR-R	TGCGGTGATTATTCGCGTCT		
HU-α-F	CGATAACCTATGAGA AGGGAAACC	474	Williams et al., 2004
HU-α-R	CTAGAAGGAAGAATTGATT GTCAAATAATG		

[a] *Forward primer.*
[b] *Reverse primer.*

MLST

Seven housekeeping genes, *dnaE, gyrB, recA, dtdS, pntA, pyrC,* and *tnaA,* were selected as target genes in this study. The primers for these genes were adopted from MLST website (http://www.pubmlst.net). The MLST primers for *recA* gene failed in most strains in the current study, and new primers for *recA* gene were adopted from a previous study (Gonzalez-Escalona et al., 2008). All primers as shown in **Table 2** were synthesized by Sango Biotech Co., Ltd. (China). Primers were diluted to 10 μM and stored at −20°C.

PCR Amplification of Housekeeping Genes

To sequence the housekeeping genes, the PCR reaction volume was set at 50 μl. The components of PCR reactions as well as the parameters for each cycle were optimized for best performance. PCR products were visualized using Agilent electrophoresis and imaging system. The bidirectional DNA sequencing was conducted by Sango Biotech Co., Ltd.

Allele and Sequence Analysis

The alignment of DNA sequences was performed by uploading each of the sequences of the seven housekeeping genes of the 72 *V. parahaemolyticus* strains to the MLST website (http://pubmlst.org/vparahaemolyticus/). If a novel allele or sequence was identified, the forward and reverse sequences were uploaded and submitted to the database administrator to obtain a serial number for the allele or sequence.

Sequence Analysis by eBURST v3.0 and START v2.0

The ST types of all the 72 strains were analyzed by eBURST v3.0 (http://eburst.mlst.net) to distinguish clonal complex (CC), group, and singleton STs. The most stringent definition was adopted to identify the homeotic complexes, each of which was defined by the presence of at least six or seven identical alleles. The single locus variant (SLV) was defined by the presence of a single allele difference between any two ST types, based on eBURST v3.0 analysis. The evolution of each allele and ST type was analyzed by using START v2.0 (http://pubmlst.org/software/analysis/start/).

RESULTS

V. parahaemolyticus colonies were round, translucent and purplish red on CHROMagar plates measuring 2–3 mm in diameter. They were round, translucent, and smooth green-colored colonies on TCBS plates. All 72 *V. parahaemolyticus* isolates were confirmed by VITEK biochemical analysis.

Serotyping

There were 25 serotypes identified among the 72 *V. parahaemolyticus* isolates. The dominant serotypes were

TABLE 2 | Primers of housekeeping genes used in MLST and PCR conditions in this study.

Locus	Primer sequence (5′ — 3′)	Annealing (°C)	Extension (S)	Length (bp)
dnaE	(F)tgtaaaacgacggccagtCGRATMAC CGCTTTCGCCG	58	60	596
	(R)caggaaacagctatgaccGAKATGT GTGAGCTGTTTGC			
gyrB	(F)tgtaaaacgacggccagtGAAGGBGG TATTCAAGC	58	60	629
	(R)caggaaacagctatgaccGAGTCACC CTCCACWATGTA			
dtdS	(F)tgtaaaacgacggccagtTGGCCATA ACGACATTCTGA	58	60	497
	(R)caggaaacagctatgaccGAGCACCA ACGTGTTTAGC			
pntA	(F)tgtaaaacgacggccagtACGGCTAC GCAAAAGAAATG	58	60	470
	(R)caggaaacagctatgaccTTGAGGCT GAGCCGATACTT			
pyrC	(F)tgtaaaacgacggccagtAGCAACCG GTAAAATTGTCG	58	60	533
	(R)caggaaacagctatgaccCAGTGTAA GAACCGGCACAA			
tnaA	(F)tgtaaaacgacggccagtTGTACGAA ATTGCCACCAAA	58	60	463
	(R)caggaaacagctatgaccAATATTT TCGCCGCATCAAC			
recA	(F) GCTTCTGGTTGAGCTGGAGA	55	60	998
	(R) GACGAGAACAAACAGAAAGCG			

The underlined lower-cased letters represent common sequences that were adapted from M13 for more efficient sequencing.

O3:K6 ($n = 8$) and O2:K28 ($n = 8$), followed by O1:KUT ($n = 6$), O5:K17 ($n = 5$), O5:KUT ($n = 5$), O1:K25 ($n = 3$), O4:K34 ($n = 3$), O10:KUT ($n = 3$), O2: KUT ($n = 3$), O1:K32 ($n = 3$). For the 21 clinical strains, nine serotypes were identifies where O3:K6 was the dominant serotype ($n = 8$), followed by O5:K17 ($n = 5$), O2:KUT ($n = 3$), and one strain of O1:K25, O11:K40, and O13:KUT (novel serotype). In addition, there was one strain (WX14115) that failed to react to either O or K antiserum (**Table 3**).

Of the 25 serotypes, six were novel serotypes. Out of the six novel serotypes, five new serotypes were recovered from environmental samples and one was from clinical sample. Specifically, the five environmental strains are O2:K25, O4:K9, O4:K42, O8:K39, and O11:31, and the single clinical isolate is O13:KUT (**Table 4**). In the present study, 25 strains of pandemic serotypes were identified from Jiangsu Province, including 16 clinical strains and 9 environmental strains. Specifically, the clinical strains were serotypes O3:K6 ($n = 8$), O5:K17 ($n = 5$), and environmental strains were serotypes O1:KUT ($n = 6$), O1:K25 ($n = 3$), O3:K29 ($n = 1$), and O3:KUT ($n = 1$) (**Table 3**).

Identification of Virulence Factor Genes *tdh, trh, GS-PCR, PGS-PCR, orf8,* and *HU-α*

The virulence genes in the 72 *V. parahaemolyticus* isolates were assessed by PCR. There were nine tdh^+ strains and four trh^+ strains, accounting for 61.9% (13/21) of the clinical strains. No strain was tdh^+trh^+. The nine tdh^+ strains included seven O3:K6, one O1:K25 strain and one O4:KUT strain. Four trh^+ strains included three O5:K20 and one O13:KUT (**Table 3**). The prevalence of the other four virulence genes, *GS-PCR, PGS-PCR, orf8, and HU-α*, are 81.9, 38.9, 16.9, and 18.9%, respectively. *GS-PCR* gene showed the highest prevalence and *orf8* gene showed the lowest prevalence for the four virulence genes in our collection (**Table 3**).

MLST Analysis

All seven housekeeping genes were amplified in the *V. parahaemolyticus* strains using specific primers, and the PCR products were sequenced. **Seventy-two** *V. parahaemolyticus* strains were classified into 48 STs by MLST analysis. Of the 48 ST types, 34 were singleton and 14 were ST groups. Each singleton represented only one strain, while each ST group included two to eight strains (**Figure 1**). Twenty-one clinical strains were classified into 13 STs. The dominant ST was ST-3 with eight strains, followed by ST-79 and ST-564, each of which covered three strains. The remainder of STs was singletons. The MLST results indicated that *V. parahaemolyticus* strains showed genetic polymorphisms with much higher incidence rate in environmental strains than in clinical strains.

Novel Allele and STs

The ST composition of the 72 *V. parahaemolyticus* strains included 32 novel STs with 32 new allele values and 34 allele spectra, accounting for 79% of total STs. All of these newly identified allele values and allele spectra were submitted to the PubMLST database (http://pubmlst.org/vparahaemolyticus/) as shown in **Table 3**. There were 13 STs among the 21 clinical strains, including seven newly identified STs accounting 53.85% (7/13) of the ST types. There were 36 STs identified among the 51 environmental strains, including 27 newly identified STs, accounting 75% (27/36) of the STs. It appears that 13 new STs were formed through allele recombination, while the other 21 new STs were created by the newly identified housekeeping gene alleles which included several types of *dnaE* ($N = 4$), *gyrB* ($N = 6$), *recA* ($n = 7$), *dtdS* ($n = 2$), *pntA* ($n = 3$), *pyrC* ($n = 9$) genes, and one *tnaA* gene.

Homologous Allele Complex and Systematic Development Analysis

The system evolution diagram was plotted by eBURST v3 software, and 48 STs were divided into one clonal complex (CC), four double combinations (D), and 38 singletons (S). The CC identified in this study was the CC3 which covered seven strains of O3:K3 ST-3 and one strain of O3:KUT ST-3; these eight strains were epidemic strains from clinical samples. The D type included ST-799-ST-1108, ST-212-ST-1002, ST-996-ST-999, and ST-993-ST-968. The ST-1108, ST-1002, ST-996, ST-999, and ST-993 were newly identified in this study. Of the 38 singletons, 28 novel STs were identified in this study, which were genetically distant to the ST-3 and other STs (**Table 3**; **Figure 1**).

TABLE 3 | Serotypes, sequence types, allele profiles, and presence of virulence genes of the 72 *V. parahaemolyticus* strains.

Strain	*dnaE*	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*	ST	O	K	*tdh*	*trh*	*GS-PCR*	*PGS-PCR*	*orf8*	*HU-α*
YZ0601	115	376	31	35	47	69	26	**968**[a]	2	28	−	−	+	+	+	+
YZ0602	81	212	75	84	63	26	47	**969**	8	39	−	−	+	+	−	−
YZ0603	28	4	82	88	63	187	1	**799**	5	UT	−	−	+	−	−	−
YZ0608	51	381	31	39	18	3	20	**988**	5	UT	−	−	+	+	+	+
YZ0612	28	4	245	88	63	187	1	**1108**	1	33	−	−	+	+	−	−
YZ0615	44	149	271	198	26	41	26	**989**	3	UT	−	−	+	+	−	+
YZ0618	104	252	272	29	26	306	23	**990**	10	UT	−	−	+	−	−	−
YZ0619	35	50	63	27	49	46	26	154	1	20	−	−	+	−	−	−
YZ0621	92	106	25	272	28	3	17	890	5	UT	−	−	+	−	−	−
YZ0625	51	29	77	13	60	8	33	356	1	33	−	−	+	−	−	−
YZ0626	131	147	60	136	90	27	23	276	1	UT	−	−	+	−	−	−
YZ0628	55	15	31	55	18	26	46	**991**	5	30	−	−	+	+	−	-
YZ0633	55	15	31	55	18	26	46	**991**	5	30	−	−	+	−	−	−
YZ0637	75	120	71	13	56	37	29	187	11	31	−	−	+	+	−	−
YZ0642	9	213	165	82	2	46	1	**992**	11	UT	−	−	+	+	+	+
YZ0646	275	376	31	35	47	69	26	**993**	2	28	−	−	−	−	−	−
YZ0647	60	104	210	126	28	226	159	**994**	4	34	−	−	+	+	−	−
YZ0650	30	276	75	171	61	73	57	**995**	2	28	−	−	+	+	−	−
YZ0652	270	371	273	120	23	238	26	**996**	5	UT	−	−	−	−	−	−
YZ0654	5	147	31	229	46	10	57	550	10	UT	−	−	+	+	−	−
YZ0656	35	50	63	27	49	46	26	154	1	20	−	−	+	−	−	−
YZ0658	175	43	274	253	194	190	9	997	2	28	−	−	+	+	−	−
YZ0659	167	2	109	293	28	307	105	**998**	2	25	−	−	+	+	−	−
YZ0663	270	371	275	120	23	238	26	**999**	5	UT	−	−	−	−	−	−
YZ0667	82	168	25	206	151	27	48	**1000**	2	28	−	−	+	−	−	−
YZ0668	175	43	274	253	194	190	9	**997**	2	28	−	−	+	−	−	−
YZ0673	69	92	69	114	54	71	24	212	1	UT	−	−	+	−	−	−
YZ0675	60	127	89	73	55	216	171	**1001**	1	32	−	−	+	−	−	−
YZ0676	69	92	69	267	54	71	24	**1002**	1	UT	−	−	−	−	−	−
YZ0684	60	217	31	18	106	150	26	**1003**	2	28	−	−	+	−	−	−
YZ0685	60	217	31	18	106	150	26	**1003**	2	28	−	−	+	+	−	−
YZ0686	60	104	210	126	28	226	159	**994**	4	34	−	−	+	−	−	−
YZ0688	60	217	31	18	106	150	26	**1003**	2	3	−	−	−	−	−	−
YZ0689	49	153	31	299	50	308	23	**1004**	10	UT	−	−	+	−	−	−
YZ0693	84	383	62	117	195	46	132	**1005**	11	UT	−	−	+	−	+	+
YZ0695	132	384	209	27	49	226	26	**1006**	1	25	−	−	−	−	−	−
YZ0696	69	92	69	114	54	71	24	212	1	32	−	−	+	−	−	−
YZ0697	44	89	31	73	46	309	86	**1007**	1	UT	−	−	+	−	−	−
HA08104	35	50	63	27	49	46	26	154	1	20	−	−	+	−	−	−
SH08108	131	147	60	136	90	27	23	276	1	25	−	−	+	−	−	−
WX08111	26	58	53	19	28	9	26	108	4	34	−	−	+	−	−	−
TC12100	31	115	22	12	4	91	68	1035	8	UT	−	−	+	−	−	−
CZ08101	270	371	273	120	23	238	26	996	5	UT	−	−	+	−	−	−
CS08103	49	153	31	299	50	308	23	**1004**	10	UT	−	−	+	−	−	−
YZ06115	84	383	62	117	195	46	132	**1005**	11	UT	−	−	+	−	−	−
KS08105	36	285	292	354	26	227	26	**1104**	4	9	−	−	+	−	−	−
RD08107	44	89	31	73	46	309	86	**1007**	1	32	−	−	+	−	−	−
SH08109	131	147	60	136	90	27	23	276	1	UT	−	−	−	−	−	−
TZ08110	283	82	31	355	53	45	13	**1105**	1	UT	−	−	+	+	−	−
YZ06114	35	352	151	47	26	325	1	**1106**	3	29	−	−	+	−	−	−
YZ06116	116	251	72	76	45	184	26	**1107**	4	42	−	−	−	+	−	−

(Continued)

TABLE 3 | Continued

Strain	*dnaE*	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*	ST	O	K	*tdh*	*trh*	*GS-PCR*	*PGS-PCR*	*orf8*	*HU-α*
WX1461	3	4	19	4	29	4	22	3	1	25	+	−	+	+	+	+
WX1465	3	4	19	4	29	4	22	3	3	6	+	−	+	+	+	+
WX1472	3	4	19	4	29	4	22	3	3	6	+	−	+	+	+	+
WX1475	80	252	160	179	26	10	23	**1109**	3	6	−	−	+	−	−	−
WX1477	3	4	19	4	29	4	22	3	3	6	+	−	+	+	+	+
WX1478	3	4	19	4	29	4	22	3	3	6	+	−	+	+	+	+
WX1483	14	30	141	78	4	37	13	332	4	UT	+	−	−	+	+	−
WX1486	3	4	19	4	29	4	22	3	3	6	+	−	+	+	−	+
WX1494	3	4	19	4	29	4	22	3	3	6	+	−	+	+	+	+
WX1498	31	366	264	339	26	45	24	**1110**	11	40	−	−	+	+	−	−
WX14102	173	406	73	47	4	116	227	**1111**	5	17	−	−	−	−	−	−
WX14103	284	343	293	191	23	326	132	**1112**	5	17	−	−	+	+	−	−
WX14105	35	43	38	21	31	35	37	79	5	17	−	+	+	−	−	−
WX14106	35	43	38	21	31	35	37	79	5	17	−	+	+	−	−	−
WX14107	34	4	216	151	201	327	33	**1113**	2	UT	−	−	−	+	−	−
WX14109	35	43	38	21	31	35	37	79	5	17	−	+	+	−	−	−
WX14113	110	407	70	76	78	328	148	**1114**	13	UT	−	+	−	−	−	−
WX14115	285	13	60	171	21	329	23	**1115**	UT	UT	−	−	+	+	+	+
WX14116	3	4	19	4	29	4	22	3	3	6	+	−	−	−	−	−
WX14118	31	106	135	74	37	212	54	564	2	UT	−	−	+	−	−	−
WX14119	31	106	135	74	37	212	54	564	2	UT	−	−	+	−	−	−

[a]*A bold-faced number refers a novel ST.*

Association of STs between Serotypes and Virulence Genes

The strains with identical serotype usually showed similar STs or were clustered together, such as the majority serotype of O3:K6 in the present study belonging to ST-3; only one O3:KUT strain shared the ST-3 with O3:K6 strains. On the other hand, some strains with identical serotypes have different STs, such as strains of O1:KUT. These were identified as ST-1007, ST-212, and ST-276 (**Figure 2**).

There were nine tdh^+ pathogenic strains identified in this study, including eight ST-3 strains (seven O3:K6 serotypes and one O1:K25 serotype), and one ST-332 strain (serotype O4:KUT). All of these 10 strains were epidemic strains from clinical samples. There were four trh^+ strains, including three ST-79 (O5:K17) strains and one ST-1114 (O13:KUT) strain. All the four trh^+ strains seemed to be epidemic strains (**Table 4**).

DISCUSSION

V. parahaemolyticus is the major foodborne pathogen. It is widely distributed with high survival and incidence rates, especially in the coastal areas of China (Su and Liu, 2007; Chao et al., 2009; Yan et al., 2015). Based on the epidemiological surveillance data from countries in Southeast Asia, *V. parahaemolyticus* infections have become a majority of foodborne pathogen (Pan et al., 1997; Wong et al., 1999; Obata et al., 2001; Liu et al., 2004; Cho et al., 2008; Letchumanan et al., 2014). In the present study, 25 serotypes and 48 STs were identified among the 72 *V. parahaemolyticus* isolates. Of the 25 serotypes and 48 STs, six additional novel serotypes and 34 novel STs were identified, indicating the *V. parahaemolyticus* population in Jiangsu Province was highly dispersed. The diversity in serotypes and STs of *V. parahaemolyticus*, especially environmental strains, is attributed to frequent recombination events in the pathogen but not by mutation (Gonzalez-Escalona et al., 2008). One example to support this is that the serotype of epidemic strains (O3:K6), which has been continuously evolving, resulted in 21 derivative serotypes of O3:K6 such as O4:K68, O1:K25, O1:KUT, O4:K12, and O5:K17 (Nair et al., 2007). All those strains demonstrated identical genotypes and molecular spectra, therefore the O3:K6 and its derivative are called O3K6 clones or pandemic strains. MLST analysis confirmed that O3:K6 and its derivatives belong to the same genetic lineage (Chowdhury et al., 2000, 2004; Matsumoto et al., 2000). Surprisingly, the 25 pandemic strains identified in this study were not exclusively from clinical samples ($n = 16$), rather, some strains were recovered from environmental samples ($n = 9$) as well.

Serotypes of O3:K6 and O5:K17 were the most common serotypes among the 21 clinical strains whereas some serotypes such as O11:K40, O4:K8, O2:KUT, and O13:KUT are rarely reported globally. It has been shown that serotype O1:KUT is closely related to pandemic strains and is seldom detected as environmental strains (Iida et al., 1997; Mahoney et al., 2010). However, in the present study, O1:KUT was identified as a common serotype with six isolates from environmental samples. More importantly, the six O1:KUT strains demonstrated extremely high genetic diversity with five different STs among

TABLE 4 | *V. parahaemolyticus* strains of pandemic serotypes and novel serotypes from clinical and environmental samples.

Strain	Sero- or sequence-type			Virulence factor						Source
	O	K	ST	*tdh*	*trh*	*GS-PCR*	*PGS-PCR*	*orf8*	*HU-α*	
PANDEMIC SEROTYPE										
YZ0626	1	UT[a]	276	−	−	+	−	−	−	Environmental
YZ0673	1	UT	212	−	−	+	−	−	−	Environmental
YZ0676	1	UT	**1002**[b]	−	−	−	−	−	−	Environmental
YZ0697	1	UT	**1007**	−	−	+	−	−	−	Environmental
SH08109	1	UT	276	−	−	−	−	−	−	Environmental
TZ08110	1	UT	**1105**	−	−	+	+	−	−	Environmental
YZ06114	3	29	**1106**	−	−	+	−	−	−	Environmental
YZ0603	5	UT	**799**	−	−	+	−	−	−	Environmental
YZ0608	5	UT	**988**	−	−	+	+	+	+	Environmental
WX1461	1	25	3	+	−	+	+	+	+	Clinical
WX1465	3	6	3	+	−	+	+	+	+	Clinical
WX1472	3	6	3	+	−	+	+	+	+	Clinical
WX1477	3	6	3	+	−	+	+	+	+	Clinical
WX1478	3	6	3	+	−	+	+	+	+	Clinical
WX1486	3	6	3	+	−	+	+	−	+	Clinical
WX1494	3	6	3	+	−	+	+	+	+	Clinical
WX1475	3	6	**1109**	−	−	+	−	−	−	Clinical
WX1483	4	UT	332	+	−	−	+	+	−	Clinical
WX14102	5	17	**1111**	−	−	−	−	−	−	Clinical
WX14103	5	17	**1112**	−	−	+	+	−	−	Clinical
WX14105	5	17	79	−	+	+	−	−	−	Clinical
WX14106	5	17	79	−	+	+	−	−	−	Clinical
WX14109	5	17	79	−	+	+	−	−	−	Clinical
WX14113	**13**	**UT**	**1114**	−	+	−	−	−	−	Clinical
WX14115	UT	UT	**1115**	−	−	+	+	+	+	Clinical
NOVEL SEROTYPE										
YZ0659	**2**	**25**	**998**	−	−	+	+	−	−	Environmental
KS08105	**4**	**9**	**1104**	−	−	+	−	−	−	Environmental
YZ06116	**4**	**42**	**1107**		−	−	+	−	−	Environmental
YZ0602	**8**	**39**	**969**	−	−	+	+	−	−	Environmental
YZ0637	**11**	**31**	187	−	−	+	+	−	−	Environmental
WX14113	**13**	**UT**	**1114**	−	+	−	−	−	−	Clinical
WX14115	UT	UT	**1115**	−	−	+	+	+	+	Clinical

[a] *UT refers to untypable.*
[b] *Bold faced letters and number refer to a novel serotype or ST.*

the six strains (**Table 4**). Furthermore, of the five STs possessed by O1:KUT strains, three were novel STs, namely ST-1002, ST-1007, and ST-1105, suggesting these O1:KUT strains were highly dispersed and evolving rapidly in the environment. In addition, some of the O3:K6-specific pandemic markers such as *GS-PCR* and *PGS-PCR* were tested positive among most of these strains of pandemic serotypes. However, the pathogenicity of those strains needs to be further confirmed by recovering those strains from patients. Nevertheless, the risk posed to the public health in China by these "environmental" pandemic strains should not be overlooked.

The exact pathogenic mechanism of *V. parahaemolyticus* remains unclear, but the *tdh* and *trh* genes are considered the main pathogenic factors. Thermostable direct hemolysin (TDH), encoded by the *tdh* gene, manifests hemolytic, intestinal, and cardiac toxicities (Iida et al., 1997; Rosec et al., 2009; Raghunath, 2014). The *trh* gene is closely associated with the production of urease (Quilici et al., 2005). An epidemiological surveillance of *V. parahaemolyticus* in Northwest Mexico showed that up to 71.74% of the environmental isolates carried the *tdh* gene (De Jesus Hernandez-Diaz et al., 2015). However, numerous reports have shown that many pathogenic strains from patients were detected with neither of the two virulence factor genes, indicating more virulence factor genes are needed as markers for identification of pandemic *V. parahaemolyticus* strains (Garcia et al., 2009; Jones et al., 2012; Liu and Chen, 2015). Additionally, the type

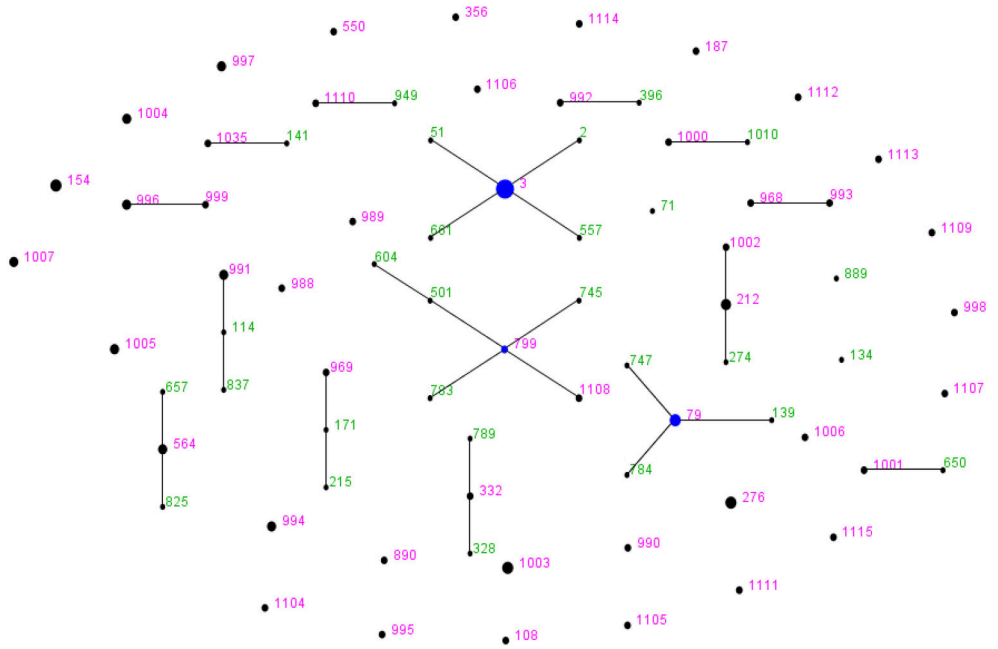


FIGURE 1 | *V. parahaemolyticus* "population snapshot" of the 72 strains in this study created by using eBURST v3. The 15 groups were defined using stringent criteria (5/7 shared alleles). The founder is labeled in blue, the number in green refers to the STs that have been existed in the query dataset; the number in pink represents the STs are among the reference and query dataset. The STs with SLVs to each other were shown connected by black lines. The sizes of the circles are relative to the numbers of strains in the ST.

III secretion system (T3SS) of *V. parahaemolyticus* has been identified as a potential strain virulence factor (Park et al., 2004; Broberg et al., 2011).

V. parahaemolyticus O3:K6 serogroup has group-specific gene sequences in the *toxRs* operon and *orf8*, one of the 10 known open reading frames (ORFs) which is unique to the O3:K6 filamentous phage f237. The *toxRs* and *orf8* genes have been used as genetic markers to differentiate O3:K6 from other serogroups (Matsumoto et al., 2000; Nasu et al., 2000). Additionally, the *HU-α*ORF, a specific biomarker for pandemic strain, which has a C-terminal amino acid sequence different from those of other strains of *V. parahaemolyticus,* was used to identify O3:K6 and other serotypes, such as O1:K25, O1:KUT, and O4:K68 (Matsumoto et al., 2000; Williams et al., 2004).

It has been reported that *V. parahaemolyticus* that lacked the *tdh* and *trh* genes were pathogenic in a study using mice (Rosec et al., 2009). In this study, most of the clinical strains (13/21) were negative for the *tdh* and *trh* genes, but possessed at least one of the four other virulence genes, *GS-PCR, PGS-PCR, orf8,* and *HU-α*. For instance, two $tdh^{-}trh^{-}$ strains, OUT:KUT (WX14115, clinical) and O5:KUT (YZ0608, environmental) were positive for the other four virulence genes (*GS-PCR, PGS-PCR, orf8,* and *HU-α*). Using a panel of six virulence genes as pathogenic markers, almost all the O3:K6 strains (except for two strains, WX1475 and WX14116) were positive for at least four of the six virulence genes, and only one clinical strain of O5:K17 (WX14102) was negative for all the six virulence genes among the 21 clinical strains. Thus, the results of this panel of virulence genes may more closely reflect the pathogenicity potential of those strains.

A high percentage (90.3%, 65/72) of strains were positive for at least one of the six virulence genes, *tdh*, *trh, GS-PCR, PGS-PCR, orf8,* and *HU-α*. Out of the six virulence genes, the *GS-PCR* gene showed the highest prevalence 81.9% (59/72). This result is very different from other investigators in China, where GS-PCR gene was seldom detected from environmental samples (Alam et al., 2009; Chao et al., 2009; Zhang et al., 2013). This difference might be a reflection of the genetic diversity between the indigenous isolates in this area (Jiangsu, China) and other areas or an artifact caused by sampling difference.

The *GS-PCR* gene has been shown to be a specific for genetic marker for the identification of pandemic *V. parahaemolyticus* strains (Li W. et al., 2014; Pazhani et al., 2014). The *trh* gene showed the lowest prevalence 5.6% (4/72), which is consistent with the results from other Asia countries (Alam et al., 2009; Chao et al., 2009; Zhang et al., 2013, 2015; Letchumanan et al., 2015a). Of note is that a number of different serotypes of $tdh^{-}trh^{-}$ strains (such as O28:K28, O5:KUT, and O11:KUT) were positive for at least three of the four virulence genes, *GS-PCR, PGS-PCR, orf8,* and *HU-α* (**Table 3**). Although these strains were isolated from the environment, it is quite possible for a non-pathogenic strain to gain pathogenicity potential after acquiring several virulence genes from pathogenic strains as indicated by the evolving path of the pandemic strain O3:K6 (Nair et al., 2007).

In this study, there were 34 new STs out of the 48 ST types, and the newly defined gene spectra accounted for 70.83%, suggesting this *V. parahaemolyticus* group is highly dispersed. The cluster diagram of MLST analysis showed that

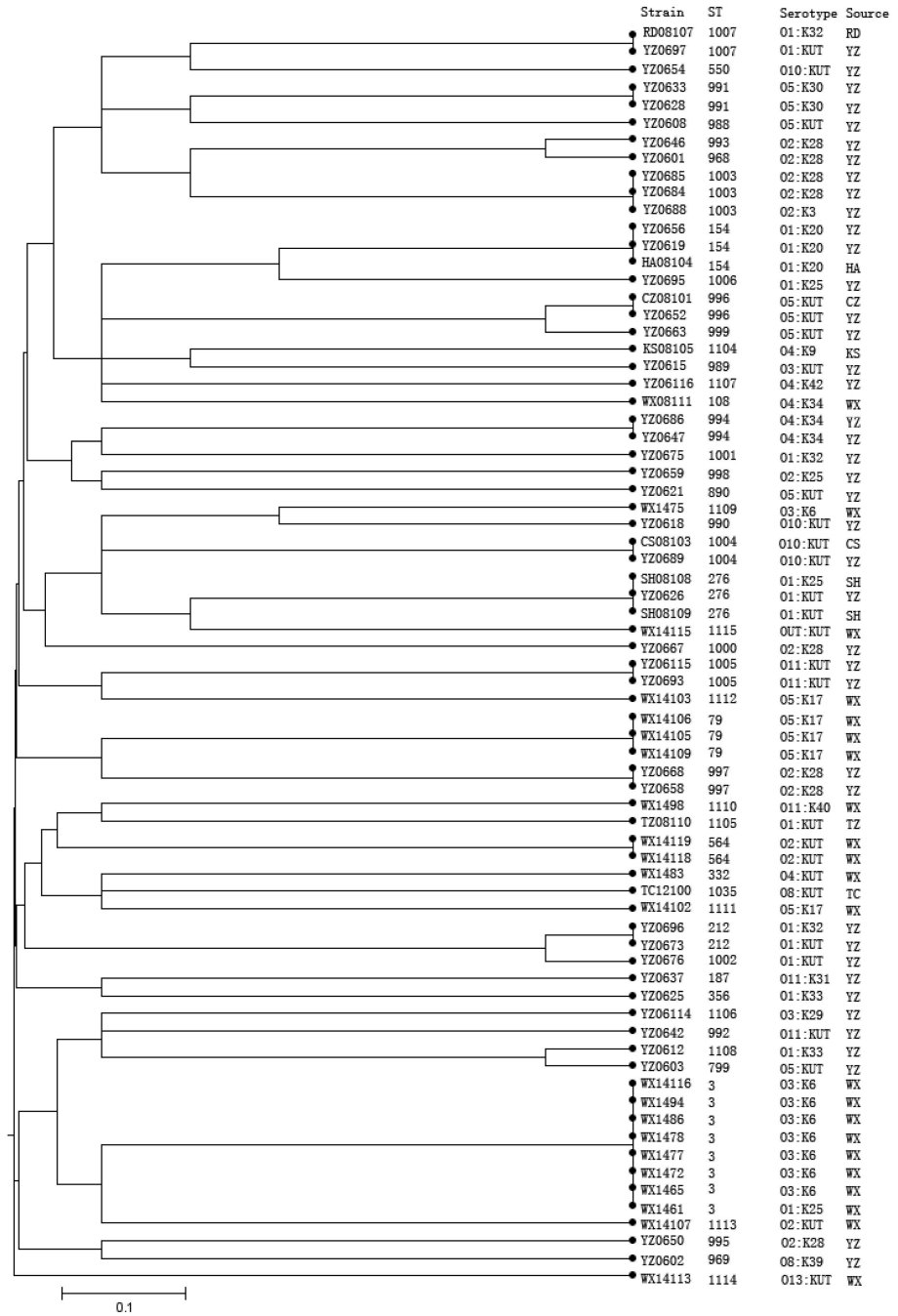


FIGURE 2 | Phylogenetic relationships of 72 *V. parahaemolyticus* strains based on the concatenated sequences of seven housekeeping genes. The dendrogram is based on the UPGMA generated from allelic profiles of *V. parahaemolyticus* strains and was performed on START (http://pubmlst.org/software/analysis/start/) written by Jolley et al. (2001). Bioinformatics, 17, 1230–1231 (Jolley et al., 2001).

V. parahaemolyticus strains in Jiangsu area demonstrated a higher level of polymorphisms within environmental strains than clinical strains. ST-3 was the dominant ST among the 21 clinical strains and all ST-3 strains ($n = 7$) belonged to pandemic O3:K6 serotype, which is in agreement with previous reports from China and other countries (Gonzalez-Escalona et al., 2008).

It is worth noting in this study that there were a number of cases in which strains with identical serotype were classified into different STs; while strains with an identical ST possessed different serotypes. For instance, several strains of O1:KUT serotype were subtyped as ST1007, ST212, and ST276 by MLST; and the same ST strains, such as ST-3 strains, included

TABLE 5 | Possible recombination scenerios of reprentive strains of *V. parahaemolyticus* that share a serotype or a ST[a].

Isolate	Allele profile							Typing			Virulence factor					
	dnaE	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*	ST	O	K	*tdh*	*trh*	*GS-PCR*	*PGS-PCR*	*orf8*	*HU-α*
YZ0658	175	43	274	253	194	190	9	997	**2**	**28**	-		+	+	-	-
YZ0668	175	43	274	253	194	190	9	997	**2**	**28**	-	-	-	+	-	-
YZ0650	30	276	75	171	61	73	57	995	**2**	**28**	-	-	+	+	-	-
YZ0667	82	168	25	206	151	27	48	1000	**2**	**28**	-	-	+	-	-	-
YZ0601	115[c]	376	31	35	47	69	26	968	**2**	**28**	-	-	+	+	+	+
YZ0646	275	376	31	35	47	69	26	993	**2**	**28**	-	-	-	-	-	-
YZ0684	60	217	31	18	106	150	26	1003	**2**	**28**	-	-	+	-	-	-
YZ0685	60	217	31	18	106	150	26	1003	2	**28**	-	-	+	+	-	-
YZ0688	60	217	31	18	106	150	26	1003	2	**3**	-	-	-	-	-	-
WX08111	26	58	53	19	28	9	26	108	**4**	**34**	-	-	+	-	-	-
YZ0686	60	104	210	126	28	226	159	994	4	34	-	-	+	-	-	-
YZ0647	60	104	210	126	28	226	159	994	**4**	**34**	-	-	+	+	-	-
WX14102	173	406	73	47	4	116	227	1111	**5**	**17**	-	-	-	-	-	-
WX14103	284	343	293	191	23	326	132	1112	**5**	**17**	-	-	+	+	-	-
WX14105	35	43	38	21	31	35	37	79	5	17	-	+	+	-	-	-
WX14106	35	43	38	21	31	35	37	79	**5**	**17**	-	+	+	-	-	-
WX14109	35	43	38	21	31	35	37	79	5	17	-	+	+	-	-	-
SH08109	131	147	60	136	90	27	23	276	1	**KUT**	-	-	-	-	-	-
YZ0626	131	147	60	136	90	27	23	276	1	**KUT**	-	-	+	-	-	-
SH08108	131	147	60	136	90	27	23	276	**1**	**25**	-	-	+	-	-	-
YZ0695	132	384	209	27	49	226	26	1006	**1**	**25**	-	-	-	-	-	-
WX1461	3	4	19	4	29	4	22	3	**1**	**25**	+	-	+	+	+	+
WX1465	3	4	19	4	29	4	22	3	3	6	+	-	+	+	+	+
WX1472	3	4	19	4	29	4	22	3	3	6	+	-	+	+	+	+
WX1477	3	4	19	4	29	4	22	3	3	6	+	-	+	+	+	+
WX1478	3	4	19	4	29	4	22	3	**3**	**6**	+	-	+	+	+	+
WX1494	3	4	19	4	29	4	22	3	3	**6**	+	-	+	+	+	+
WX1486	3	4	19	4	29	4	22	3	3	**6**	+	-	+	+	-	+
WX14116	3	4	19	4	29	4	22	3	3	**6**	+	-	-	-	-	-
WX1475	80	252	160	179	26	10	23	1109	**3**	**6**	-	-	+	-	-	-
HA08104	35	50	63	27	49	46	26	154	1	**20**	-		+	-	-	-
YZ0656	35	50	63	27	49	46	26	154	1	**20**	-		+	-	-	-
YZ0619	35	50	63	27	49	46	26	154	1	**20**	-		+	-	-	-
YZ0695	132	384	209	27	49	226	26	1006	1	**25**			-	-	-	-

[a] *ST, sequence type; O, O-antigen; K, K-antigen.*
[b] *An arrow refers to an O- and K-antigen or K antigen exchange between strains of different serotypes or STs.*
[c] *A blue-highlighted number indicates a new allelic gene.*

O3:K6 and O3:KUT serotypes, whereas ST-276 strains included O1K:25 and O1K:KUT serotypes (**Table 5**). This observation not only indicates the advantages and limitations of serotyping and MLST analysis, but also implies that frequent mutation and/or recombination occur on the O- and K-antigens on the chromosome.

In this study, we used serotyping and MLST methods to differentiate the *V. parahaemolyticus* isolates and assessed the presence of six virulence factors, *tdh*, *trh*, *orf8, GS-PCR, PGS-PCR,* and *HU-α*. A total of 25 serotypes and 48 STs were identified among the 72 *V. parahaemolyticus* isolates; six novel serotypes and 34 novel STs were identified; and highly variable profiles of six virulence factor genes were detected among the isolates, suggesting this *V. parahaemolyticus* group was a highly dispersed group and was evolving rapidly. This information not only can enrich the MLST database but also can serve as a valuable set of matrices to trace the gene mutation and HGT (or recombination) among the *V. parahaemolyticus* population.

Data from **Table 5** indicate that the O- and K-antigens move together more often than to move independently; and the K-antigen is more likely to be swapped than O-antigen. For example, the ST-3 strains included, in addition to the seven O3:K6 strains, a single strain of O1:K25 (WX1461). TheO1:K25 strain (belonged to ST-3) can serve as an example for O- and K-antigen exchange between serotypes O3:K6 and O1:K25, i.e., the O3:K6 antigens of strain WX1461 were replaced by O1:K25 antigens as evidenced by its identical allele and virulence gene profiles with seven O3:K6 (ST-3) strains. Another example is WX1475 which is serotype O3:K6 but was subtyped as ST-1109 by MLST based on different allele and virulent gene profiles than with those other O3:K6 serotype strains (**Table 5**, lower middle). The third example is that strains with different STs acquired the O2:K28 antigens from a strain with different ST (ST-1003; Top of **Table 5**). Specifically, a serotype O2:K28 isolate (YZ0864) was subtyped as ST-1003 with another serotype O2:K28 isolate (YZ0685).

In this study, there were eight strains with serotype O2:K28 but were differentiated into six different STs, based on the high discriminatory power of MLST (**Table 5**). Serotype O2:K28 was initially found in 1977 (Libinzon et al., 1977) and is generally considered as an environmental strain (Martinez-Urtaza et al., 2004). Drastic differences were found between the results derived from the two subtyping methods. It is hard to pinpoint the exact cause(s) that led to the differences in allele profiles among those isolates during their evolution path. However, a plausible scenario is that the O- and K-antigens of the six strains (O2:K28) of different STs might have been acquired from strains such as YZ0684 (O2:K28; ST-1003) over years (**Table 5**).

Similar virulence gene profiles exist between the O2:K28 strains of ST-1003 and the six different STs strains (lacking *tdh* and *trh* genes but are positive for *GS-PCR*) seem to corroborate this notion (**Table 5**). In addition to the genetic evidence generated in this study, our hypothesis is also supported by the findings on bacterial antigens, virulence genes, and genetic traits of *V. parahaemolyticus* (Chowdhury et al., 2000; Gonzalez-Escalona et al., 2008; Chao et al., 2009; Mala et al., 2016). As shown in **Table 5**, serotype conversion (from one serotype to a different serotype) occurred more frequently than ST change (from one ST to a different ST) among the listed strains, whose serotype was shared by strains of different STs or whose ST was shared by strains of different serotypes. It seemed that O- and K-antigen conversion occurred simultaneously more often than independently, suggesting that the O- and K-antigens are actively evolving and the two antigens are mostly moving together by horizontal gene transfer (HGT) (or recombination). This presumption is well in agreement with the recent findings on *V. parahaemolyticus* genomic evolution that HGT is 10–1000 times more attributable than single nucleotide variants to genome diversification. This may be the underlying drive that is responsible for the high diversity among the *V. parahaemolyticus* studied. This hypothesis is consistent with other researchers on the diversity and pathogenicity of *V. parahaemolyticus* (Chowdhury et al., 2000; Gonzalez-Escalona et al., 2008; Chao et al., 2009; Mala et al., 2016). Furthermore, other studies on K-antigen and comparative genomic analysis of *V. parahaemolyticus* (Chen et al., 2010, 2011), have demonstrated that the O- and K-antigens are at two adjacent loci on chromosome II and, thus the O- and K-antigens could be swapped via a single recombination event to create both novel O- and K-antigens (Chen et al., 2010). Moreover, the human upper intestine is believed to be a particularly suitable niche for the intra- and inter-specific lateral transfer of genetic material necessary to enhance bacterial pathogenicity (Larocque et al., 2005; Okada et al., 2009; Hasan et al., 2010; Wang et al., 2011). Therefore, our model is supported not only by multiple lines of genetic evidence from *V. parahaemolyticus*, but also by the presence of suitable ecological niche that can facilitate HGT. With this notion, we can better interpret the scenarios we encountered here, i.e., the pandemic *V. parahaemolyticus* strains recovered not only from patients, but also from nine "environmental" seafood samples; and the high genetic diversity among the 72 isolates.

In summary, *V. parahaemolyticus* in Jiangsu, China, were highly dispersed and widely distributed in the environment. In light of that *V. parahaemolyticus* has become one of the major foodborne pathogens in China in recent years (Li Y. et al., 2014; Qi et al., 2016), the new threat to the public health posed by these "environmental" pandemic strains should not be overlooked. T. The findings of this study provide new insight into the phylogenic relationship between *V. parahaemolyticus* strains of pandemic serotypes from clinical and environmental sources; the information on the genetic diversity among isolates enriches the MLST database; and our proposed possible O- and K- antigen evolving paths of *V. parahaemolyticus* may help understand how the serotypes of this dispersed bacterial population evolve. Our findings also underscores the necessity for more epidemiological studies and more comprehensive surveillances on *V. parahaemolyticus* in order to efficiently prevent the diseases caused by this organism.

AUTHOR CONTRIBUTIONS

JL performed the experiments. YJ, FX, and BL conceived and designed the study. XZ, DZ, JL, FX, and BL analyzed the data. ZY and GC provided the isolates. JL, YJ, FX, and BL wrote the manuscript. All the authors reviewed the manuscript.

ACKNOWLEDGMENTS

We would like to thank Yanhong Wan, Lu Gao and Haibo Zhou for their useful suggestions to this work. The study was supported by the National Natural Science Foundation of China (31301460) the National Science and Technology Support Program of 2012BAK17B10, National 10000 Talents—Youth Top-Notch Talent Support Program, and Jiangsu Province Science and Technology Support Program of BE2013733.

REFERENCES

Alam, M., Chowdhury, W. B., Bhuiyan, N. A., Islam, A., Hasan, N. A., Nair, G. B., et al. (2009). Serogroup, virulence, and genetic traits of *Vibrio parahaemolyticus* in the estuarine ecosystem of Bangladesh. *Appl. Environ. Microbiol.* 75, 6268–6274. doi: 10.1128/AEM.00266-09

Ansaruzzaman, M., Lucas, M., Deen, J. L., Bhuiyan, N. A., Wang, X. Y., Safa, A., et al. (2005). Pandemic serovars (O3:K6 and O4:K68) of *Vibrio parahaemolyticus* associated with diarrhea in Mozambique: spread of the pandemic into the African continent. *J. Clin. Microbiol.* 43, 2559–2562. doi: 10.1128/JCM.43.6.2559-2562.2005

Bogdanovich, T., Carniel, E., Fukushima, H., and Skurnik, M. (2003). Use of O-antigen gene cluster-specific PCRs for the identification and O-genotyping of *Yersinia pseudotuberculosis* and *Yersinia pestis*. *J. Clin. Microbiol.* 41, 5103–5112. doi: 10.1128/JCM.41.11.5103-5112.2003

Broberg, C. A., Calder, T. J., and Orth, K. (2011). *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. *Microbes Infect.* 13, 992–1001. doi: 10.1016/j.micinf.2011.06.013

Chao, G., Jiao, X., Zhou, X., Yang, Z., Huang, J., Pan, Z., et al. (2009). Serodiversity, pandemic O3:K6 clone, molecular typing, and antibiotic susceptibility of foodborne and clinical *Vibrio parahaemolyticus* isolates in Jiangsu, China. *Foodborne Pathog. Dis.* 6, 1021–1028. doi: 10.1089/fpd.2009.0295

Chen, M., Guo, D., Wong, H. C., Zhang, X., Liu, F., Chen, H., et al. (2012). Development of O-serogroup specific PCR assay for detection and identification of *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.* 159, 122–129. doi: 10.1016/j.ijfoodmicro.2012.08.012

Chen, Y., Dai, J., Morris, J. G. Jr., and Johnson, J. A. (2010). Genetic analysis of the capsule polysaccharide (K antigen) and exopolysaccharide genes in pandemic *Vibrio parahaemolyticus* O3:K6. *BMC Microbiol.* 10:274. doi: 10.1186/1471-2180-10-274

Chen, Y., Stine, O. C., Badger, J. H., Gil, A. I., Nair, G. B., Nishibuchi, M., et al. (2011). Comparative genomic analysis of *Vibrio parahaemolyticus*: serotype conversion and virulence. *BMC Genomics* 12:294. doi: 10.1186/1471-2164-12-294

Cho, S. H., Shin, H. H., Choi, Y. H., Park, M. S., and Lee, B. K. (2008). Enteric bacteria isolated from acute diarrheal patients in the Republic of Korea between the year 2004 and 2006. *J. Microbiol.* 46, 325–330. doi: 10.1007/s12275-008-0015-4

Chowdhury, N. R., Chakraborty, S., Ramamurthy, T., Nishibuchi, M., Yamasaki, S., Takeda, Y., et al. (2000). Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerg. Infect. Dis.* 6, 631–636. doi: 10.3201/eid0606.000612

Chowdhury, N. R., Stine, O. C., Morris, J. G., and Nair, G. B. (2004). Assessment of evolution of pandemic *Vibrio parahaemolyticus* by multilocus sequence typing. *J. Clin. Microbiol.* 42, 1280–1282. doi: 10.1128/JCM.42.3.1280-1282.2004

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., et al. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973–1998. *J. Infect. Dis.* 181, 1661–1666. doi: 10.1086/315459

De Jesus Hernandez-Diaz, L., Leon-Sicairos, N., Velazquez-Roman, J., Flores-Villasenor, H., Guadron-Llanos, A. M., Martinez-Garcia, J. J., et al. (2015). A pandemic *Vibrio parahaemolyticus* O3:K6 clone causing most associated diarrhea cases in the Pacific Northwest coast of Mexico. *Front. Microbiol.* 6:221. doi: 10.3389/fmicb.2015.00221

Flores-Primo, A., Pardio-Sedas, V. T., Lopez-Hernandez, K., Lizarraga-Partida, L., and Uscanga-Serrano, R. (2015). [Growth and survival of total and pathogenic *Vibrio parahaemolyticus* in American oyster (*Crassostrea virginica*) under cold storage]. *Salud Publica Mex.* 57, 211–218.

Garcia, K., Torres, R., Uribe, P., Hernandez, C., Rioseco, M. L., Romero, J., et al. (2009). Dynamics of clinical and environmental *Vibrio parahaemolyticus* strains during seafood-related summer diarrhea outbreaks in southern Chile. *Appl. Environ. Microbiol.* 75, 7482–7487. doi: 10.1128/AEM.01662-09

Gonzalez-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R. T., Jaykus, L. A., and Depaola, A. (2008). Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. *J. Bacteriol.* 190, 2831–2840. doi: 10.1128/JB.01808-07

Hasan, N. A., Grim, C. J., Haley, B. J., Chun, J., Alam, M., Taviani, E., et al. (2010). Comparative genomics of clinical and environmental *Vibrio mimicus*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21134–21139. doi: 10.1073/pnas.1013825107

Iida, T., Suthienkul, O., Park, K. S., Tang, G. Q., Yamamoto, R. K., Ishibashi, M., et al. (1997). Evidence for genetic linkage between the ure and trh genes in *Vibrio parahaemolyticus*. *J. Med. Microbiol.* 46, 639–645. doi: 10.1099/00222615-46-8-639

Jolley, K. A., Feil, E. J., Chan, M. S., and Maiden, M. C. (2001). Sequence type analysis and recombinational tests (START). *Bioinformatics* 17, 1230–1231. doi: 10.1093/bioinformatics/17.12.1230

Jones, J. L., Ludeke, C. H., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B., et al. (2012). Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *J. Clin. Microbiol.* 50, 2343–2352. doi: 10.1128/JCM.00196-12

Kelly, M. T., and Stroh, E. M. (1988). Occurrence of *Vibrionaceae* in natural and cultivated oyster populations in the Pacific Northwest. *Diagn. Microbiol. Infect. Dis.* 9, 1–5. doi: 10.1016/0732-8893(88)90054-5

Kimura, B., Sekine, Y., Takahashi, H., Tanaka, Y., Obata, H., Kai, A., et al. (2008). Multiple-locus variable-number of tandem-repeats analysis distinguishes *Vibrio parahaemolyticus* pandemic O3:K6 strains. *J. Microbiol. Methods* 72, 313–320. doi: 10.1016/j.mimet.2007.12.014

Larocque, R. C., Harris, J. B., Dziejman, M., Li, X., Khan, A. I., Faruque, A. S., et al. (2005). Transcriptional profiling of *Vibrio cholerae* recovered directly from patient specimens during early and late stages of human infection. *Infect. Immun.* 73, 4488–4493. doi: 10.1128/IAI.73.8.4488-4493.2005

Letchumanan, V., Chan, K. G., and Lee, L. H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front. Microbiol.* 5:705. doi: 10.3389/fmicb.2014.00705

Letchumanan, V., Pusparajah, P., Tan, L. T., Yin, W. F., Lee, L. H., and Chan, K. G. (2015a). Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* from Shellfish in Selangor, Malaysia. *Front. Microbiol.* 6:1417. doi: 10.3389/fmicb.2015.01417

Letchumanan, V., Yin, W. F., Lee, L. H., and Chan, K. G. (2015b). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Front. Microbiol.* 6:33. doi: 10.3389/fmicb.2015.00033

Li, B., Jackson, S. A., Gangiredla, J., Wang, W., Liu, H., Tall, B. D., et al. (2015). Genomic evidence reveals numerous *Salmonella enterica* serovar Newport reintroduction events in Suwannee watershed irrigation ponds. *Appl. Environ. Microbiol.* 81, 8243–8253. doi: 10.1128/AEM.02179-15

Li, B., Vellidis, G., Liu, H., Jay-Russell, M., Zhao, S., Hu, Z., et al. (2014). Diversity and antimicrobial resistance of *Salmonella enterica* isolates from surface water in Southeastern United States. *Appl. Environ. Microbiol.* 80, 6355–6365. doi: 10.1128/AEM.02063-14

Li, W., Mei, L., Tang, Z., Yang, X., Li, X., Pei, X., et al. (2014). [Analysis of molecular features of clinical *Vibrio parahaemolyticus* strains in China]. *Zhonghua Yu Fang Yi Xue Za Zhi* 48, 44–52.

Li, Y., Xie, X., Shi, X., Lin, Y., Qiu, Y., Mou, J., et al. (2014). *Vibrio parahaemolyticus*, Southern Coastal Region of China, 2007-2012. *Emerg. Infect. Dis.* 20, 685–688. doi: 10.3201/eid2004.130744

Libinzon, A. E., Demina, A. I., Kulov, G. I., Shestialtynova, I. S., and Manuk'ian, G. V. (1977). [*Halophilic vibrios* isolated from the Sea of Azov]. *Zh. Mikrobiol. Epidemiol. Immunobiol.* 6, 77–80.

Liu, M., and Chen, S. (2015). A novel adhesive factor contributing to the virulence of *Vibrio parahaemolyticus*. *Sci. Rep.* 5:14449. doi: 10.1038/srep14449

Liu, X., Chen, Y., Wang, X., and Ji, R. (2004). [Foodborne disease outbreaks in China from 1992 to 2001 national foodborne disease surveillance system]. *Wei Sheng Yan Jiu* 33, 725–727.

Ma, C., Deng, X., Ke, C., He, D., Liang, Z., Li, W., et al. (2014). Epidemiology and etiology characteristics of foodborne outbreaks caused by *Vibrio parahaemolyticus* during 2008-2010 in Guangdong province, China. *Foodborne Pathog. Dis.* 11, 21–29. doi: 10.1089/fpd.2013.1522

Mahoney, J. C., Gerding, M. J., Jones, S. H., and Whistler, C. A. (2010). Comparison of the pathogenic potentials of environmental and clinical *Vibrio parahaemolyticus* strains indicates a role for temperature regulation in virulence. *Appl. Environ. Microbiol.* 76, 7459–7465. doi: 10.1128/AEM.01450-10

Maiden, M. C. (2006). Multilocus sequence typing of bacteria. *Annu. Rev. Microbiol.* 60, 561–588. doi: 10.1146/annurev.micro.59.030804.121325

Mala, W., Alam, M., Angkititrakul, S., Wongwajana, S., Lulitanond, V., Huttayananont, S., et al. (2016). Serogroup, virulence, and molecular traits of *Vibrio parahaemolyticus* isolated from clinical and cockle sources in northeastern Thailand. *Infect. Genet. Evol.* 39, 212–218. doi: 10.1016/j.meegid.2016.01.006

Martinez-Urtaza, J., Lozano-Leon, A., Depaola, A., Ishibashi, M., Shimada, K., Nishibuchi, M., et al. (2004). Characterization of pathogenic *Vibrio parahaemolyticus* isolates from clinical sources in Spain and comparison with Asian and North American pandemic isolates. *J. Clin. Microbiol.* 42, 4672–4678. doi: 10.1128/JCM.42.10.4672-4678.2004

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., et al. (2000). Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. *J. Clin. Microbiol.* 38, 578–585.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K., and Yamai, S. (1969). *In vitro* hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J. Bacteriol.* 100, 1147–1149.

Miyamoto, Y., Nakamura, K., and Takizawa, K. (1962). Seasonal distribution of *oceanomonas* spp., halophilic bacteria, in the coastal sea. Its significance in epidemiology and marine industry. *Jpn. J. Microbiol.* 6, 141–158.

Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Dutta, B., Takeda, Y., and Sack, D. A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *Clin. Microbiol. Rev.* 20, 39–48. doi: 10.1128/CMR.00025-06

Nasu, H., Iida, T., Sugahara, T., Yamaichi, Y., Park, K. S., Yokoyama, K., et al. (2000). A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. *J. Clin. Microbiol.* 38, 2156–2161.

Obata, H., Kai, A., and Morozumi, S. (2001). [The trends of *Vibrio parahaemolyticus* foodborne outbreaks in Tokyo: 1989-2000]. *Kansenshogaku Zasshi* 75, 485–489. doi: 10.11150/kansenshogakuzasshi1970.75.485

Okada, N., Iida, T., Park, K. S., Goto, N., Yasunaga, T., Hiyoshi, H., et al. (2009). Identification and characterization of a novel type III secretion system in trh-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity of pathogenic machinery beyond the species level. *Infect. Immun.* 77, 904–913. doi: 10.1128/IAI.01184-08

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A. K., et al. (1997). Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35, 3150–3155.

Okura, M., Osawa, R., Iguchi, A., Takagi, M., Arakawa, E., Terajima, J., et al. (2004). PCR-based identification of pandemic group *Vibrio parahaemolyticus* with a novel group-specific primer pair. *Microbiol. Immunol.* 48, 787–790. doi: 10.1111/j.1348-0421.2004.tb03596.x

Pan, T. M., Wang, T. K., Lee, C. L., Chien, S. W., and Horng, C. B. (1997). Food-borne disease outbreaks due to bacteria in Taiwan, 1986 to 1995. *J. Clin. Microbiol.* 35, 1260–1262.

Park, K. S., Ono, T., Rokuda, M., Jang, M. H., Okada, K., Iida, T., et al. (2004). Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect. Immun.* 72, 6659–6665. doi: 10.1128/IAI.72.11.6659-6665.2004

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., et al. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *Int. J. Food Microbiol.* 128, 354–361. doi: 10.1016/j.ijfoodmicro.2008.09.019

Pazhani, G. P., Bhowmik, S. K., Ghosh, S., Guin, S., Dutta, S., Rajendran, K., et al. (2014). Trends in the epidemiology of pandemic and non-pandemic strains of *Vibrio parahaemolyticus* isolated from diarrheal patients in Kolkata, India. *PLoS Negl. Trop. Dis.* 8:e2815. doi: 10.1371/journal.pntd.0002815

Qi, X. L., Wang, H. X., Bu, S. R., Xu, X. G., Wu, X. Y., and Lin, D. F. (2016). Incidence rates and clinical Symptoms of *Salmonella*, *Vibrio parahaemolyticus*, and *Shigella* infections in China, 1998-2013. *J. Infect. Dev. Ctries.* 10, 127–133. doi: 10.3855/jidc.6835

Quilici, M. L., Robert-Pillot, A., Picart, J., and Fournier, J. M. (2005). Pandemic *Vibrio parahaemolyticus* O3:K6 spread, France. *Emerg. Infect. Dis.* 11, 1148–1149. doi: 10.3201/eid1107.041008

Raghunath, P. (2014). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Front. Microbiol.* 5:805. doi: 10.3389/fmicb.2014.00805

Rosec, J. P., Simon, M., Causse, V., and Boudjemaa, M. (2009). Detection of total and pathogenic *Vibrio parahaemolyticus* in shellfish: comparison of PCR protocols using pR72H or toxR targets with a culture method. *Int. J. Food Microbiol.* 129, 136–145. doi: 10.1016/j.ijfoodmicro.2008.11.017

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

Sun, H., Li, Y., Shi, X., Lin, Y., Qiu, Y., Zhang, J., et al. (2015). Association of CRISPR/Cas evolution with *Vibrio parahaemolyticus* virulence factors and genotypes. *Foodborne Pathog. Dis.* 12, 68–73. doi: 10.1089/fpd.2014.1792

Velazquez-Roman, J., Leon-Sicairos, N., De Jesus Hernandez-Diaz, L., and Canizalez-Roman, A. (2013). Pandemic *Vibrio parahaemolyticus* O3:K6 on the American continent. *Front. Cell. Infect. Microbiol.* 3:110. doi: 10.3389/fcimb.2013.00110

Wang, D., Wang, H., Zhou, Y., Zhang, Q., Zhang, F., Du, P., et al. (2011). Genome sequencing reveals unique mutations in characteristic metabolic pathways and the transfer of virulence genes between *V. mimicus* and *V. cholerae*. *PLoS ONE* 6:e21299. doi: 10.1371/journal.pone.0021299

Williams, T. L., Musser, S. M., Nordstrom, J. L., Depaola, A., and Monday, S. R. (2004). Identification of a protein biomarker unique to the pandemic O3:K6 clone of *Vibrio parahaemolyticus*. *J. Clin. Microbiol.* 42, 1657–1665. doi: 10.1128/JCM.42.4.1657-1665.2004

Wong, H. C., and Lin, C. H. (2001). Evaluation of typing of *Vibrio parahaemolyticus* by three PCR methods using specific primers. *J. Clin. Microbiol.* 39, 4233–4240. doi: 10.1128/JCM.39.12.4233-4240.2001

Wong, H. C., Liu, C. C., Pan, T. M., Wang, T. K., Lee, C. L., and Shih, D. Y. (1999). Molecular typing of *Vibrio parahaemolyticus* isolates, obtained from patients involved in food poisoning outbreaks in Taiwan, by random amplified polymorphic DNA analysis. *J. Clin. Microbiol.* 37, 1809–1812.

Wong, H. C., Lu, K. T., Pan, T. M., Lee, C. L., and Shih, D. Y. (1996). Subspecies typing of *Vibrio parahaemolyticus* by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 34, 1535–1539.

Yan, W. X., Dai, Y., Zhou, Y. J., Liu, H., Duan, S. G., Han, H. H., et al. (2015). Risk factors for sporadic *Vibrio parahaemolyticus* gastroenteritis in east China: a matched case-control study. *Epidemiol. Infect.* 143, 1020–1028. doi: 10.1017/S0950268814001599

Zhang, C., Hu, X., Luo, J., Wu, Z., Wang, L., Li, B., et al. (2015). Degradation dynamics of glyphosate in different types of citrus orchard soils in China. *Molecules* 20, 1161–1175. doi: 10.3390/molecules20011161

Zhang, H., Sun, S., Shi, W., Cui, L., and Gu, Q. (2013). Serotype, virulence, and genetic traits of foodborne and clinical *Vibrio parahaemolyticus* isolates in Shanghai, China. *Foodborne Pathog. Dis.* 10, 796–804. doi: 10.1089/fpd.2012.1378

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 May 2016
doi: 10.3389/fmicb.2016.00764

Recovery and Growth Potential of *Listeria monocytogenes* in Temperature Abused Milkshakes Prepared from Naturally Contaminated Ice Cream Linked to a Listeriosis Outbreak

Yi Chen[1], Emma Allard[2], Anna Wooten[3], Minji Hur[4], Ishani Sheth[1], Anna Laasri[1], Thomas S. Hammack[1] and Dumitru Macarisin[1]*

[1] Office of Regulatory Science, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD, USA, [2] College of Life Sciences and Agriculture, University of New Hampshire, Durham, NH, USA, [3] Joint Institute for Food Safety and Applied Nutrition, University of Maryland, College Park, MD, USA, [4] Department of Food Science, Gachon University, Seoul, South Korea

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università degli Studi di Teramo, Italy

Reviewed by:
Antonio Valero, University of Cordoba, Spain
Alexandra Lianou, Agricultural University of Athens, Greece

***Correspondence:**
Yi Chen
yi.chen@fda.hhs.gov

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

Received: *29 March 2016*
Accepted: *05 May 2016*
Published: *18 May 2016*

Citation:
Chen Y, Allard E, Wooten A, Hur M, Sheth I, Laasri A, Hammack TS and Macarisin D (2016) Recovery and Growth Potential of Listeria monocytogenes in Temperature Abused Milkshakes Prepared from Naturally Contaminated Ice Cream Linked to a Listeriosis Outbreak. Front. Microbiol. 7:764. doi: 10.3389/fmicb.2016.00764

The recovery and growth potential of *Listeria monocytogenes* was evaluated in three flavors of milkshakes (vanilla, strawberry, and chocolate) that were prepared from naturally contaminated ice cream linked to a listeriosis outbreak in the U.S. in 2015, and were subsequently held at room temperature for 14 h. The average lag phase duration of *L. monocytogenes* was 9.05 h; the average generation time was 1.67 h; and the average population level increase per sample at 14 h was 1.14 log CFU/g. Milkshake flavors did not significantly affect these parameters. The average lag phase duration of *L. monocytogenes* in milkshakes with initial contamination levels $\leq$ 3 CFU/g (9.50 h) was significantly longer ($P < 0.01$) than that with initial contamination levels $>$ 3 CFU/g (8.60 h). The results highlight the value of using samples that are contaminated with very low levels of *L. monocytogenes* for recovery and growth evaluations. The behavior of *L. monocytogenes* populations in milkshakes prepared from naturally contaminated ice cream linked to the listeriosis outbreak should be taken into account when performing risk based analysis using this outbreak as a case study.

Keywords: *L. monocytogenes*, ice cream, milkshake, growth potential, temperature abuse, outbreak

INTRODUCTION

In March 2015, a listeriosis outbreak (outbreak I) was reported from a hospital (hospital X) involving five highly susceptible elderly patients who were hospitalized for other medical conditions prior to exposure to *Listeria monocytogenes* (Centers for Disease Control and Prevention, 2015). DNA fingerprinting [pulsed field gel electrophoresis (PFGE) and whole genome sequencing (WGS)] of *L. monocytogenes* isolates from these patients and various ice cream products linked four patients to the consumption of three flavors of milkshakes (vanilla, chocolate, and strawberry) served at lunch or dinner in hospital X and prepared with the contaminated

ice cream scoops that were produced in production line A (Centers for Disease Control and Prevention, 2015; Karl Klontz, personal communication). It is expected that very little post-contamination growth occurred in ice cream because this product is kept frozen all along the production and distribution chain. Thus, enumeration of *L. monocytogenes* in these products would provide a relatively clear estimation of the amount of *L. monocytogenes* consumed by the case patients and shed some light on the risk associated with *L. monocytogenes* contamination and its infective dose. Therefore, Chen (2015) enumerated the levels of *L. monocytogenes* in 2,320 individually wrapped scoops of ice cream produced in production line A. The enumeration study demonstrated that *L. monocytogenes* was present in 99.4% of all tested products produced between November 2014 and March 2015, and among them *L. monocytogenes* was present in 100% of tested products produced between November 2014 and January 2015. The levels of *L. monocytogenes* in individual scoops were below 20 and 50 most probable number (MPN)/g in 92.3 and 98.4% of tested scoops, respectively; and they were homogeneously low among different production dates with a geometric mean concentration of 0.15 to 7.1 MPN/g (Chen, 2015).

However, the case patients consumed milkshakes prepared from contaminated ice cream scoops, and thus, the enumeration data cannot be directly used for risk assessment without taking into account the possible growth of *L. monocytogenes* in milkshakes prior to human consumption. Another listeriosis outbreak (outbreak II) linked to contaminated ice cream products occurred in another state in 2015 (Rietberg et al., 2015), involving two patients from a hospital (hospital Y) who were served milkshakes prepared from contaminated ice cream. No evidence of temperature abuse of milkshakes in hospital X or Y was reported (Centers for Disease Control and Prevention, 2015; Rietberg et al., 2015), and no samples were available from outbreak II (Rietberg et al., 2015). Milkshake is a commonly consumed commodity, and these two outbreaks called for studies on the behavior of *L. monocytogenes* in milkshakes prepared from ice cream products, especially those naturally contaminated with *L. monocytogenes*. However, such studies have never been reported. We were able to obtain ice cream samples produced from the production line that was implicated in outbreak I, and therefore, the objective of the present study was to evaluate the recovery and growth potential of *L. monocytogenes* in milkshakes prepared from naturally contaminated ice cream scoops linked to outbreak I. Assuming an extreme scenario that a milkshake was unintentionally left at room temperature for an extended period of time, we evaluated the recovery and growth of *L. monocytogenes* in the course of 14 h at room temperature.

MATERIALS AND METHODS

Ice Cream Samples

Individually wrapped scoops of ice cream produced from production line A were made available by the company. The scoops produced between November 2014 and January 2015 were used to prepare the milkshakes.

Preparation of Milkshakes

Vanilla, strawberry and chocolate milkshakes were prepared using the recipe from hospital X. Vanilla milkshakes were made from two scoops of ice cream (80–85 g/scoop) and 118 mL of 1% fat milk. Strawberry milkshakes were made from two scoops of ice cream, 118 mL of 1% fat milk and 15 mL of strawberry syrup. Chocolate milkshakes were made from two scoops of ice cream, 118 mL of 1% fat milk and 15 mL of chocolate syrup. Milk, strawberry syrup, and chocolate syrup were purchased from a local supermarket. Ice cream scoops were briefly left at room temperature to soften, and milkshakes were then prepared using a sterilized commercial drink mixer (Model, HMD200, Hamilton Beach Inc., Southern Pines, NC, USA). For each milkshake flavor, 10 milkshake samples were prepared from randomly picked ice cream scoops, and held at room temperature (22.5 $\pm$ 0.5°C). To facilitate thorough mixing of the milkshakes, the entire portion of milkshakes were aseptically transferred to sterilized stainless steel laboratory blenders (Model, Waring® 7011S, Conair Corporation, East Windsor, NJ, USA) after preparation, and blended prior to each hourly sampling. The temperature increases of 10 randomly selected milkshakes used for growth curve construction were measured every half hour using thermometers certified by U.S. National Institute of Standards and Technology (Cat. #ACC10033BLSFC, Thermo Fisher Scientific Inc., Waltham, MA, USA). Ten additional vanilla milkshakes were prepared as described above, aseptically transferred to plastic cups and incubated at room temperature. Their temperatures were monitored to investigate the effect of container material and the absence of blending on the temperature increase. These 10 additional samples were not used to evaluate the growth potential of *L. monocytogenes* and thus were not subject to hourly blending.

Growth Curves

Enumeration of *L. monocytogenes* in milkshakes was conducted hourly in the course of 14 h. During the first 10 h of sampling 2 g of each milkshake was directly plated onto 5 RAPID'*L. mono* agar (Cat. No. 3563694, Bio-Rad Laboratories, Hercules, CA, USA) plates (400 μl/plate) using the easySpiral® automatic spiral plater (Interscience, Inc., France) set to constant volume plating, and this plating yielded a limit of detection (LOD) of 0.5 CFU/g. After 10 h, 1 g of samples was directly plated onto 5 RAPID'*L. mono* agar plates (200 μl/plate), and this plating yielded a LOD of 1 CFU/g. All plates were left to dry before incubation. Representative colonies were confirmed according to the *L. monocytogenes* chapter of the FDA *Bacteriological Analytical Manual* (Hitchins et al., 2016). The *L. monocytogenes* level change (in CFU/g) of each sample was then used to construct the growth curve. The lag phase was determined as the time for the initial population level to increase twofold (Buchanan and Solberg, 1972). The data were not transferred to log values due to very low values (<10 CFU/g) of most of the data points. The generation time was calculated as the time required for cells to double during the exponential phase.

Statistical Analysis

Comparison of lag phase durations and generation times of *L. monocytogenes* in different flavors of milkshakes with different initial levels of *L. monocytogenes* were performed using one way ANOVA analysis (Bewick et al., 2004) or *t*-test (Whitley and Ball, 2002).

RESULTS AND DISCUSSION

The growth evaluation was performed on naturally contaminated ice cream products that had varying initial levels of *L. monocytogenes*. Combining results from milkshakes of all three flavors, the initial *L. monocytogenes* levels ranged from 1 to 20.5 CFU/g with 50% (15/30) of the milkshakes having initial *L. monocytogenes* levels $\leq$ 3 CFU/g and 76.7% (23/30) of the milkshakes having initial *L. monocytogenes* levels $\leq$ 5 CFU/g. The final *L. monocytogenes* population after 14 h of incubation at 22.5°C ranged from 9 to 422 CFU/g with 73.3% (22/30) of the milkshakes having final *L. monocytogenes* levels $\leq$ 100 CFU/g and 93.3% (28/30) of the milkshakes having final *L. monocytogenes* levels $\leq$ 150 CFU/g. Two samples having initial *L. monocytogenes* levels of 12 and 20.5 CFU/g yielded final levels of 233 and 422 CFU/g, respectively. The average level increase per sample at 14 h was 1.23 $\pm$ 0.26 (average $\pm$ standard deviation), 1.01 $\pm$ 0.19, and 1.19 $\pm$ 0.15 log CFU/g for vanilla, strawberry, and chocolate milkshakes, respectively, with an average of 1.14 $\pm$ 0.22 log CFU/g for all milkshakes (**Table 1**). This indicated that the milkshakes were in the early stage of the exponential phase, and therefore, the growth curve was presented as the change of CFU/g and lag phase duration was determined as the time required for initial cell level to increase twofold (**Figure 1**; Buchanan and Solberg, 1972). The average lag phase durations of *L. monocytogenes* were 8.85 $\pm$ 0.78, 9.50 $\pm$ 0.82, and 8.80 $\pm$ 0.95 h for vanilla, strawberry, and chocolate milkshakes, respectively (**Table 1**; **Figure 1**), which were not statistically different ($P > 0.05$) from each other. The average lag phase duration was 9.05 $\pm$ 0.88 h for all milkshakes (**Table 1**). The average generation times of *L. monocytogenes* were 1.65 $\pm$ 0.39, 1.79 $\pm$ 0.36, and 1.57 $\pm$ 0.18 h for vanilla, strawberry, and chocolate milkshakes, respectively (**Table 1**), which were not statistically different ($P > 0.05$) from each other. The average generation time was 1.67 $\pm$ 0.33 h for all milkshakes. Because of the relatively long lag phase, no growth of *L. monocytogenes* was observed for 7.30 $\pm$ 0.79, 8.05 $\pm$ 0.96, and 7.40 $\pm$ 0.70 h for vanilla, strawberry, and chocolate milkshakes, respectively (**Table 1**; **Figure 1**). To our knowledge, this is the first report of the recovery and growth potential of *L. monocytogenes* in milkshakes prepared with ice cream products that were naturally contaminated with very low levels of bacteria and produced from a production line that was implicated in a listeriosis outbreak. The lag phase duration and generation time of *L. monocytogenes* determined in the present study contributed to a better understanding of the behavior of *L. monocytogenes* in the temperature abused milkshake prepared with naturally contaminated ice cream. The data can be combined with previously generated enumeration data for risk based characterization of *L. monocytogenes* contamination in this commodity. There is a possibility that the average generation time of the entire exponential phase may be different from the generation time observed in the present study; however, we chose to calculate the lag phase duration and generation time within 14 h of incubation because that value was relevant in assessing the recovery and growth of *L. monocytogenes*, had the milkshakes been unintentionally left at room temperature during the day of serving. A probabilistic analysis between direct plating and MPN for the ice cream samples showed agreement of the two methods in 96.1% of the samples with direct plating providing an underestimate in 0.8% of the samples and MPN providing an underestimate in 3.1% of the samples. This indicated that the injury status of *L. monocytogenes* in these ice cream products did not prevent the cells from recovering and growing on RAPID'*L. mono* agar (Chen, 2015). WGS analysis using the Center for Food Safety and Applied Nutrition (CFSAN) Single Nucleotide Polymorphism (SNP) pipeline (Pettengill et al., 2014; Davis et al., 2015) showed that the randomly picked isolates from these ice cream products all matched the clinical specimen of the patients in hospital X (unpublished data).

It is very important to keep in mind that during the first few hours of the exposure to room temperature, the temperature of the milkshakes was low. Milkshakes were −2 to 0°C when freshly prepared and reached 17 to 19°C after being held at room temperature for 3 h (**Figure 2**) in stainless steel cups, and thus, after milkshakes reached room temperature, it took *L. monocytogenes* another 6.05 h to reach the exponential phase. Milkshakes in plastic cups that were not subject to hourly blending reached 17 to 19°C after 4 h at room temperature. Therefore the use of stainless steel container and hourly blending facilitated a slightly faster temperature increase of milkshakes than plastic cups without hourly blending.

Studies which examined growth kinetics of *L. monocytogenes* in various enrichment broths and foods under various conditions often had the technical limitation (Gnanou Besse et al., 2006) set by the sensitivity of enumeration methods. In some cases, the variability in the enumeration of low levels of bacterial cells

TABLE 1 | Lag phase duration, generation time, time before growth was observed, level increase per sample for the 30 milkshakes (10 vanilla milkshakes, 10 strawberry milkshakes, and 10 chocolate milkshakes) analyzed in the present study.

Milkshake flavor	Lag phase duration (h)	Generation time (h)	Time before growth was observed (h)	Level increase per sample at 14 h (Log CFU/g)
Vanilla	8.85 $\pm$ 0.78	1.65 $\pm$ 0.39	7.30 $\pm$ 0.79	1.23 $\pm$ 0.26
Strawberry	9.50 $\pm$ 0.82	1.79 $\pm$ 0.36	8.05 $\pm$ 0.96	1.01 $\pm$ 0.19
Chocolate	8.80 $\pm$ 0.95	1.57 $\pm$ 0.18	7.40 $\pm$ 0.70	1.19 $\pm$ 0.15
Total	9.05 $\pm$ 0.88	1.67 $\pm$ 0.33	7.58 $\pm$ 0.86	1.14 $\pm$ 0.22

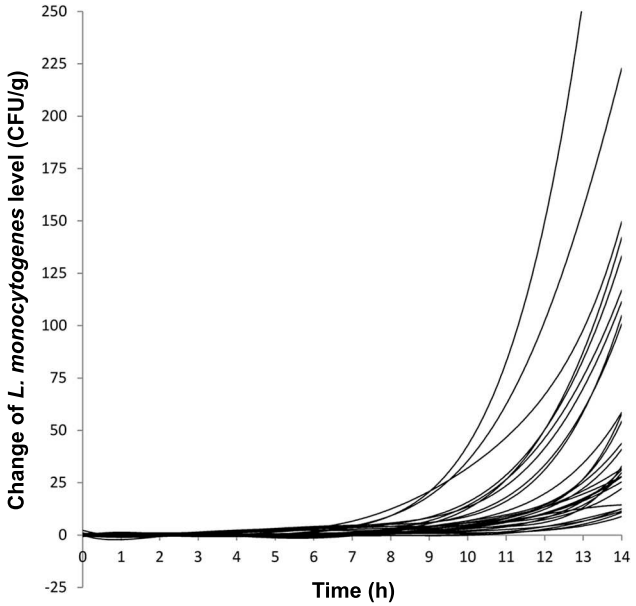


FIGURE 1 | Growth curves of 30 milkshake samples (10 vanilla milkshakes, 10 strawberry milkshakes, and 10 chocolate milkshakes) held at 22.5°C for 14 h. Each line represents the growth curve of a milkshake sample.

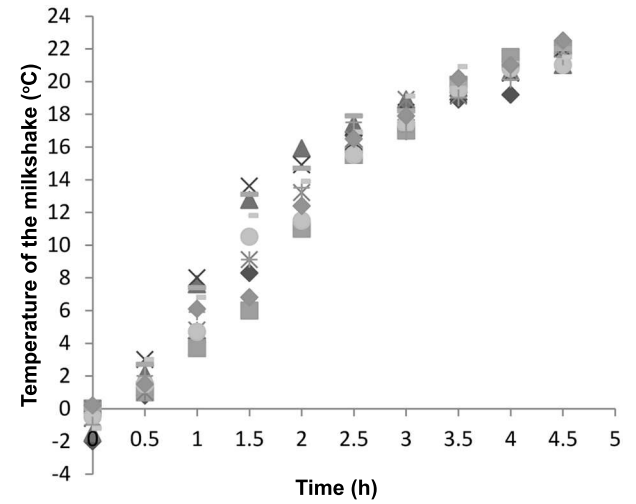


FIGURE 2 | Temperature increase of 10 randomly selected milkshakes held in stainless steel containers at 22.5°C that were used for growth curve construction. Different symbols represent different milkshake samples.

could compromise the reliability of any statistical analysis (Duffy et al., 1994). This is probably why quite a few studies used foods artificially inoculated with 10^2 to 10^4 CFU/g or even higher levels of bacteria for the convenience of subsequent enumeration and statistical analysis (Xanthiakos et al., 2006; Panagou and Nychas, 2008; Schvartzman et al., 2014; Huang et al., 2015; Luo et al., 2015). However, these inoculum sizes do not reflect the low bacterial contamination levels usually found in food. Indeed, if the bacterial levels are low, a large number of agar plates or an MPN scheme with a large number of MPN tubes per level and a large number of biological replicates would have to be used to accurately determine the lag phase duration. This would be impractical for hourly monitoring of bacterial growth, especially for an extended period of time. The milkshake samples in the present study could be homogenized with no dilutions in any buffer; and they were viscous and could stay off the edge of the plates. As a result, as much as 400 μl of milkshake could be evenly spread onto one plate with the help of the automatic spiral plater, and this allowed us to plate up to 2 g per sample and to obtain reliable data to perform statistical analysis.

The average lag phase duration of *L. monocytogenes* in 15 milkshakes having initial *L. monocytogenes* levels $\leq$ 3 CFU/g was 9.50 $\pm$ 0.76 h, and it was significantly higher ($P < 0.01$) than that of 15 milkshakes having initial *L. monocytogenes* levels > 3 CFU/g, which was 8.60 $\pm$ 0.78 h. Among the 15 milkshakes having initial *L. monocytogenes* levels > 3 CFU/g, eight milkshakes had initial *L. monocytogenes* levels of 3.5 to 5 CFU/g and seven milkshakes had initial *L. monocytogenes* levels of 5.5 to 20.5 CFU/g. Their average lag phase durations were 8.44 $\pm$ 0.78 and 8.79 $\pm$ 0.81 h, respectively, which were not significantly different ($P > 0.05$) from each other. The enumeration study revealed that 7.7, 1.6, and 0.2% of all tested ice cream scoops had *L. monocytogenes* of more than 20, 50, and 100 MPN/g, respectively (Chen, 2015). The milkshakes were made of two 80–85 g scoops and then diluted in 118 mL of milk, and thus, in order to obtain milkshakes containing *L. monocytogenes* of more than 30 CFU/g the addition of numeric values of the *L. monocytogenes* levels in the two scoops needed to exceed 100. The probability of this happening was very low, and therefore, we would not have been able to obtain sufficient biological replicates of milkshakes containing *L. monocytogenes* of more than 30 CFU/g even if we had prepared a much larger number of milkshakes. Robinson et al. (2001) showed that when the inoculum levels of *L. monocytogenes* were below 100 to 1,000 cells per sample, the lag time increased as the inoculum size decreased, especially under suboptimal growth conditions, partially due to the variability in the lag phase of individual cells; and the effect of inoculum size on lag phase duration started to disappear when the inoculum levels were higher. Aguirre et al. (2013) found that under experimental conditions, the lag phase durations of *L. innocua* with initial levels of 0.7 to 20 CFU/sample were higher than those with initial levels of 20 to 200 CFU/sample, and there was no statistical difference in lag phase durations among populations with initial levels between 20 and 200 CFU/sample. This phenomenon was also observed by Baranyi (1998) and Pin and Baranyi (2006) in other bacteria. The results from different studies may not be directly compared due to differences in the *Listeria* species/strains, growth media, and applied methodologies, however, they could partially explain why we observed a longer lag phase duration ($P < 0.01$) of cells in milkshakes with initial levels $\leq$ 3 CFU/g than that of cells with initial levels between 3 and 20.5 CFU/g, and no significant difference ($P > 0.05$) in lag phase durations between cells with initial levels of 3.5 to 5 CFU/g and those with initial

levels of 5.5 to 20.5 CFU/g. The finding in the present study, combined with those reported previously, suggests that under certain growth conditions the average lag phage duration of cells that are sparsely distributed in foods could be significantly longer than that indicated from foods inoculated with high levels of inoculum.

No studies have been performed on the behavior of *L. monocytogenes* in milkshakes made from ice cream, but a few studies analyzed artificially inoculated dairy products. Alavi et al. (1999) reported that the generation time of *L. monocytogenes* with an initial inoculum level of 1,000 CFU/mL in liquid whole milk stored at 22.5°C was 1.40 h. Xanthiakos et al. (2006) reported that with an initial inoculum level of 10^3 to 10^4 CFU/mL in pasteurized milk stored at 16°C, the lag phase duration of *L. monocytogenes* was 7.12 h and the generation time was 1.27 h. Panagou and Nychas (2008) found that with an initial inoculum level of 100 CFU/g, *L. monocytogenes* in a vanilla cream dessert-type product held at 15°C had an average lag phase duration of 7.85 h and an average generation time of 1.45 h. Gougouli et al. (2008) studied the behavior of *L. monocytogenes* in artificially inoculated ice cream extensively under multiple chilling and freezing conditions. A pro-longed (500 h) chilling-freezing experiment monitoring the recovery and growth of *L. monocytogenes* every 20 to 40 h demonstrated that *L. monocytogenes* cells in ice cream did not suffer significant injury in freezing conditions up to −30°C, and during consecutive freezing and thawing cycles. This suggested that the relatively long lag phase and generation time determined in the present study were not direct results of cell injury. Due to the overall low prevalence of *L. monocytogenes* in foods, most studies that used naturally contaminated samples could only yield a small number of positive samples and thus not enough biological replicates could be generated (Jorgensen and Huss, 1998; Encinas et al., 1999; Lappi et al., 2004; Beaufort et al., 2007). The 100% prevalence rate of *L. monocytogenes* (Chen, 2015) in the ice cream samples used in the present study allowed the use of a large number of naturally contaminated biological replicates to generate the unique and highly confident research data.

CONCLUSION

This is the first report on the characterization of the behavior of *L. monocytogenes* in milkshakes prepared from the ice cream products naturally contaminated with low levels of *L. monocytogenes*. This provides relevant information for future risk assessment using the 2015 U.S. ice cream listeriosis outbreak as a case study, especially since the ice cream scoops used in the present study were produced from the production line that was implicated in the outbreak. The results demonstrated the value of using samples contaminated with very low levels of *L. monocytogenes* to perform recovery and growth evaluations of *L. monocytogenes* in foods. It is important to keep in mind that the rather long lag phase and generation time observed only pertained to the strains/samples in the present study, and our conclusions by no means undermine the critical importance of stringent temperature control by food handlers.

AUTHOR CONTRIBUTIONS

YC is the principle investigator (PI) of the project and wrote the manuscript. TH and DM are co-PIs of the project. EA, AW, MH, IS, and AL are research scientists working on the experiments.

ACKNOWLEDGMENTS

This research was funded in part by the University of Maryland Joint Institute for Food Safety and Applied Nutrition through a cooperative agreement with the U.S. Food and Drug Administration (FDA), #FDU001418, and in part by an appointment to the Research Participation Program at the FDA Center for Food Safety and Applied Nutrition, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. FDA.

REFERENCES

Aguirre, J. S., Gonzalez, A., Ozcelik, N., Rodriguez, M. R., and Garcia De Fernando, G. D. (2013). Modeling the *Listeria innocua* micropopulation lag phase and its variability. *Int. J. Food Microbiol.* 164, 60–69. doi: 10.1016/j.ijfoodmicro.2013.03.003

Alavi, S. H., Puri, V. M., Knabel, S. J., Mohtar, R. H., and Whiting, R. C. (1999). Development and validation of a dynamic growth model for *Listeria monocytogenes* in fluid whole milk. *J. Food Prot.* 62, 170–176.

Baranyi, J. (1998). Comparison of stochastic and deterministic concepts of bacterial lag. *J. Theor. Biol.* 192, 403–408. doi: 10.1006/jtbi.1998.0673

Beaufort, A., Rudelle, S., Gnanou-Besse, N., Toquin, M. T., Kerouanton, A., Bergis, H., et al. (2007). Prevalence and growth of *Listeria monocytogenes* in naturally contaminated cold-smoked salmon. *Lett. Appl. Microbiol.* 44, 406–411. doi: 10.1111/j.1472-765X.2006.02096.x

Bewick, V., Cheek, L., and Ball, J. (2004). Statistics review 9: one-way analysis of variance. *Crit. Care* 8, 130–136. doi: 10.1186/cc2597

Buchanan, R. L., and Solberg, M. (1972). Interaction of sodium nitrite, oxygen and PH on growth of *Staphylococcus aureus*. *J. Food Sci* 37, 81–85. doi: 10.1111/j.1365-2621.1972.tb03391.x

Centers for Disease Control and Prevention (2015). *Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update)*. Available at: http://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/

Chen, Y. (2015). "Enumeration of *Listeria monocytogenes* in ice cream samples linked to a recent multi-state outbreak," in *Annual Meeting of International Association for Food Protection*, Portland, OR.

Davis, S., Pettengill, J., Luo, Y., Payne, J., Shpuntoff, A., Rand, H., et al. (2015). CFSAN SNP Pipeline: an automated method for constructing SNP matrices from next-generation sequence data. *PeerJ Comput. Sci.* 1:e20. doi: 10.7717/peerj-cs.20

Duffy, G., Sheridan, J. J., Buchanan, R. L., Mcdowell, D. A., and Blair, I. S. (1994). The effect of aeration, initial inoculum and meat microflora on the growth kinetics of *Listeria monocytogenes* in selective enrichment broths. *Food Microbiol.* 11, 429–438. doi: 10.1006/fmic.1994.1048

Encinas, J. P., Sanz, J. J., Garcia-Lopez, M. L., and Otero, A. (1999). Behaviour of *Listeria* spp. in naturally contaminated chorizo (Spanish fermented sausage). *Int. J. Food Microbiol.* 46, 167–171. doi: 10.1016/S0168-1605(98)00184-6

Gnanou Besse, N., Audinet, N., Barre, L., Cauquil, A., Cornu, M., and Colin, P. (2006). Effect of the inoculum size on *Listeria monocytogenes*

growth in structured media. *Int. J. Food Microbiol.* 110, 43–51. doi: 10.1016/j.ijfoodmicro.2006.03.002

Gougouli, M., Angelidis, A. S., and Koutsoumanis, K. (2008). A study on the kinetic behavior of *Listeria monocytogenes* in ice cream stored under static and dynamic chilling and freezing conditions. *J. Dairy Sci.* 91, 523–530. doi: 10.3168/jds.2007-0255

Hitchins, A. D., Jinneman, K., and Chen, Y. (2016). *Food and Drug Administration Bacteriological Analytical Manual Chapter 10, Detection and Enumeration of Listeria monocytogenes in foods*. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm

Huang, J., Luo, Y., and Nou, X. (2015). Growth of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut cantaloupe under different temperature abuse scenarios. *J. Food Prot.* 78, 1125–1131. doi: 10.4315/0362-028X.JFP-14-468

Jorgensen, L. V., and Huss, H. H. (1998). Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *Int. J. Food Microbiol.* 42, 127–131. doi: 10.1016/S0168-1605(98)00071-3

Lappi, V. R., Ho, A., Gall, K., and Wiedmann, M. (2004). Prevalence and growth of *Listeria* on naturally contaminated smoked salmon over 28 days of storage at 4 degrees C. *J. Food Prot.* 67, 1022–1026.

Luo, K., Hong, S. S., and Oh, D. H. (2015). Modeling the effect of storage temperatures on the growth of *Listeria monocytogenes* on ready-to-eat ham and sausage. *J. Food Prot.* 78, 1675–1681. doi: 10.4315/0362-028X.JFP-15-053

Panagou, E. Z., and Nychas, G. J. (2008). Dynamic modeling of *Listeria monocytogenes* growth in pasteurized vanilla cream after postprocessing contamination. *J. Food Prot.* 71, 1828–1834.

Pettengill, J. B., Luo, Y., Davis, S., Chen, Y., Gonzalez-Escalona, N., Ottesen, A., et al. (2014). An evaluation of alternative methods for constructing phylogenies from whole genome sequence data: a case study with *Salmonella*. *PeerJ* 2:e620. doi: 10.7717/peerj.620

Pin, C., and Baranyi, J. (2006). Kinetics of single cells: observation and modeling of a stochastic process. *Appl. Environ. Microbiol.* 72, 2163–2169. doi: 10.1128/AEM.72.3.2163-2169.2006

Rietberg, K., Lloyd, J., Melius, B., Wyman, P., Treadwell, R., Olson, G., et al. (2015). Outbreak of *Listeria monocytogenes* infections linked to a pasteurized ice cream product served to hospitalized patients. *Epidemiol. Infect.* 1, 1–4. doi: 10.1017/S0950268815003039

Robinson, T. P., Aboaba, O. O., Kaloti, A., Ocio, M. J., Baranyi, J., and Mackey, B. M. (2001). The effect of inoculum size on the lag phase of *Listeria monocytogenes*. *Int. J. Food Microbiol.* 70, 163–173. doi: 10.1016/S0168-1605(01)00541-4

Schvartzman, M. S., Gonzalez-Barron, U., Butler, F., and Jordan, K. (2014). Modeling the growth of *Listeria monocytogenes* on the surface of smear- or mold-ripened cheese. *Front. Cell Infect. Microbiol.* 4:90. doi: 10.3389/fcimb.2014.00090

Whitley, E., and Ball, J. (2002). Statistics review 5: comparison of means. *Crit. Care* 6, 424–428. doi: 10.1186/cc1820

Xanthiakos, K., Simos, D., Angelidis, A. S., Nychas, G. J., and Koutsoumanis, K. (2006). Dynamic modeling of *Listeria monocytogenes* growth in pasteurized milk. *J. Appl. Microbiol.* 100, 1289–1298. doi: 10.1111/j.1365-2672.2006.02854.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.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the U.S. FDA.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 May 2016
doi: 10.3389/fmicb.2016.00591

Biofilm-Forming Capacity in Biogenic Amine-Producing Bacteria Isolated from Dairy Products

*Maria Diaz, Victor Ladero *, Beatriz del Rio, Begoña Redruello, María Fernández, M. Cruz Martin and Miguel A. Alvarez*

Department of Technology and Biotechnology of Dairy Products, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Villaviciosa, Spain

Biofilms on the surface of food industry equipment are reservoirs of potentially food-contaminating bacteria—both spoilage and pathogenic. However, the capacity of biogenic amine (BA)-producers to form biofilms has remained largely unexamined. BAs are low molecular weight, biologically active compounds that in food can reach concentrations high enough to be a toxicological hazard. Fermented foods, especially some types of cheese, accumulate the highest BA concentrations of all. The present work examines the biofilm-forming capacity of 56 BA-producing strains belonging to three genera and 10 species (12 *Enterococcus faecalis*, 6 *Enterococcus faecium*, 6 *Enterococcus durans*, 1 *Enterococcus hirae*, 12 *Lactococcus lactis*, 7 *Lactobacillus vaginalis*, 2 *Lactobacillus curvatus*, 2 *Lactobacillus brevis*, 1 *Lactobacillus reuteri,* and 7 *Lactobacillus parabuchneri*), all isolated from dairy products. Strains of all the tested species - except for *L. vaginalis*—were able to produce biofilms on polystyrene and adhered to stainless steel. However, the biomass produced in biofilms was strain-dependent. These results suggest that biofilms may provide a route via which fermented foods can become contaminated by BA-producing microorganisms.

Keywords: biofilm, biogenic amines, histamine, tyramine, putrescine, lactic acid bacteria, dairy industry

OPEN ACCESS

Edited by:
Giovanna Suzzi, University of Teramo, Italy

Reviewed by:
Giorgia Perpetuini, University of Teramo, Italy
Fausto Gardini, University of Bologna, Italy

****Correspondence:***
Victor Ladero
ladero@ipla.csic.es

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

Received: *24 February 2016*
Accepted: *11 April 2016*
Published: *12 May 2016*

Citation:
Diaz M, Ladero V, del Rio B, Redruello B, Fernández M, Martin MC and Alvarez MA (2016) Biofilm-Forming Capacity in Biogenic Amine-Producing Bacteria Isolated from Dairy Products. Front. Microbiol. 7:591. doi: 10.3389/fmicb.2016.00591

INTRODUCTION

Food safety is a major priority in today's food industry. Bacterial biofilms on industrial surfaces are a cause for concern since they may act as reservoirs of contaminating microorganisms (Winkelströter et al., 2014). Dairy products in particular are susceptible to such contamination (Srey et al., 2013), with equipment surfaces one of its major sources (Kumar and Anand, 1998). Stainless steel type 304 is the most common material in contact with food in the dairy industry (Zottola and Sasahara, 1994). While inert, easy to clean and highly resistant to corrosion, it can, however, develop small cracks and crevices where biofilm formation is facilitated (Winkelströter et al., 2014). In addition, some parts of food processing equipment may have inaccessible areas where bacteria can evade cleaning treatments. The main biofilm-related risk is the growth of pathogens and spoilage microorganisms.

The capacity of biogenic amine (BA)-producers to form biofilms has not been investigated. BAs are low-molecular weight organic compounds derived from their corresponding amino acids via enzymatic decarboxylation. Although, BAs play an important role in human physiology, the ingestion of food containing them in large quantities can have toxicological effects on the digestive, circulatory, and respiratory systems (ten Brink et al., 1990; Shalaby, 1996; Ladero et al., 2010a). The

most important BAs (both qualitatively and quantitatively) in foods and beverages are histamine, tyramine, and putrescine. Together with fish and wine, dairy products—especially cheese—can develop BA concentrations that may exceed 1000 mg kg^{-1} (Linares et al., 2012).

BAs form in food via the activity of bacteria with aminoacyl decarboxylase activity (Halasz et al., 1994). Their appearance in dairy products has mainly been attributed to Gram positive bacteria of the lactic acid bacteria (LAB) group. These can be present in the microbiota of milk, as part of the starter culture, or be introduced by contamination during manufacturing (Linares et al., 2011), with equipment surfaces a potentially important source of contamination (Novella-Rodríguez et al., 2004). The post-ripening processing of cheese, particularly grating, extends the contact of food with equipment surfaces, increasing the number of histamine-producing bacteria present in the final product (Ladero et al., 2009) and therefore the histamine concentration that may be reached.

The dairy histamine-producing species *Lactobacillus parabuchneri* has been reported to produce biofilms (Diaz et al., 2016b), but this capacity has not been studied in other BA producers. The aim of the present work was to test the ability of BA-producing bacteria isolated from different cheeses to form biofilms on polystyrene and adhere to stainless steel surfaces.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

In this work we have examined the biofilm-forming capacity of 56 BA-producing strains belonging to three genera and 10 species (12 *Enterococcus faecalis*, 6 *Enterococcus faecium*, 6 *Enterococcus durans*, 1 *Enterococcus hirae*, 12 *Lactococcus lactis*, 7 *Lactobacillus vaginalis*, 2 *Lactobacillus curvatus*, 2 *Lactobacillus brevis*, 1 *Lactobacillus reuteri,* and 7 *Lactobacillus parabuchneri*), all isolated from dairy products. The ability of all (except of the *Lactobacillus parabuchneri* strains) to produce BAs was known from previous work (Ladero et al., 2010b,c, 2011a,b, 2012a,b; Diaz et al., 2015; del Rio et al., 2015). Given the reported ability of *L. parabuchneri* to form biofilms (Diaz et al., 2016b), and the apparent importance of the species in the accumulation of histamine in cheese (Diaz et al., 2016a), seven new strains isolated from cheese (following the protocol of Diaz et al., 2015) were included among those examined. These were identified at the species level by *16S rRNA* sequencing (Diaz et al., 2015) and their ability to produce histamine checked by UHPLC (Redruello et al., 2013).

Lactobacilli were grown at 37°C in MRS (Oxoid, Basingstoke, UK), while enterococci and lactococci were grown at 30°C in M17 (Oxoid) supplemented with 0.5% (w/v) glucose (GM17). To test the individual capacity of the *L. parabuchneri* strains to produce histamine, the culture medium was supplemented with 5 mM histidine.

Analysis of Biofilm Formation on Polystyrene

The ability of the test strains to produce a biofilm on polystyrene was performed as described by Diaz et al. (2016b). Briefly, MRS or GM17 overnight cultures were diluted to approximately 10^6 cfu mL^{-1} with fresh medium and used to fill polystyrene 96-well microtitre plates (Nunc™ MicroWell™ Plates with a Nunclon™ Delta Surface; Thermo Fisher Scientific, Waltham, MA, USA). Negative controls consisted of wells filled with the corresponding uninoculated culture medium. All plates were then incubated at 30 or 37°C depending on the species. Biofilm biomass was determined using the crystal violet staining method (CV assay) (Kubota et al., 2008). After 24, 30, or 48 h of incubation, the supernatant was removed and the wells rinsed with PBS buffer to eliminate non-adhered cells. The potential biofilm present was then stained with 0.5% (w/v) CV in distilled water (dH_2O); the excess dye was removed with dH_2O. The bound dye was then extracted using acetone/ethanol (80:20, v:v) and quantified by absorbance at 595 nm using a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA, USA). The mean ± SD of the optical density (OD) of three replicates was calculated for each strain. ANOVA with *post-hoc* Bonferroni correction was used to analyse all data. Significance was set at $p < 0.05$. All statistical calculations were undertaken using SPSS v.15.0 software (SPSS Inc., IL, USA). Biofilm production capacity was expressed using cut-off values (Extremina et al., 2011). The cut-off value between biofilm-producers and non-producers (ODc) was defined as the mean of the negative controls (ODnc) plus three SDs. The strains were then classified as belonging to one of the following categories: ODc < OD≤ 2 × ODc = weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc = moderate biofilm producer, and OD > 4 × ODc = strong biofilm producer.

Analysis of Bacterial Adherence to Stainless Steel

The test surfaces used were 1 cm^2 stainless steel (type AISI 304) coupons. These were washed with soap and dH_2O, rinsed with dH_2O, and then immersed in acetone for 30 min to remove any grease or fingerprints. They were then rinsed once again in dH_2O, autoclaved, and immersed singly in tubes containing MRS or GM17 broth inoculated with 10^6 cfu mL^{-1} of the assayed strain (performed in triplicate). Each coupon was then incubated at 30 or 37°C for 24 or 48 h before removal using sterile forceps. Non-adhered cells were removed by rinsing the coupon three times in PBS buffer. The coupons were then re-immersed in 5 mL PBS buffer, and the adhered cells detached from the coupon by sonication in an ultrasonic bath (Ultrasons-H, Selecta, Spain) for 15 min. The bacterial suspension produced was serially diluted in PBS, and 100 μL of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions were plated on MRS or GM17 and incubated for 48 h (Kruszewski et al., 2013). Three replicates were performed for each strain using independent bacterial cultures. Bacterial counts were expressed as $\log_{10}$ cfu cm^{-2} (mean ± SD of three replicates). To confirm the tolerance of the cells to sonication, bacterial suspensions of all the examined strains were sonicated for 15 min. Pre- and post-sonication suspensions were serially diluted in PBS, plated, incubated for 48 h, and the cells enumerated; no significant differences were seen between pre- and post-sonication cell counts confirming that no cells were killed by this procedure.

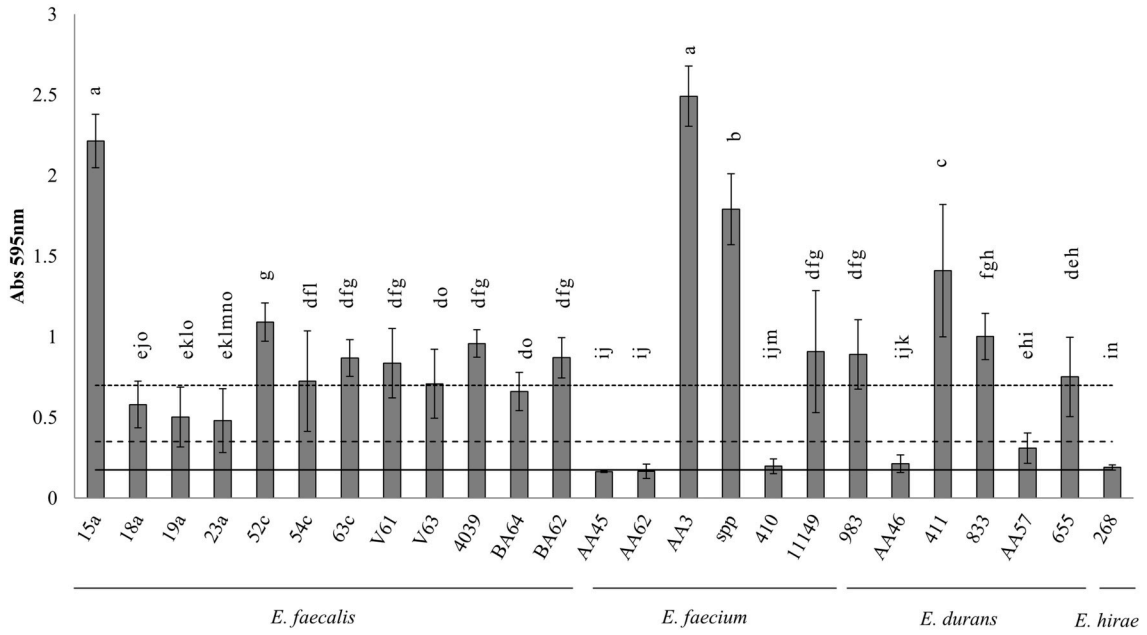


FIGURE 1 | Biofilm-producing capacity on polystyrene of the biogenic amine-producing *Enterococcus* strains after 30 h of incubation at 30°C. Data represent means ± SD (error bars) of three experiments. Values marked with the same letter do not differ significantly ($p > 0.05$ according to the Bonferroni *post-hoc* test). (___) Cut-off line (ODc); (__ __ __) 2 × ODc; (.....) 4 × ODc.

Scanning Electron Microscopy of Cells Adhered to Stainless Steel

The method of Kubota et al. (2008) was followed, with some modifications, to observe by scanning electron microscopy (SEM) the cells adhered to the stainless steel coupons. Briefly, the latter were rinsed twice in PBS and then fixed in 2.5% glutaraldehyde (Sigma-Aldrich) in PBS for 16 h at room temperature. The fixed bacteria were then dehydrated using a graded series of acetone solutions (50–100% v/v), and the coupons dried with CO_2 using a CPD-030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). They were then coated with gold using a SCD 004 Sputtering Coater (Bal-Tec AG, Balzers, Liechtenstein) and observed using a JSM-6610LV SEM (JEOL USA, Inc, Peabody, MA, USA).

RESULTS

Biofilm Formation on Polystyrene

Maximum biofilm biomass values were obtained at different times of incubation depending on the species. For all the *Enterococcus* strains assayed, biofilm biomass was maximal at 30 h; for all the *L. lactis*, *L. curvatus,* and *L. brevis* strains, maximum values were reached at 24 h; and for the *L. reuteri* and *L. parabuchneri* strains, maxima were recorded at 48 h. All incubation time results shown are those at which maximum biomass was reached.

All the *E. faecalis* strains were able to form biofilms on polystyrene. Six were classified as strong biofilm producers, and six as moderate biofilm producers (**Figure 1**). The only *E. hirae* strain assayed was a weak biofilm producer (**Figure 1**). All the *E. durans* strains tested were able to form biofilms; four were strong biofilm producers, and two were weak producers (**Figure 1**). Of the six *E. faecium* strains analyzed, three were strong biofilm producers, one was a weak producer, and two were unable to form a biofilm (**Figure 1**).

All the *L. lactis* strains were able to produce biofilms on polystyrene (**Figure 2**). Three *L. lactis* subsp. *cremoris* and two *L. lactis* subsp. *lactis* were strong biofilm producers. The remaining strains—two *L. lactis* subsp. *cremoris* and five *L. lactis* subsp. *lactis* strains—were weak producers.

None of the *L. vaginalis* strains were able to form a biofilm (**Figure 3**). All the *L. curvatus, L. brevis*, and *L. reuteri* strains were, however, strong biofilm producers (**Figure 3**). Two out of seven *L. parabuchneri* strains were strong biofilm producers, while the remaining strains were only weak producers (**Figure 3**).

Bacterial Adherence to Stainless Steel

The strains selected for this assay were the strongest biofilm producers in the polystyrene surface assay, i.e., *E. faecalis* 15a, *E. hirae* 268, *E. durans* 411, *E. faecium* AA3, *L. lactis* subsp. *cremoris* CECT 8666, *L. lactis* subsp. *lactis* 1AA59, *L. curvatus* VI6, *L. brevis* 3811, *L. parabuchneri* IPLA 11150, and *L. reuteri* IPLA 11078. Although, no *L. vaginalis* strain was able to form a biofilm on polystyrene, *L. vaginalis* IPLA 11064 was tested with the steel surface. Two incubation times (24 and 48 h) were tested, but no differences were observed. **Figure 4** shows the number of adhered

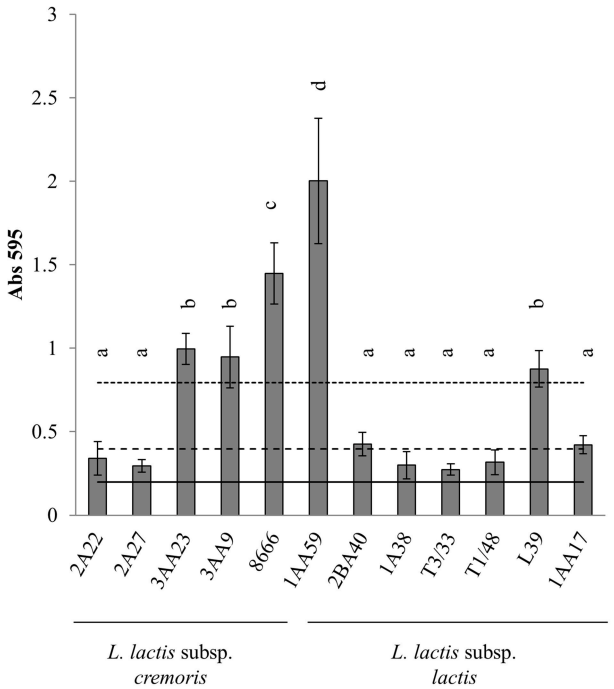


FIGURE 2 | Biofilm-producing capacity on polystyrene of the biogenic amine-producing *Lactococcus* strains after 24 h of incubation at 30°C. Data represent means ± SD (error bars) of three experiments. Values marked with the same letter do not differ significantly ($p > 0.05$ according to the Bonferroni *post-hoc* test). (___) Cut-off line (ODc); (__ __ __) 2 × ODc; (.....) 4 × ODc.

cells of each strain after incubation for 48 h ($>10^4$ cfu cm^{-2} for all strains assayed).

Scanning Electron Microscopy Imaging of Cells

SEM photomicrographs of cells adhered to the stainless steel coupons were captured for the same strains as used in the previous assay. Although, no differences were seen in the counts at different incubation times (24 and 48,h), differences in aggregation type and bacterial ultrastructure were observed.

E. faecalis 15a (**Figure 5A1**), *E. faecium* AA3 (**Figure 5B1**) and *E. durans* 411 returned clearer images after 24 h of incubation (data not shown) and appeared uniformly spread on the coupons. No adhering *E. hirae* 268 cells were observed at either 24 or 48 h. The *E. faecalis* cells were observed embedded in an extracellular matrix (**Figure 5A2**); this was not observed for the other two species. Structures that might be involved in anchoring to the surface were observed on *E. faecium* cells (**Figure 5B2**).

L. lactis subsp. *cremoris* CECT 8666 and *L. lactis* subsp. *lactis* 1AA59 returned clearer images after 24 h of incubation. The strain 8666 was uniformly spread across the coupon surface (**Figure 6A1**), while 1AA59 formed more compact aggregates (**Figure 6B1**). In both cases, an extracellular matrix was observed, but with a different appearance (see **Figures 6A2,A3,B2**). Similar structures to those observed in *E. faecium*, and that might be involved in anchoring to the surface, were also observed for both *L. lactis* strains (**Figures 6A3,B3**).

L. parabuchneri IPLA 11150, *L. reuteri* IPLA 11078, and *L. brevis* 3811 returned clearer images after 48 h of incubation. *L. vaginalis* IPLA 11064 and *L. curvatus* VI6 showed no adhered cells at either 24 or 48 h. *L. parabuchneri* was distributed across the coupon, showing aggregations with an extracellular matrix (**Figures 7A1,A2**). *L. reuteri* was distributed across the coupons in small aggregations (**Figure 7B1**) in a clear extracellular matrix (**Figure 7B2**). *L. brevis* was seen only in the fissures of the coupon (**Figures 7C1,C2**).

DISCUSSION

The formation of BA in fermented foods by bacteria, especially cheese, is a cause of much concern. The industrial equipment used in cheesemaking and processing is a source of contamination, and the ability of microorganisms to adhere to the surfaces of such equipment increases their contamination potential. In this work, the ability of 56 BA-producing dairy LAB strains to adhere to a polystyrene surface was examined using the CV assay, a technique that allows easy quantification of total biofilm biomass. The strongest biofilm producers of each species were then examined for their ability to adhere to stainless steel coupons, and the adhered cells observed by SEM.

The formation of biofilms by clinical *Enterococcus* isolates has been much studied since it affects pathogenic potential (Langsrud, 2009). However, enterococci are also a cause of concern for the food industry, and the biofilm-forming ability of several food-related *Enterococcus* species has also been studied (Gomes et al., 2008; Jahan and Holley, 2014; da Silva Fernandes et al., 2015). In the present study, the biofilm-forming capacity of tyramine- or tyramine and putrescine-producing *Enterococcus* species was examined. The results of the CV assay showed that all the strains analyzed, except for two *E. faecium* strains, were able to form biofilms, although the total biomass produced differed. These results agree with those of previous studies that showed several foodborne *E. faecalis* and *E. faecium* isolates to be either weak, moderate or strong biofilm producers, while some isolates formed no biofilm at all (Gomes et al., 2008; Jahan and Holley, 2014). *E. durans* has also been described as a strong or moderate biofilm producer (Amel et al., 2015; Pieniz et al., 2015). The only strain of *E. hirae* analyzed in the present work was a weak biofilm producer; to our knowledge, the capacity of this species to form biofilms has not been previously studied.

All the *Enterococcus* strains selected to see whether they could adhere to stainless steel did so, with counts reaching 10^4 cfu cm^{-2}. The capacity of *E. faecalis, E. faecium,* and *E. durans* to adhere to stainless steel has been previously reported (Amel et al., 2015; da Silva Fernandes et al., 2015). The *Enterococcus* strains that adhered to the stainless steel coupons were observed by SEM, except for *E. hirae* 268; - this strain attached only weakly to the metal surface and was unable to resist the treatment required prior to observation. Of the strains that covered the surface of the steel coupons (**Figure 5**), none formed the complex three-dimensional structures reported

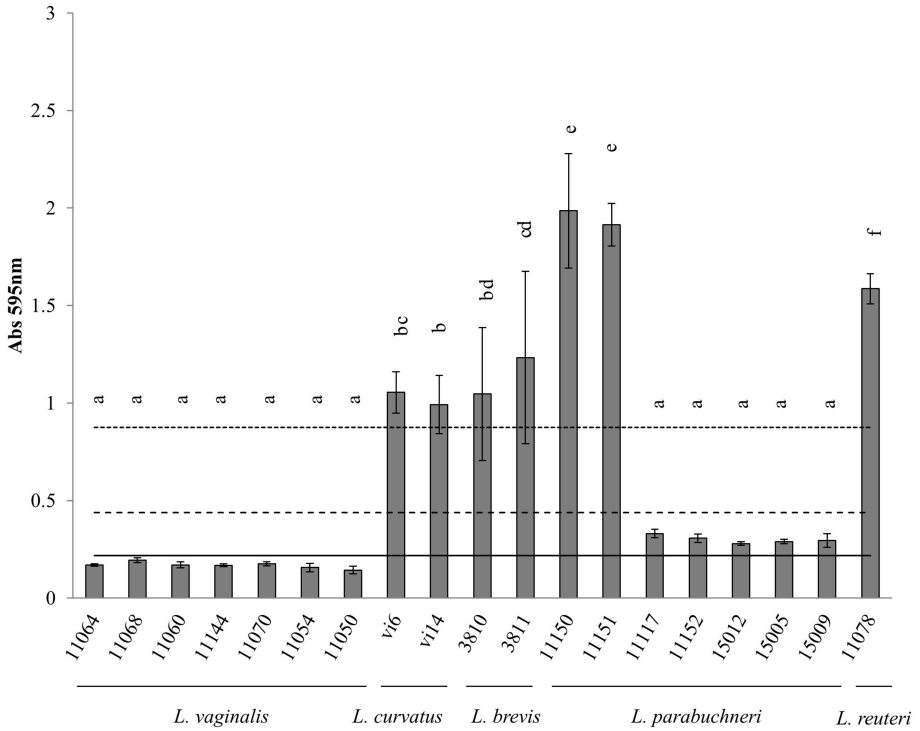


FIGURE 3 | Biofilm-producing capacity on polystyrene of the biogenic amine-producing *Lactobacillus* strains incubated at 37°C. The *L. vaginalis, L. curvatus* and *L. brevis* strains were incubated for 24 h. The *L. parabuchneri* and *L. reuteri* strains were incubated for 48 h. Data represent means ± SD (error bars) of three experiments. Values marked with the same letter do not differ significantly ($p > 0.05$ according to the Bonferroni *post-hoc* test). (___) Cut-off line (ODc); (__ __ __) 2 × ODc; (.....) 4 × ODc.

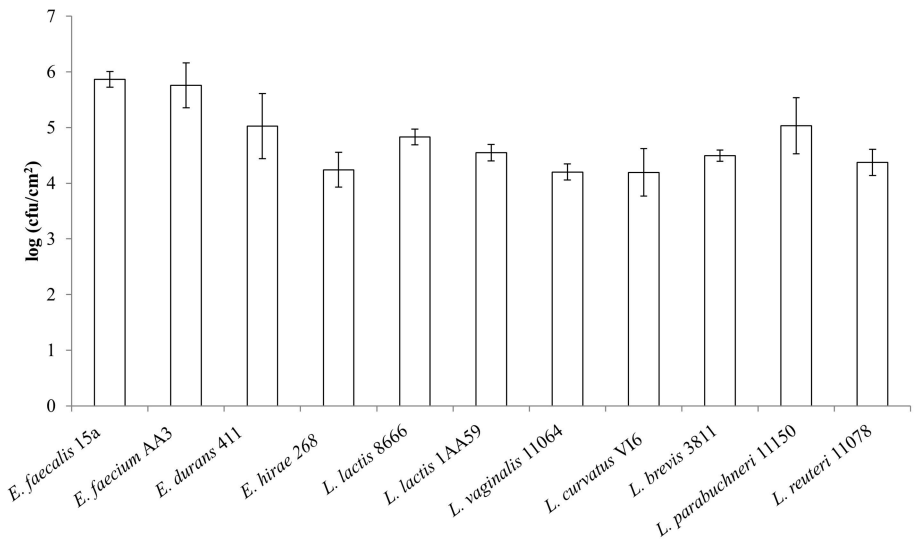


FIGURE 4 | Adherence to stainless steel coupons by biofilm-producing strains. Data are expressed as $\log_{10}$ cfu/cm^2 and represent the mean ± SD (error bars) of three experiments.

by da Silva Fernandes et al. (2015). Some aggregations of *E. faecalis* 15a cells were observed (**Figure 5A2**), while *E. faecium* AA3 cells appeared separated from one another, but all cells were sufficiently well adhered not to be detached by the PBS washes or the treatment required prior to SEM observations. To our knowledge, this is the first time that images of *E. durans* cells adhered to stainless steel have been captured.

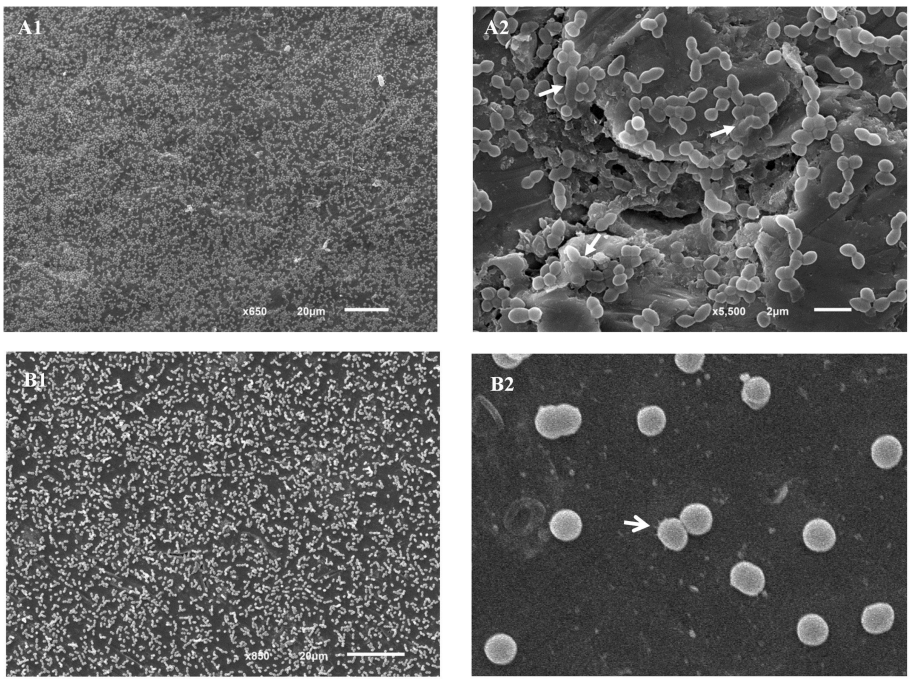


FIGURE 5 | Scanning electron photomicrographs of *Enterococcus* strains adhered to stainless steel coupons after 24 h of incubation. (A) *E. faecalis* 15a, **(B)** *E. faecium* AA3. Closed arrows point to the extracellular matrix; open arrows point to anchoring structures.

The ability of *L. lactis* to form a biofilm on a surface has been related to the latter's physicochemical properties (Giaouris et al., 2009; Oxaran et al., 2012). However, few studies have ever been performed on *L. lactis* biofilm formation. In the present work, 12 putrescine-producing *L. lactis* strains were shown by the CV assay to be either weak or strong biofilm producers. The two strains that most strongly formed biofim on polystyrene—*L. lactis* subsp. *cremoris* CECT 8666 and *L. lactis* subsp. *lactis* 1AA59—also adhered to stainless steel, reaching counts of 10^4 cfu cm^{-2}. SEM images showed cells of both strains to be embedded in an extracellular matrix and to be clearly adhered to the coupons. This is the first time that *L. lactis* adhered on stainless steel have been observed by SEM. Unlike that seen for enterococci, the *L. lactis* CECT 8666 cells showed tridimensional structures including filamentous ones that might help them adhere to the surface (**Figures 6A2,A3**). The extracellular material formed a pod-like covering over the cells (**Figures 6A2,A3**). *L. lactis* 1AA59 cell clusters also appeared to be attached via the extracellular matrix (**Figure 6B2**). For both *L. lactis* strains (**Figures 6A3,B3**) examined, and *E. faecium* AA3 (**Figure 5B2**), small protuberances of the cell surface were observed, which may have helped anchor the cells to the coupons. Certainly, aggregation and biofilm formation in *L. lactis* has been associated with the production of functional pili (Oxaran et al., 2012). Although, the adhesion of the putrescine-producing strains analyzed in this work would be undesirable for the food industry, allowing the development of *L. lactis* biofilms has been proposed as a means of preventing the growth of pathogens on food industry surfaces (Leriche et al., 1999; Zhao et al., 2004).

The presence of several Lactobacillus species (*L. curvatus*, *Lactobacillus fermentum*, *Lactobacillus delbruekii*, *Lactobacillus paracasei*, *Lactobacillus plantarum,* and *L. reuteri*) on the surfaces of dairy equipment has been reported (Somers et al., 2001; Scatassa et al., 2015). In the present work, two tyramine and putrescine-producing *L. brevis* strains were strong biofilm producers on polystyrene. This agrees with previous results for *L. brevis* strains isolated from onions (Kubota et al., 2008). The two tyramine-producing *L. curvatus* strains tested were also strong biofilm producers. Biofilm formation by *L. curvatus* has been previously described, although it was found to be a weaker biofilm producer than in the present work (Pérez Ibarreche et al., 2014). Fifteen histamine-producing strains, seven belonging to *L. vaginalis*, seven to *L. parabuchneri* and one to *L. reuteri*, were examined by the CV assay. The seven histamine-producing *L. vaginalis* strains were unable to form biofilms. All the *L. parabuchneri* strains were able to form biofilms, and were either weak or strong biofilm producers, depending on the strain. This agrees with the results of previous reports (Diaz et al., 2016b). The *L. reuteri* strain examined was a strong biofilm producer. Numerous studies have described the formation of biofilms by *L. reuteri*, some strains of which are considered probiotics (Leccese Terraf et al., 2014). A system regulating biofilm formation in *L. reuteri* was recently characterized (Su and Ganzle, 2014). In the present work, *L. reuteri* IPLA 11078, along with *L. vaginalis* IPLA 11064, *L. curvatus* VI6, *L. brevis* 3811 and *L. parabuchneri* IPLA 11150 were all able to adhere to the stainless steel coupons, and reached counts of over 10^4 cfu cm^{-2}. However, when the coupons were observed under the SEM, only *L. parabuchneri*

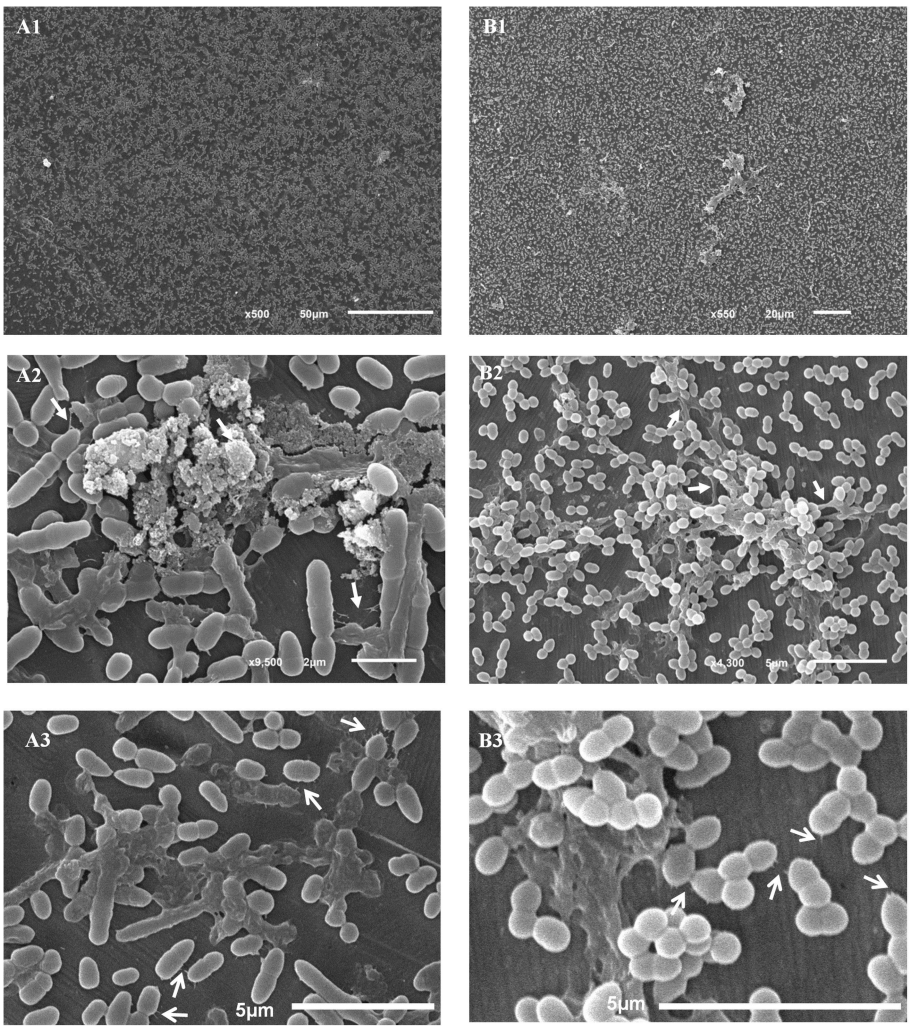


FIGURE 6 | Scanning electron photomicrographs of *Lactococcus lactis* strains adhered to stainless steel coupons after 24 h of incubation. (A) *L. lactis* subsp *cremoris* CECT8666, **(B)** *L. lactis* subsp. *lactis* 1AA59. Closed arrows point to the extracellular matrix; open arrows point to anchoring structures.

IPLA 11150 (**Figure 7A**) and *L. reuteri* IPLA 11078 (**Figure 7B**) cells were seen attached. For *L. parabuchneri* IPLA 11150, the cells aggregated into clumps composed of long chains of undivided cells. A previous study on *Pseudomonas aeruginosa* showed that elongated cells are inclined to form cohesive clumps (Yoon et al., 2011). According to the CV assay, the present *L. parabuchneri* and *L. reuteri* strains were strong biofilm producers. However, while the same assay suggested *L. curvatus* VI6 and *L. brevis* 3811 to be strong biofilm producers, the absorbance measured was very close to the lower limit for such classification. The few cells seen attached to the coupons might be the result of them not being able to resist the treatment required prior to SEM observation. This would appear to be supported by the fact that *L. brevis* 3811 was not homogenously attached to the surface of the coupons, but seen in their fissures. This is an important finding for the food industry since steel surfaces do develop cracks and these appear able to protect bacteria from cleaning procedures.

The present results show all the species examined, except *L. vaginalis*, had BA-producing strains able to form biofilms and that they could adhere to stainless steel, a material commonly used to make equipment in the food industry. Although, the cells attached to the stainless steel coupons commonly showed none of the three-dimensional structures reported by da Silva Fernandes et al. (2015), counts of over 10^4 cfu cm^{-2} were recorded in all cases, with *E. faecalis, E. faecium, E. durans,* and *L. parabuchneri* exceeding 10^5 cfu cm^{-2}. Given the large surface area of industrial equipment, BA-producing bacteria that can adhere to steel clearly pose a food contamination threat. This problem is of particular concern in the dairy industry since post-ripening treatments such as cheese grating bring food into close and prolonged contact with equipment surfaces. In fact, it has already been shown that cheese that has undergone post-ripening processing (cutting, slicing, or grating) has higher histamine levels than non-processed cheese (Ladero et al., 2009).

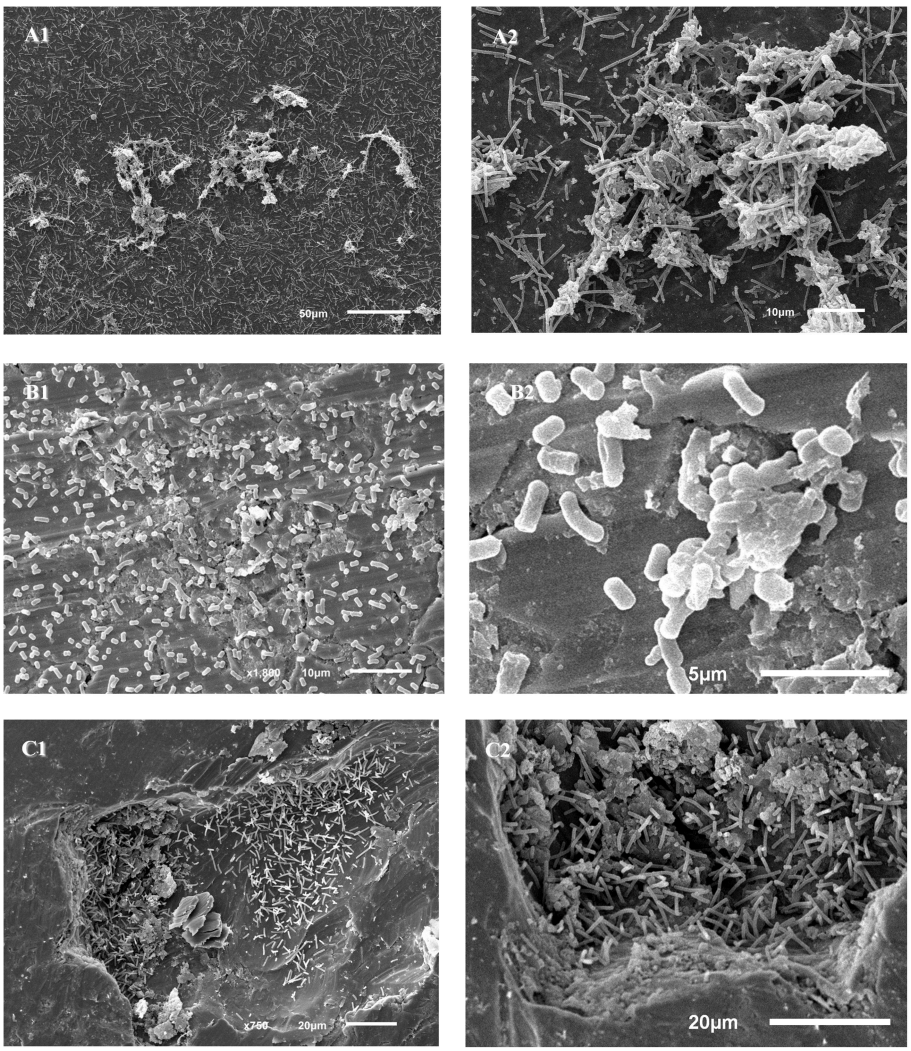


FIGURE 7 | Scanning electron photomicrographs of *Lactobacillus* strains adhered to stainless steel coupons after 48 h of incubation. (A) *L. parabuchneri* IPLA 11150, **(B)** *L. reuteri* IPLA 11078, **(C)** *L. brevis* 3811.

It is therefore important that our knowledge of the adhesion and biofilm forming capacities of BA-producers be improved, to prevent food contamination by these spoilage bacteria and eventually the accumulation of biogenic amines in food.

AUTHOR CONTRIBUTIONS

MD carried out the experiments and drafted the manuscript; BR, VL collaborated in conducting some experiments; VL, Bd, and MF participated in the study design and writing of the manuscript; MC, MA provided the general concept, designed the experiments, and supervised the experimental work and the manuscript. All authors contributed to the discussion of the research and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Christian Magni for providing some of the strains used in this work, the *Unidad de Microscopía Electrónica y Microanálisis* of the University of Oviedo for assistance with the SEM techniques, and Adrian Burton for language assistance. This work was funded by the Spanish Ministry of the Economy and Competitiveness (AGL2013-45431-R) and the Plan for Science, Technology and Innovation 2013–2017 financed by the European Regional Development Fund and the Principality of Asturias (GRUPIN14-137). MD is a beneficiary of an FPI fellowship from the Spanish Ministry of the Economy and Competitiveness.

REFERENCES

Amel, A. M., Farida, B., and Djamila, S. (2015). Anti-adherence potential of *Enterococcus durans* cells and its cell-free supernatant on plastic and stainless steel against foodborne pathogens. *Folia Microbiol. (Praha)* 60, 357–363. doi: 10.1007/s12223-014-0367-6

da Silva Fernandes, M., Kabuki, D. Y., and Kuaye, A. Y. (2015). Biofilms of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the processing of ricotta and the control of these pathogens through cleaning and sanitization procedures. *Int. J. Food Microbiol.* 200, 97–103. doi: 10.1016/j.ijfoodmicro.2015.02.004

del Rio, B., Ladero, V., Redruello, B., Linares, D. M., Fernández, M., Martin, M. C., et al. (2015). Lactose-mediated carbon catabolite repression of putrescine production in dairy *Lactococcus lactis* is strain dependent. *Food Microbiol.* 48, 163–170. doi: 10.1016/j.fm.2014.11.018

Diaz, M., del Rio, B., Ladero, V., Redruello, B., Fernández, M., Martin, M. C., et al. (2015). Isolation and typification of histamine-producing *Lactobacillus vaginalis* strains from cheese. *Int. J. Food Microbiol.* 215, 117–123. doi: 10.1016/j.ijfoodmicro.2015.08.026

Diaz, M., del Rio, B., Ladero, V., Redruello, B., Sanchez-Llana, E., Fernandez, M., et al. (2016b). Histamine-producing *Lactobacillus parabuchneri* strains isolated from grated cheese can form biofilms on stainless steel. *Food Microbiol.*

Diaz, M., Ladero, V., Redruello, B., Sanchez-Llana, E., del Rio, B., Fernandez, M., et al. (2016a). A PCR-DGGE method for the identification of histamine-producing bacteria in cheese. *Food Control* 63, 216–223. doi: 10.1016/j.foodcont.2015.11.035

Extremina, C. I., Costa, L., Aguiar, A. I., Peixe, L., and Fonseca, A. P. (2011). Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *J. Microbiol. Methods* 84, 167–173. doi: 10.1016/j.mimet.2010.11.007

Giaouris, E., Chapot-Chartier, M. P., and Briandet, R. (2009). Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. *Int. J. Food Microbiol.* 131, 2–9. doi: 10.1016/j.ijfoodmicro.2008.09.006

Gomes, B. C., Esteves, C. T., Palazzo, I. C., Darini, A. L., Felis, G. E., Sechi, L. A., et al. (2008). Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. *Food Microbiol.* 25, 668–675. doi: 10.1016/j.fm.2008.03.008

Halasz, A., Barath, A., Simon-Sarkadi, L., and Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* 5, 42–49. doi: 10.1016/0924-2244(94)90070-1

Jahan, M., and Holley, R. A. (2014). Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25 degrees C and 37 degrees C. *Int. J. Food Microbiol.* 170, 65–69. doi: 10.1016/j.ijfoodmicro.2013.11.002

Kruszewski, K. M., Nistico, L., Longwell, M. J., Hynes, M. J., Maurer, J. A., Hall-Stoodley, L., et al. (2013). Reducing *Staphylococcus aureus* biofilm formation on stainless steel 316L using functionalized self-assembled monolayers. *Mater. Sci. Eng. C Mater. Biol. Appl.* 33, 2059–2069. doi: 10.1016/j.msec.2013.01.023

Kubota, H., Senda, S., Nomura, N., Tokuda, H., and Uchiyama, H. (2008). Biofilm formation by lactic acid bacteria and resistance to environmental stress. *J. Biosci. Bioeng.* 106, 381–386. doi: 10.1263/jbb.106.381

Kumar, C. G., and Anand, S. K. (1998). Significance of microbial biofilms in food industry: a review. *Int. J. Food Microbiol.* 42, 9–27. doi: 10.1016/S0168-1605(98)00060-9

Ladero, V., Calles-Enriquez, M., Fernandez, M., and Alvarez, M. A. (2010a). Toxicological effects of dietary biogenic amines. *Curr. Nutr. Food Sci.* 6, 145–156. doi: 10.2174/157340110791233256

Ladero, V., Canedo, E., Perez, M., Martin, M. C., Fernandez, M., and Alvarez, M. A. (2012a). Multiplex qPCR for the detection and quantification of putrescine-producing lactic acid bacteria in dairy products. *Food Control* 27, 307–313. doi: 10.1016/j.foodcont.2012.03.024

Ladero, V., Coton, M., Fernández, M., Buron, N., Martin, M. C., Guichard, H., et al. (2011a). Biogenic amines content in Spanish and French natural ciders: application of qPCR for quantitative detection of biogenic amine-producers. *Food Microbiol.* 28, 554–561. doi: 10.1016/j.fm.2010.11.005

Ladero, V., Fernández, M., and Alvarez, M. A. (2009). Effect of post-ripening processing on the histamine and histamine-producing bacteria contents of different cheeses. *Int. Dairy J.* 19, 759–762. doi: 10.1016/j.idairyj.2009.05.010

Ladero, V., Fernández, M., Calles-Enriquez, M., Sanchez-Llana, E., Canedo, E., Martin, M. C., et al. (2012b). Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiol.* 30, 132–138. doi: 10.1016/j.fm.2011.12.016

Ladero, V., Fernández, M., Cuesta, I., and Alvarez, M. A. (2010b). Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. *Food Microbiol.* 27, 933–939. doi: 10.1016/j.fm.2010.05.026

Ladero, V., Martinez, N., Martin, M. C., Fernandez, M., and Alvarez, M. A. (2010c). qPCR for quantitative detection of tyramine-producing bacteria in dairy products. *Food Res. Int.* 43, 289–295. doi: 10.1016/j.foodres.2009.10.007

Ladero, V., Rattray, F. P., Mayo, B., Martín, M. C., Fernández, M., and Alvarez, M. A. (2011b). Sequencing and transcriptional analysis of the biosynthesis gene cluster of putrescine-producing *Lactococcus lactis*. *Appl. Environ. Microbiol.* 77, 6409–6418. doi: 10.1128/AEM.05507-11

Langsrud, S. (2009). "Biofilm formation by Gram-positive bacteria including *Staphylococcus aureus*, *Mycobacterium avium* and *Enterococcus* spp. in food processing environments," in *Biofilms in the Food and Beverage Industries*, eds P. Fratamico, B. Annous, and N. Gunther (Cambridge: Woodhead Publishing), 250–269.

Leccese Terraf, M. C., Mendoza, L. M., Juárez Tomás, M. S., Silva, C., and Nader-Macías, M. E. F. (2014). Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli. *J. Appl. Microbiol.* 117, 1761–1772. doi: 10.1111/jam.12642

Leriche, V., Chassaing, D., and Carpentier, B. (1999). Behaviour of *L. monocytogenes* in an artificially made biofilm of a nisin-producing strain of Lactococcus lactis. *Int. J. Food Microbiol.* 51, 169–182. doi: 10.1016/S0168-1605(99)00128-2

Linares, D. M., del Río, B., Ladero, V., Martínez, N., Fernández, M., Martín, M. C., et al. (2012). Factors influencing biogenic amines accumulation in dairy products. *Front. Microbiol.* 3:180. doi: 10.3389/fmicb.2012.00180

Linares, D. M., Martín, M. C., Ladero, V., Alvarez, M. A., and Fernández, M. (2011). Biogenic amines in dairy products. *Crit. Rev. Food Sci. Nutr.* 51, 691–703. doi: 10.1080/10408398.2011.582813

Novella-Rodríguez, S. N., Veciana-Nogues, M. T., Roig-Sagués, A. X., Trujillo-Mesa, A. J., and Vidal-Carou, M. C. (2004). Evaluation of biogenic amines and microbial counts throughout the ripening of goat cheeses from pasteurized and raw milk. *J. Dairy Res.* 71, 245–252. doi: 10.1017/S0022029904000147

Oxaran, V., Ledue-Clier, F., Dieye, Y., Herry, J. M., Péchoux, C., Meylheuc, T., et al. (2012). Pilus biogenesis in *Lactococcus lactis*: molecular characterization and role in aggregation and biofilm formation. *PLoS ONE* 7:e50989. doi: 10.1371/journal.pone.0050989

Pérez Ibarreche, M., Castellano, P., and Vignolo, G. (2014). Evaluation of anti-Listeria meat borne *Lactobacillus* for biofilm formation on selected abiotic surfaces. *Meat Sci.* 96, 295–303. doi: 10.1016/j.meatsci.2013.07.010

Pieniz, S., De Moura, T. M., Cassenego, A. P. V., Andreazza, R., Frazzon, A. P. G., Camargo, F. A. O., et al. (2015). Evaluation of resistance genes and virulence factors in a food isolated *Enterococcus durans* with potential probiotic effect. *Food Control* 51, 49–54. doi: 10.1016/j.foodcont.2014.11.012

Redruello, B., Ladero, V., Cuesta, I., Álvarez-Buylla, J. R., Martin, M. C., Fernandez, M., et al. (2013). A fast, reliable, ultra high performance liquid chromatography method for the simultaneous determination of amino acids, biogenic amines and ammonium ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent. *Food Chem.* 139, 1029–1035. doi: 10.1016/j.foodchem.2013.01.071

Scatassa, M. L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., et al. (2015). Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. *Food Microbiol.* 52, 31–41. doi: 10.1016/j.fm.2015.06.008

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Res. Int.* 29, 675–690. doi: 10.1016/S0963-9969(96)00066-X

Somers, E. B., Johnson, M. E., and Wong, A. C. (2001). Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the

dairy environment. *J. Dairy Sci.* 84, 1926–1936. doi: 10.3168/jds.S0022-0302(01)74634-6

Srey, S., Jahid, I. K., and Ha, S. D. (2013). Biofilm formation in food industries: a food safety concern. *Food Control* 31, 572–585. doi: 10.1016/j.foodcont.2012.12.001

Su, M. S., and Gänzle, M. G. (2014). Novel two-component regulatory systems play a role in biofilm formation of *Lactobacillus reuteri* rodent isolate 100-23. *Microbiology* 160, 795–806. doi: 10.1099/mic.0.071399-0

ten Brink, B., Damink, C., Joosten, H. M., and Huis in ′T Veld, J. H. J. (1990). Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* 11, 73–84. doi: 10.1016/0168-1605(90)90040-C

Winkelströter, L. K., Teixeira, F. B., Silva, E. P., Alves, V. F., and de Martinis, E. C. (2014). Unraveling microbial biofilms of importance for food microbiology. *Microb. Ecol.* 68, 35–46. doi: 10.1007/s00248-013-0347-4

Yoon, M. Y., Lee, K. M., Park, Y., and Yoon, S. S. (2011). Contribution of cell elongation to the biofilm formation of *Pseudomonas aeruginosa* during anaerobic respiration. *PLoS ONE* 6:e16105. doi: 10.1371/journal.pone.0016105

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

Zottola, E. A., and Sasahara, K. C. (1994). Microbial biofilms in the food processing industry-should they be a concern? *Int. J. Food Microbiol.* 23, 125–148. doi: 10.1016/0168-1605(94)90047-7

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 April 2016
doi: 10.3389/fmicb.2016.00549

Prevalence, Molecular Characterization, and Antibiotic Susceptibility of *Vibrio parahaemolyticus* from Ready-to-Eat Foods in China

Tengfei Xie[1,2], *Xiaoke Xu*[2], *Qingping Wu*[2]*, *Jumei Zhang*[2] and *Jianheng Cheng*[2]

[1] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China, [2] Guangdong Institute of Microbiology, State Key Laboratory of Applied Microbiology Southern China: Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangzhou, China

OPEN ACCESS

Edited by:
Maria Schirone,
University of Teramo, Italy

Reviewed by:
Aoife Boyd,
National University of Ireland Galway, Ireland
Learn-Han Lee,
Monash University Malaysia, Malaysia

***Correspondence:**
Qingping Wu
wuqp203@163.com

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

Received: 16 January 2016
Accepted: 04 April 2016
Published: 21 April 2016

Citation:
Xie T, Xu X, Wu Q, Zhang J and Cheng J (2016) Prevalence, Molecular Characterization, and Antibiotic Susceptibility of Vibrio parahaemolyticus from Ready-to-Eat Foods in China. Front. Microbiol. 7:549. doi: 10.3389/fmicb.2016.00549

Vibrio parahaemolyticus is the leading cause of foodborne outbreaks, particularly outbreaks associated with consumption of fish and shellfish, and represents a major threat to human health worldwide. This bacterium harbors two main virulence factors: the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH). Additionally, various serotypes have been identified. The extensive use of antibiotics is a contributing factor to the increasing incidence of antimicrobial-resistant *V. parahaemolyticus.* In the current study, we aimed to determine the incidence and features of *V. parahaemolyticus* in ready-to-eat (RTE) foods in China. We found 39 *V. parahaemolyticus* strains on Chinese RTE foods through investigation of 511 RTE foods samples from 24 cities in China. All isolates were analyzed for the presence of *tdh* and *trh* gene by PCR, serotyping was performed using multiplex PCR, antibiotic susceptibility analysis was carried out using the disk diffusion method, and molecular typing was performed using enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) typing and multilocus sequence typing (MLST). The results showed that none of the isolates were positive for *tdh* and *trh.* Most of the isolates (33.3%) were serotype O2. Antimicrobial susceptibility results indicated that most strains were resistant to streptomycin (89.7%), cefazolin (51.3%), and ampicillin (51.3%). The isolates were grouped into five clusters by ERIC-PCR and four clusters by MLST. We updated 10 novel loci and 33 sequence types (STs) in the MLST database. Thus, our findings demonstrated the presence of *V. parahaemolyticus* in Chinese RTE foods, provided insights into the dissemination of antibiotic-resistant strains, and improved our knowledge of methods of microbiological risk assessment in RTE foods.

Keywords: ***Vibrio parahaemolyticus,*** **ready-to-eat foods, antibiotic resistance, ERIC-PCR, MLST, serotype, virulence gene**

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative halophilic bacterium that naturally occurs worldwide in estuarine environments. This microorganism is recognized as one of the leading causes of foodborne illness worldwide and has been shown to cause acute gastroenteritis in humans. Complications such as septicemia can sometimes lead to death in patients with *V. parahaemolyticus* infection (Qadri et al., 2005; Lopatek et al., 2015). Previous studies have focused on the prevalence of *V. parahaemolyticus* in shellfish, oysters, water, seafood, and shrimp (Khouadja et al., 2013; Lopatek et al., 2015). However, no reports have identified isolates of *V. parahaemolyticus* on ready-to-eat (RTE) foods such as cooked meat, roasted poultry, and cold vegetable dishes in sauce, that are highly popular in China. Various foodborne pathogens may be present in RTE foods and may cause illnesses in consumers because RTE foods do not require heat treatment or other forms of curing before eating (Shi et al., 2015). Moreover, the high genetic diversity on RTE foods can facilitate identification of strain relatedness and epidemiological investigations.

Traditionally, *V. parahaemolyticus* is considered susceptible to antimicrobials. However, during the past few decades, antimicrobial resistance has emerged and evolved in many bacterial genera owing the excessive use of antimicrobials in human, agriculture, and aquaculture systems (Cabello, 2006; Kang et al., 2016). For example, tetracyclines are recommended as antibiotics in the treatment of severe *Vibrio* infections, and third-generation cephalosporins with doxycycline or fluoroquinolone alone are also used on occasion. Moreover, antibiotics are commonly used to treat fish (Han et al., 2007; Devi et al., 2009). Some *V. parahaemolyticus* isolates from seafood and other environments are commonly resistant to ampicillin, aminoglycosides (streptomycin and gentamicin), ciprofloxacin, chloramphenicol, and other antibiotics (Oh et al., 2011; Raissy et al., 2012; Shaw et al., 2014; Lopatek et al., 2015). Therefore, the potential presence of antibiotic-resistant *V. parahaemolyticus* on RTE foods may be an important public health problem related to disease management and control.

The virulence of *V. parahaemolyticus* is mainly attributed to the production of two major factors: thermo-stable direct hemolysin (TDH) encoded by the *tdh* gene and TDH-related hemolysin encoded by the *trh* gene (Honda and Iida, 1993). Clinical strains commonly contain either these genes, and the presence of these genes is associated with the pathogenicity of the strain in humans (Su and Liu, 2007; Jones et al., 2012; Pazhani et al., 2014). Detection of *V. parahaemolyticus* virulence factors is typically based on molecular biological analysis and amplification of *V. parahaemolyticus*-specific sequences (Shirai et al., 1990; West et al., 2013). To date, there are 13 O-serogroups and over 70 K-serogroups, differentiated on the basis of somatic (O) and capsular (K) antigens in *V. parahaemolyticus* (Ishibashi et al., 2000; Jones et al., 2012). The emergence of the first pandemic strain belonging to serovar O3:K6 (Okuda et al., 1997) supported the view that the serotype of *V. parahaemolyticus* is correlated with virulence. A multiplex polymerase chain reaction (PCR)-based O-antigen serotyping method has been adopted because other antiserum-based approaches are time consuming, expensive and can be associated with the risk of cross-reactions (Chen M. et al., 2012; Xu et al., 2014).

Molecular typing of *V. parahaemolyticus*, including pulsed field gel electrophoresis (PFGE) (Marshall et al., 1999), repetitive extragenic palindromic sequence PCR (REP-PCR) (Wong and Lin, 2001), and ribotyping (Bag et al., 1999), has been shown to be a useful tool for providing genetic relatedness information (Olive and Bean, 1999). Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) has been proven useful for subtyping of *V. parahaemolyticus* strains with highly conserved repetitive intergenic consensus sequences (De Bruijn, 1992; Chen W. et al., 2012). Additionally, multilocus sequence typing (MLST), which is based on sequence analysis of selected housekeeping genes (e.g., *recA*, *gyrB*, *dnaE*, *dtdS*, *pntA*, *pyrC*, and *tnaA*), is becoming an important method for investigation of the evolution and epidemiology of *V. parahaemolyticus* owing to its high repeatability (González-Escalona et al., 2008; Banerjee et al., 2014).

V. parahaemolyticus is not frequently found in RTE foods, but is still considered hazardous in humans. Therefore, the objectives of the current study were as follows: (i) to determine the prevalence and contamination level of *V. parahaemolyticus* in RTE foods in China and (ii) to determine the genetic variation and phenotypic characteristics of *V. parahaemolyticus* isolates from RTE foods. The information generated in this study will provide insights into the distribution and population of *V. parahaemolyticus* across Chinese RTE foods and differentiation of *V. parahaemolyticus* strains on different RTE foods.

MATERIALS AND METHODS

Sample Collection

From November 2011 to May 2014, a total of 511 RTE food samples were collected from retail markets in 24 cities, covering most provincial capitals of China (**Figure 1**). The samples consisted of 371 deli meat samples, 97 cold vegetable dishes or noodles in sauce, and 43 fried rice or noodle samples. The samples were placed in sterile sealed plastic bags, transported to the laboratory in a cold box below 4°C, and analyzed immediately.

Qualitative Detection

The bacteriological media used herein, unless indicated, were purchased from Guangdong Huankai Co. Ltd. (Guangzhou, China). For qualitative detection, the *V. parahaemolyticus* was isolated according to GB 4789.7-2013 for food microbiological examination of *V. parahaemolyticus* (National Food Safety Standards of China) with minor modifications. In brief, 25 g of each sample was homogenized for 60 s in a stomacher bag (Huankai Co. Ltd., Guangzhou, China) with 225 mL of alkaline peptone water (APW) containing 3% NaCl. Homogenates were incubated at 37°C for 16−18 h. After incubation, a loopful from the top 1 cm was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and incubated at 37°C for 18−24 h. Presumptive colonies (green or blue green colonies, 2−3 mm in diameter) were streaked onto Chromogenic Vibrio Medium and

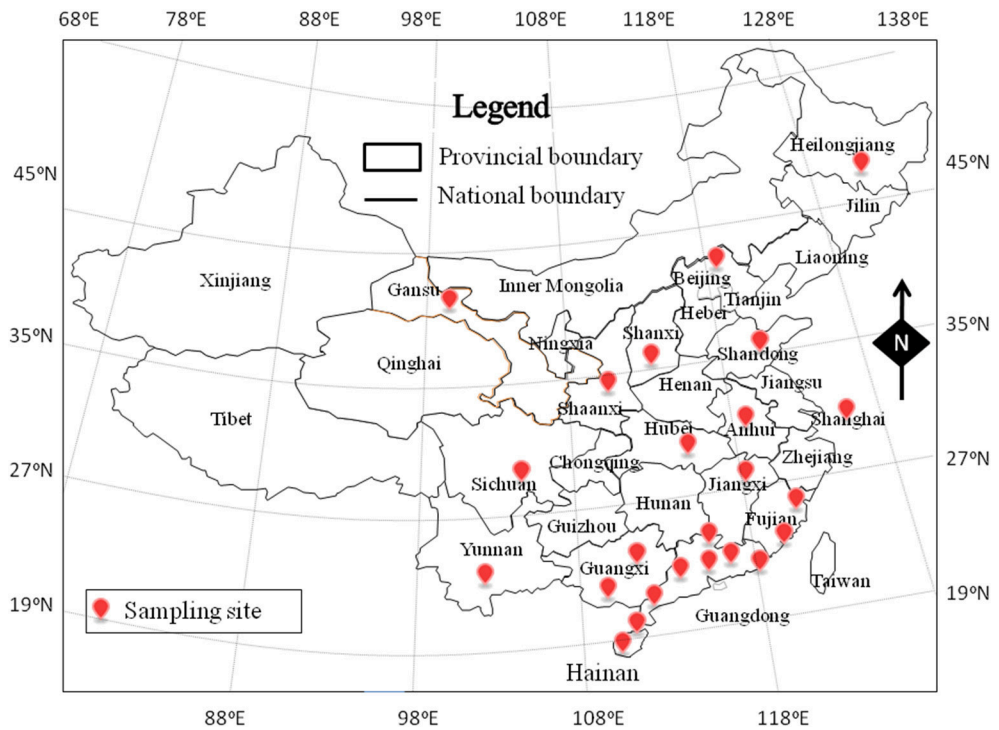


FIGURE 1 | The sampling site of ready-to-eat (RTE) foods in China.

incubated at 37°C for 24 h. The mauve colonies were selected for further *V. parahaemolyticus* identification by analysis of oxidase activity, Gram staining, 3.5% NaCl triple sugar iron (TSI) tests, halophilism tests, and API 20E diagnostic strips (Biomerieux Company, France).

MPN Enumeration (MPN) of *V. parahaemolyticus*

Enumeration of *V. parahaemolyticus* in RTE food samples was performed using the MPN method according to the Bacteriological Analytical Manual standard method (Kaysner and Depaola, 2004) and a previous study (Xu et al., 2014).

Detection of tdh and *trh* Genes

Detection of the *V. parahaemolyticus tdh* and *trh* genes was performed by PCR as described previously (Gutierrez West et al., 2013). The oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China) (Tdh-F: CTGTCCCTT TTCCTGCCCCCG, Tdh-R: AGCCAGACACCGCTGCCATTG; Trh-F: ACCTTTTCCTTCTCCWGGKTCSG, Trh-F: CCGCTC TCATATGCYTCGACAKT). Each reaction mixture included the following (total volume, 25 μL): 2 × PCR Mix (Qiagen), 12.5 μL; 0.5 μM each primer, dd H_2O, 9.5 μL; and DNA template, 1 μL. Both genes were amplified using the following thermal-cycling program: denaturation at 95°C for 5 min; 40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 2 min. PCR was conducted in a Bio-Rad PTC-200 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The amplified products were then analyzed electrophoretically on a 2% agarose gel containing Gold View. The images were captured digitally and analyzed using a Gel Image system (Bio-Rad). *V. parahaemolyticus* strains ATCC 33847 (*tdh*+) and ATCC 17802 (*trh*+) were used as positive controls, and distilled water was used as the negative control.

Multiplex Serotyping PCR

The serotypes of *V. parahaemolyticus* isolates were identified using the PCR-based O-antigen serotyping technique. The primer concentrations and amplification conditions used were as described previously (Chen M. et al., 2012). The primers used for this assay were synthesized by Sangon Biotech (Shanghai, China). The 12 primer pairs were divided into two groups to amplify target DNA; PCR group 1 was used to detect serogroups O1, O2, O4, O5, O6, and O10, whereas PCR group 2 was used to detect serogroups O3/O13, O7, O8, O9, O11, and O12. The PCR was performed in a 25-μL reaction mixture containing the following: 2 × PCR mix (Dongshen, Guangzhou, China), 12.5 μL; 0.5 μM each primer, dd H_2O, 9.5 μL; and DNA template, 1 μL. All amplifications were carried out with the following protocol: 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min. The thermal cycler was prewarmed to 80°C before all the reaction tubes were added in order to reduce nonspecific amplification. PCR was conducted in a Bio-Rad PTC-200 Thermal Cycler (Bio-Rad, California, USA). The amplified products were then analyzed electrophoretically on a 2% agarose gel containing Gold View. The images were captured digitally and

analyzed using the Gel Image system (Bio-Rad, California, USA). *V. parahaemolyticus* ATCC 17802 and ATCC 33847 were used as control strains.

Antimicrobial Susceptibility

The susceptibility of the *V. parahaemolyticus* isolates to antibiotics was examined by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012) and a previous study (Xie et al., 2015). Briefly, Muller-Hinton agar and a panel of 12 antibiotics disks were selected for resistance tests. These 12 antibiotic disks (Oxoid, Hampshire, UK) were ampicillin (10 μg), azithromycin (15 μg), cefazolin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), trimethoprim-sulfamethoxazole (25 μg), and tetracycline (30 μg). The results were expressed as sensitive (S), intermediate (I), or resistant (R) following the methods of the CLSI. *Escherichia coli* ATCC 25922 and *V. parahaemolyticus* ATCC 17802 were used as quality control organisms.

ERIC-PCR Analysis

ERIC-PCR analysis was performed on the *V. parahaemolyticus* isolates using a previously described protocol with some modifications (Chen W. et al., 2012; Xie et al., 2015). The reaction mixture (25 μL per reaction) consisted of 12.5 μL 2 × Long Taq Mix (Dongsheng Biotech, Guangzhou, China), 0.6 μM of each primer (5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′), and 100 ng template DNA. PCR was performed in a DNA thermocycler (Applied Biosystems, CA, USA) using the following procedure: one cycle of denaturation at 95°C for 5 min; 35 cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 3 min; and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 2.0% agarose gels followed by Goldview staining (0.005%, v/v; SBS Genetech, Beijing) and imaging with a UV Imaging System (GE Healthcare, WI, USA). The images were captured in TIFF file format for further analysis.

The size of each band in the ERIC patterns was determined, and the data were coded as 0 (absence) or 1 (presence). Cluster analysis was performed with NTSYS-pc (Version 2.10), a numerical taxonomy and multivariate analysis software package (Rohlf, 2000), based on Dice's similarity coefficient (SD), with a 1% position tolerance and the unweighted pair group method using arithmetic averages (UPGMA).

MLST

MLST analysis was conducted via the *V. parahaemolyticus* MLST website and database (http://pubmlst.org/vparahaemolyticus/) (Jolley et al., 2004). PCR conditions were denaturation at 96°C for 1 min; primer (Table S1; synthesized by Sangon Biotech, Shanghai, China) annealing at 58°C for 1 min; and extension at 72°C for 1 min, for 30 cycles; with a final extension step at 72°C for 10 min. PCR was performed with a Bio- Rad PTC-200 Thermal Cycler (Bio-Rad, California, USA) according to the manufacturer's directions. PCR products were sequenced on a BGI instrument (Shenzhen, China). The alignments of these sequences were determined using BioEdit. The sequences were analyzed online (http://pubmlst.org/vparahaemolyticus/) to assign allele numbers and define STs. New sequences for alleles and new ST profiles were submitted to the *V. parahaemolyticus* MLST database.

The evolution tree of the concatenated sequences of the seven loci was built based on the method of the Kimura-2-parameter in Mega 6.0 (Tamura et al., 2013). The ratio between the number of synonymous and nonsynonymous substitutions, showing the type of selection at each locus, was calculated using the method of Nei and Gojobori in Mega 6. The hypotheses of neutrality (dS = dN), purifying selection (dS/dN >1), and positive selection (dS/dN < 1) were tested using DNAsp 5.10 (Lüdeke et al., 2015).

RESULTS

V. parahaemolyticus in RTE Food Samples

Of the 511 samples tested, eight were positive by both qualitative and MPN methods; 12 showed positive results by the qualitative method only, while 10 were positive with the MPN method only. Thirty (5.9%) samples positive for *V. parahaemolyticus* were detected among 511 samples after qualitative and MPN analyses, including 22 (5.9%) of the 371 deli meat samples, seven (7.2%) of the 97 samples of cold vegetable dishes or noodles in sauce, and one (2.3%) of the 43 fried rice or noodle samples. In 18 positive samples detected by the MPN method, *V. parahaemolyticus* densities ranged between 3 and 100 MPN/g in all of the samples. The densities of rest positive samples were below 3.0 MPN/g. According to the National Food Safety Standards of China (GB 2727-2005, GB2726-2005), the pathogenic bacteria should not be detected in deli meat such as cooked meat or roasted meat.

Detection of *tdh* and *trh* Genes in *V. parahaemolyticus* Isolates

A total of 39 *V. parahaemolyticus* isolates were confirmed and tested for the presence of the *trh* and *tdh* genes. None of the isolates possessed the *tdh* and *trh* genes.

Serotyping by Multiplex PCR

With the exception of serotypes O9, O10, and O11, all other serotypes were detected among the isolates. Serotype O2 was the most prevalent (13 isolates), followed by serotype O4 (eight isolates). The results of the O-antigen serotyping for all 39 isolates are shown in **Table 1**. The serotypes of *V. parahaemolyticus* ATCC 17802 and ATCC 33847 were O1 and O4, respectively (Figure S2).

Antimicrobial Susceptibility

The susceptible, intermediate, and resistance rates of the 39 examined *V. parahaemolyticus* isolates with respect to 12 antibiotics are shown in **Table 2**. The resistance to streptomycin (S), ampicillin (AMP), cefazolin (KZ), cephalothin (KF), kanamycin (K), gentamicin (CN), and trimethoprim-sulfamethoxazole (SXT) reached 89.7, 51.3, 51.3, 41.0, 41.0, 2.6, and 2.6%, respectively. None of the strains showed resistance to azitromycin (AZM), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), or tetracycline (TE). Most of the isolates

TABLE 1 | Results of the PCR-based O-antigen serotyping of 39 *V. parahaemolyticus* isolates.

Serogroup		Product size (bp)	No. of isolates
Group 1	O1	474	3
	O2	238	13
	O4	671	8
	O5	852	3
	O6	1409	1
Group 2	O3/O13	868	1
	O7	385	1
	O8	680	2
	O12	256	7
Total			39

TABLE 2 | Antimicrobial resistance profiles of 39 *Vibrio parahaemolyticus* isolates.

Antimicrobial agent	*Vibrio parahaemolyticus* ($n = 39$)		
	NO.(%) of R	NO.(%) of I	NO.(%) of S
Ampicillin (AMP)	20 (51.3)	14 (35.9)	5 (12.8)
Azitromycin (AZM)	0 (0.0)	14 (35.9)	25 (64.1)
Cefazolin (KZ)	20 (51.3)	18 (46.1)	1 (2.6)
Cephalothin (KF)	16 (41.0)	22 (56.4)	1 (2.6)
Chloramphenicol (C)	0 (0.0)	1 (2.6)	38 (97.4)
Ciprofloxacin (CIP)	0 (0.0)	13 (33.3)	26 (66.7)
Gentamicin (CN)	1 (2.6)	18 (46.1)	20 (51.3)
Kanamycin (K)	16 (41.0)	20 (51.3)	3 (7.7)
Nalidixic acid (NA)	0 (0.0)	2 (5.1)	37 (94.9)
Streptomycin (S)	35 (89.7)	4 (10.3)	0 (0.0)
Trimethoprim-sulfamethoxazole (SXT)	1 (2.6)	4 (10.3)	34 (87.1)
Tetracycline (TE)	0 (0.0)	4 (10.3)	35 (89.7)

**R, resistant; I, intermediate resistance; S, susceptibility.*

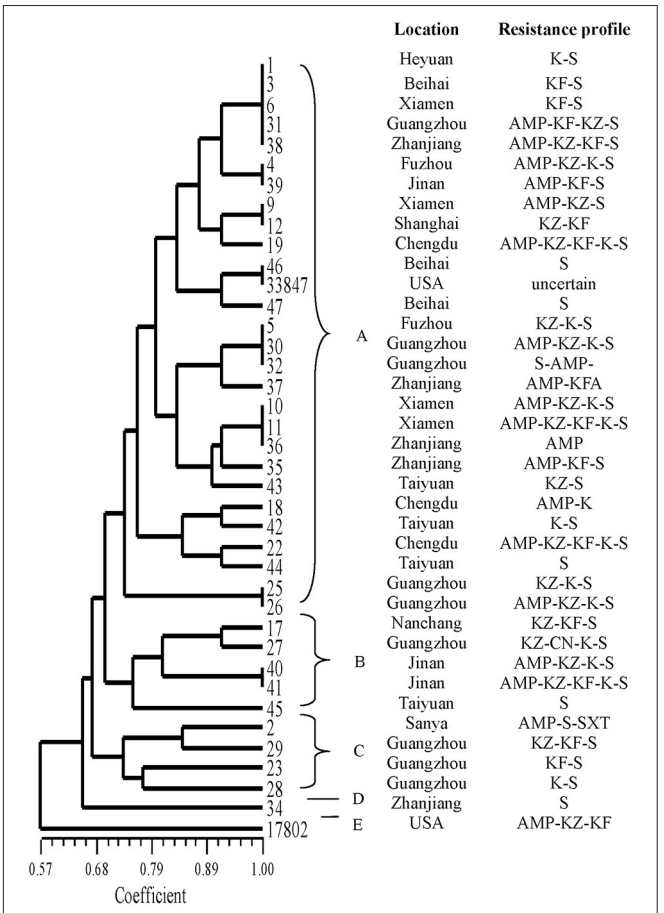


FIGURE 2 | ERIC-PCR DNA fingerprint analysis of *V. parahaemolyticus* isolates in RTE foods from China.

were resistant to streptomycin, with resistance and intermediate rates of 89.3 and 10.3%, respectively, which was consistent with a previous study (Xie et al., 2015). The next-highest susceptible rates were observed for trimethoprim-sulfamethoxazole (87.1%) and gentamicin (51.3%). In addition, there were four multidrug-resistant isolates showing resistance to five antibiotics, of which one isolate was collected from deli meat in Xiamen, two isolates were obtained from deli meat in Chengdu, and one isolate was obtained from deli meat in Jinan (Table S2).

ERIC-PCR

The results of ERIC-PCR analysis of the 39 isolates are shown in **Figure 2**. ERIC-PCR resulted in 3–8 amplification bands, with sizes ranging from 130 to about 6000 bp. The ERIC image shows that bands with molecular sizes of 500, 1500, and 2500 bp were common (Figure S1). All the isolates were classified into five clusters (designated as A, B, C, D, and E). Most isolates were distributed on cluster A. Only one strain (no. 34) from Zhanjiang divided into cluster D. The standard strain ATCC 17802 was in cluster E alone. The other standard strain (ATCC 33847) was belonged on cluster A and on the same sub-cluster along with isolates nos. 46 and 47.

MLST

All *V. parahaemolyticus* isolates were analyzed by MLST using the sequences generated from internal fragments of seven HK genes. Numbers for alleles and sequence types (STs) were assigned according to the database created for *V. parahaemolyticus* on submitting the sequence results (**Table 3**). The potential novel loci would appear some mismatching base. Seven gene locus mismatching the ST types on database maybe the novel one. The administrator of the website was notified when we obtained novel loci and ST types; after confirmation, new numbers will be given. The numbers of alleles observed for each MLST locus in our study were distributed as follows: 22 (*dna E*), 22 (*gyr B*), 27 (*rec A*), 20 (*dtd S*), 18 (*pnt A*), 24 (*pyr C*), and 18 (*tna A*). Thirty-three STs were observed among the 39 isolates. There were ten novel loci: *dna E* 302, 303; *gyr B* 428; *rec A* 306, 307; d*td S* 371; *pnt A* 213; *pyr C* 339, 340; and *tna A* 238. Most of the isolates (26) were novel STs, namely 1228–1251, 1301, and 1302. Among all 3682 sites, the number of variable sites was 230; the total number of mutations was 245; the haplotype

TABLE 3 | The MLST result of *Vibrio parahaemolyticus.*

NO.	Name	Source	dnaE	gyrB	recA	dtdS	pntA	pyrC	tnaA	ST
1	462tf	Deli meat	51	4	77	67	213*	8	24	1228*
2	VP002	Deli meat	60	170	133	145	2	130	26	291
3	642B3	Deli meat	42	88	113	242	18	99	23	1229*
4	VP004	Fried rice	60	284	4	53	43	63	23	1013
5	743tf	Deli meat	152	57	4	14	213*	54	14	1230*
6	VP006	Cold vegetable dishes in sauce	69	92	69	114	54	71	24	212
7	VP007	Cold vegetable dishes in sauce	69	92	69	114	54	71	24	212
8	VP008	Cold vegetable dishes in sauce	69	92	69	114	54	71	24	212
9	VP009	Deli meat	47	58	53	19	50	37	26	162
10	VP010	Deli meat	47	58	53	19	50	37	26	162
11	VP011	Deli meat	47	58	53	19	50	37	26	162
12	VP811	Deli meat	47	58	53	13	50	37	26	1231*
17	VP943	Deli meat	5	106	31	214	2	142	106	1232*
18	VP1029	Deli meat	31	106	135	19	26	62	54	1233*
19	VP1044B3	Deli meat	112	104	307*	13	23	339*	54	1301*
22	VP1409B3	Deli meat	302*	133	286	151	18	340*	37	1234*
23	VP1409	Cold vegetable dishes in sauce	11	75	64	151	124	7	50	1235*
25	VP1586A2	Deli meat	295	104	3	176	28	37	51	1236*
26	VP1588	Deli meat	26	428*	19	220	23	278	9	1237*
27	VP1588A1	Deli meat	5	106	307*	13	2	142	106	1238*
28	VP1588B3	Deli meat	44	260	31	67	26	200	99	1302*
29	VP1588C1	Deli meat	148	355	74	103	127	101	202	1239*
30	VP1589	Cold vegetable dishes in sauce	9	21	15	185	4	10	26	1240*
31	VP1609B1	Cold vegetable dishes in sauce	303*	25	246	185	31	252	73	1241*
32	VP1636	Deli meat	303*	25	306*	185	31	252	73	1242*
34	1787A3	Deli meat	132	16	286	371*	26	76	54	1243*
35	1787B2	Deli meat	11	106	192	19	71	73	17	1244*
36	1787C3	Deli meat	11	106	306*	220	71	73	17	1245*
37	1810	Cold noodles in sauce	36	131	44	67	102	5	37	1246*
38	1810C3	Cold noodles in sauce	126	25	123	103	103	7	26	1247*
39	VP039	Cold noodles in sauce	9	213	165	185	2	46	1	396
40	VP040	Cold noodles in sauce	9	213	165	185	2	46	1	396
41	2011A3	Deli meat	9	213	257	185	2	46	1	1248*
42	2138B2	Deli meat	44	58	257	371*	4	3	238*	1249*
43	VP043	Deli meat	148	355	74	19	127	101	202	847
44	VP044	Deli meat	44	58	257	371*	4	3	238*	1249*
45	2138C1	Deli meat	19	106	81	372*	31	5	33	1250*
46	VP046	Deli meat	14	30	67	46	27	11	13	300
47	2384A2	Deli meat	5	303	173	373*	152	318	23	1251*

""The novel locis and STs.*

number was 33; and the haplotype diversity was 0.989. All loci showed ratios of synonymous and nonsynonymous substitutions (dN/dS) below 1. A minimum evolution tree was constructed using the concatenated sequences of each allele (**Figure 3**).

DISCUSSION

V. parahaemolyticus is a major seafood-borne gastroenteritis-causing bacterium that is frequently isolated from aquatic products (Letchumanan et al., 2015b). In our study, we analyzed 511 samples from Chinese RTE foods and isolated 39 *V. parahaemolyticus* strains. In our study, some samples only detected positive in the qualitative method. It may be related to the variation in culture concentration. Enrichment culture was streaked onto TCBS agar plates with undiluted liquid in qualitative method, while culture represent 1/10, 1/100, 1/1000 dilution were streaked onto TCBS agar plates in MPN method. For some samples were only positive by the MPN method, the reason maybe attribute to the incomplete homogeneous of the sample culture solution. When a loopful (10 μL) of bacterial suspension was streaked onto the TCBS

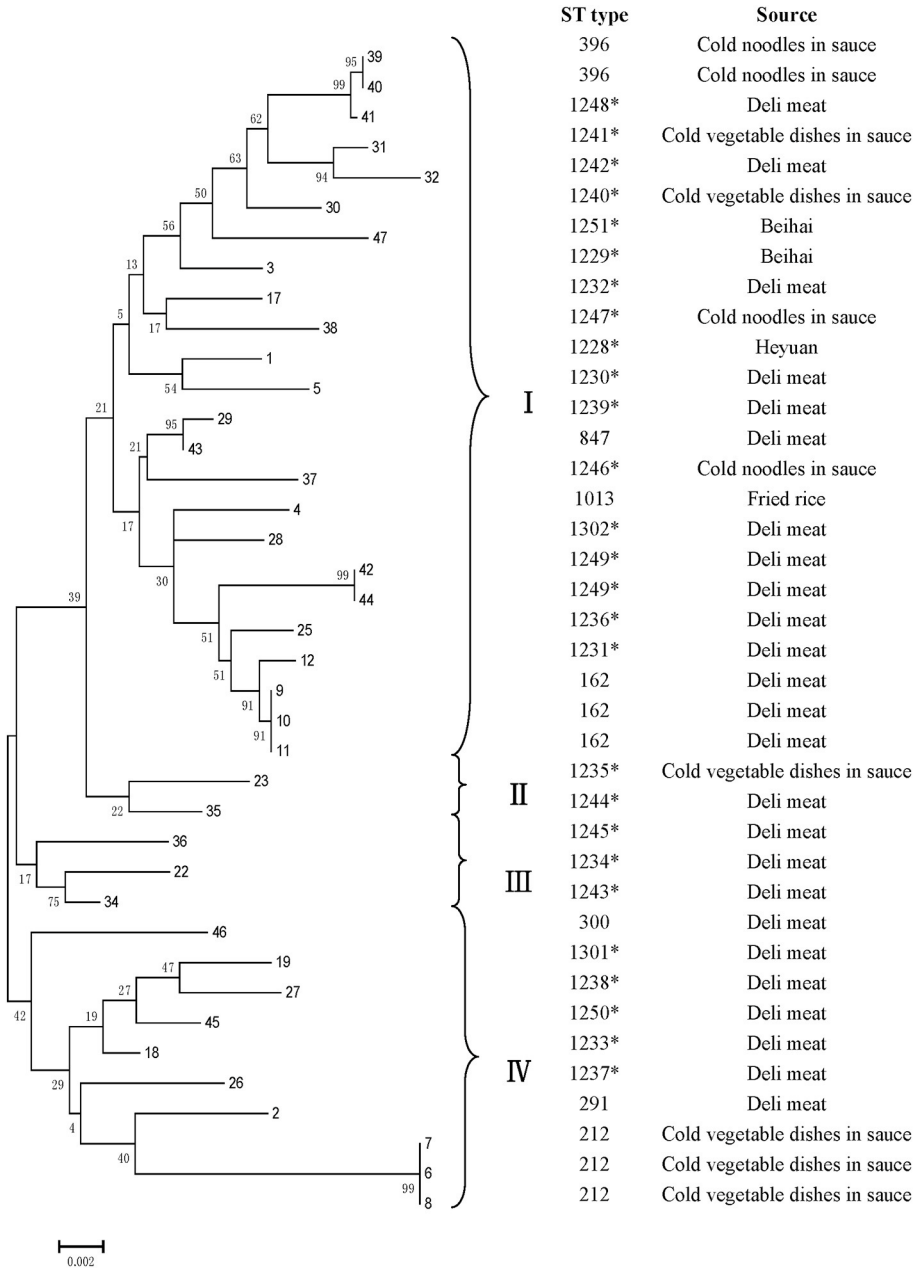


FIGURE 3 | MLST minimum evolution tree of the 39 V. *parahaemolyticus* RTE foods isolates. The tree was built with Mega six software using concatenated sequences. * indicates the novel STs.

agar plate could not certain to form colonies on the agar. A combination of qualitative and MPN methods avoid missing detection of *V. parahaemolyticus* effectively. Unlike *Listeria monocytogenes* (Shi et al., 2015) and *Salmonella* (Yang et al., 2016), *V. parahaemolyticus* was not frequently detected on RTE foods. Previous study reported that no *V. parahaemolyticus* was positive in 145 samples of RTE food in Korea (Chung et al., 2010), similar result was found in Iran (Zarei et al., 2012). However, a report indicated that the prevalence of *V. parahaemolyticus* in RTE foods was an important cause of food poisoning in Shanghai, China (Tian et al., 2008). As we know, RTE foods do not need further processing before consumption. Thus, identification of contamination in RTE foods is critical for assuring food safety. Our research can provide insights into food safety in RTE foods.

TDH and TRH are considered major virulence factors in *V. parahaemolyticus* (Ceccarelli et al., 2013; Letchumanan et al., 2014; Raghunath, 2014). Moreover, the presence of *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains represents a major public health risk. In our study, strains identified in Chinese RTE foods strains were negative for both *trh* and *tdh* genes. These results

are consistent with the findings of a previous study reported in India (Raghunath et al., 2008), but this was contradictory to most findings from other previous studies (Zhao et al., 2011; Letchumanan et al., 2015a). The overall mechanism of *V. parahaemolyticus* pathogenesis remains unclear (Ceccarelli et al., 2013); although TDH and TRH have been shown to be correlated with pathogenic strains, they do not fully explain the pathogenicity of *V. parahaemolyticus* (Lynch et al., 2005). Several studies have reported the presence of clinical strains without *tdh* and *trh* (Shirai et al., 1990). Thus, even in the absence of *tdh* or *trh*, *V. parahaemolyticus* still remains pathogenic, and some environmental isolates lacking *tdh* and/or *trh* can produce putative virulence factors. For example, some oyster isolates will contain T3SS1 genes without *tdh* and/or *trh* (Mahoney et al., 2010; Jones et al., 2012).

In 1996, pandemic O3:K6 serovars were shown to be responsible for *V. parahaemolyticus* outbreaks. The serotype O3:K6 *V. parahaemolyticus* emerged from India and spread throughout the world, including to China and the USA (Okuda et al., 1997; Honda et al., 2008; Tan et al., 2010). Our study indicated that serovar O2 was the predominant serotype among the strains isolated from RTE foods. These findings are in contrast with the results of a previous study, in which the O3 serotype was identified as the predominant serotype in China (Zhang et al., 2006; Zhao et al., 2011). O3:K6, O1:Kut, O4:K8, and O2:K3 were also the dominant serovars identified in outbreaks of *V. parahaemolyticus* in China (Zhang et al., 2013; Ma et al., 2014). The O3 isolate was the same as the most frequent serotype among our clinical isolates (source: Shenzhen Centres for Disease Control). We also showed the presence of other O-type serovars of *V. parahaemolyticus* from RTE foods; the results demonstrated the diverse distributions in different RTE food types and locations in China. The changes in pandemic serogroups of *V. parahaemolyticus* have been reported to occur over time, an increasing number of nonpandemic serogroups have been shown to carry pandemic marker genes (Matsumoto et al., 2000). Therefore, these O2 strains from Chinese RTE foods may have the potential for pathogenesis in humans.

With the steady expansion of the Asian aquaculture industry, in order to increase production, aquaculture farmers are using different antibiotics to prevent (prophylactic use) and treat (therapeutic use) pathogenic bacterial infections in aquatic products (Cabello et al., 2013; Huang et al., 2015; Tan et al., 2016). Furthermore, the continuous and extensive use of antibiotics in humans, has led to the emergence of antimicrobial-resistant *V. parahaemolyticus* strains worldwide (Sani et al., 2013; Yano et al., 2014). In our study, the highest resistance rate was observed for streptomycin. Similarly, previous study have demonstrated the occurrence of streptomycin- and ampicillin-resistant *V. parahaemolyticus* isolates (Pazhani et al., 2014). We also found a small number of isolates showing resistance to gentamicin and trimethoprim-sulfamethoxazole, which are first-line drugs used in clinic treatment. Moreover, we found that most of the strains (21/39 isolates) were multidrug resistant. As RTE foods are eaten without cooking, the presence of these strains will increase the health risks of consuming such foods in humans. Thus, it may be important to evaluate variations in antimicrobial susceptibility profiles in *V. parahaemolyticus* strains.

Molecular subtyping is widely used for the analysis of genetic diversity. ERIC-PCR provides discriminatory values and can be used for rapid *V. parahaemolyticus* typing (Khan et al., 2002). Compared to *V. parahaemolyticus* isolates from the 1997 Canadian outbreak using three method, they found that ERIC-PCR and ribotyping were the most informative typing methods (Marshall et al., 1999). Using this approach, the isolates were classified into five clusters at 0.65 similarity. Most of the strains were on cluster A; which showed they were may be genetically related. The reference strain ATCC 17804 harbors *trh* grouped into a single cluster E, exhibiting differences with other strains. Strain (no. 34), from Zhanjiang, was grouped into cluster D alone and was genetically diverse from other isolates. Clustering based on ERIC-PCR did not coincide with the isolation sources or patterns of antibiotic resistance. The result was similar to other studies, showing the high genetic diversity in *V. parahaemolyticus* strains. MLST is a good method for typing owing to its reproducibility, as shown in the sequencing of seven housekeeping genes; this method has been widely used for analysis of the *V. parahaemolyticus* sequence (Lüdeke et al., 2015). In our study, all the isolates could be grouped into four main clusters (I, II, III, and IV). ST 291, ST 396, and ST 300 were identified in a public database as environmental isolates from China; ST1013 was separated from environmental samples from USA. Thus, some *V. parahaemolyticus* isolates from RTE foods were similar to environment strains. Additionally, one ST162 strain was reported as a clinical isolate from the USA. With the identification of 10 novel loci and 22 novel STs, this study substantially contributed to the diversity in the MLST database. As most strains identified in RTE foods *V. parahaemolyticus* stains were novel STs, these results suggested that *V. parahaemolyticus* strains found in RTE foods may be distinct from other stains. In our study, both ERIC-PCR and MLST confirmed the genetic diversity within strains.

In summary, this is the first comprehensive study that described the prevalence, serotype, virulence, antibiotic resistance phenotype, and molecular subtyping of *V. parahaemolyticus* from RTE foods in China. This study showed that none of the isolates possessed *tdh* and *trh*, and serotype O2 was found to be prevalent. The antimicrobial-resistance patterns revealed that the streptomycin-resistant were widespread (89.7%) and the isolates resistance to some clinical antibiotics such as cephalothin, gentamicin. ERIC-PCR and MLST typing showed genetic diversity. The novel loci and STs indicated genetically diverse on RTE foods isolates. As RTE foods are common and popular food choices in China, therefore the continuous monitoring of food-borne pathogens such as *V. parahaemolyticus* are vital to ensure the safety of these food products.

AUTHOR CONTRIBUTIONS

TX, XX are the common first authors, finsh the article experiment and write the article together. QW (Corresponding Author) give

the idea and experiments support. JZ, JC help to finish the experiment on article.

ACKNOWLEDGMENTS

XX, TX contributed equally to this work. This work was supported by research grants from the Key Projects in the National Science & Technology Pillar Program during the Twelfth 5-year Plan Period (No. 2013BAD16B05), Guangdong province science and technology plan projects (No.2012A020100010), Natural Science Foundation of Guangdong Province (No. S2012030006235).We would like to thank Editage for providing editorial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00549

REFERENCES

Bag, P. K., Nandi, S., Bhadra, R. K., Ramamurthy, T., Bhattacharya, S. K., Nishibuchi, M., et al. (1999). Clonal diversity among recently emerged strains of *Vibrio parahaemolyticus* O3: K6 associated with pandemic spread. *J. Clin. Microbiol.* 37, 2354–2357.

Banerjee, S. K., Kearney, A. K., Nadon, C. A., Peterson, C.-L., Tyler, K., Bakouche, L., et al. (2014). Phenotypic and genotypic characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from 2000 to 2009. *J. Clin. Microbiol.* 52, 1081–1088. doi: 10.1128/JCM.03047-13

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* 8, 1137–1144. doi: 10.1111/j.1462-2920.2006.01054.x

Cabello, F. C., Godfrey, H. P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., et al. (2013). Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. *Environ. Microbiol.* 15, 1917–1942. doi: 10.1111/1462-2920.12134

Ceccarelli, D., Hasan, N. A., Huq, A., and Colwell, R. R. (2013). Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Front. Cell. Infect. Microbiol.* 3:97. doi: 10.3389/fcimb.2013.00097

Chen, M., Guo, D., Wong, H.-C., Zhang, X., Liu, F., Chen, H., et al. (2012). Development of O-serogroup specific PCR assay for detection and identification of *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.* 159, 122–129. doi: 10.1016/j.ijfoodmicro.2012.08.012

Chen, W., Xie, Y., Xu, J., Wang, Q., Gu, M., Yang, J., et al. (2012). Molecular typing of *Vibrio parahaemolyticus* isolates from the middle-east coastline of China. *Int. J. Food Microbiol.* 153, 402–412. doi: 10.1016/j.ijfoodmicro.2011.12.001

Chung, M. S., Kim, C. M., and Ha, S. (2010). Detection and enumeration of microorganisms in ready-to-eat foods, ready-to-cook foods and fresh-cut produce in Korea. *J. Food Saf.* 30, 480–489. doi: 10.1111/j.1745-4565.2010.00221.x

CLSI (2012). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Approved Standard M45-A*. Wayne, PA: Clinical and Laboratory Standards Institute.

De Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil *bacteria*. *Appl. Environ. Microbiol.* 58, 2180–2187.

Devi, R., Surendran, P., and Chakraborty, K. (2009). Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from shrimp farms along the southwest coast of India. *World J. Microbiol. Biotechnol.* 25, 2005–2012. doi: 10.1007/s11274-009-0101-8

González-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R. T., Jaykus, L.-A., and Depaola, A. (2008). Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. *J. Bacteriol.* 190, 2831–2840. doi: 10.1128/JB.01808-07

Gutierrez West, C. K., Klein, S. L., and Lovell, C. R. (2013). High frequency of virulence factor genes *tdh*, *trh*, and *tlh* in *Vibrio parahaemolyticus* strains isolated from a pristine estuary. *Appl. Environ. Microbiol.* 79, 2247–2252. doi: 10.1128/AEM.03792-12

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

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., Iida, T., Akeda, Y., and Kadama, T. (2008). Sixty Years of *Vibrio parahaemolyticus* Research-Despite decades of research following a severe foodborne outbreak in Japan, this pathogen remains mysterious at the molecular level. *Microbe* 3, 462.

Huang, Y., Zhang, L., Tiu, L., and Wang, H. H. (2015). Characterization of antibiotic resistance in commensal bacteria from an aquaculture ecosystem. *Front. Microbiol.* 6:00914. doi: 10.3389/fmicb.2015.00914

Ishibashi, M., Ohta, K., Shimada, T., Honda, T., Sugiyama, J., Miwatani, T., et al. (2000). Current status of OK serotype combinations of *Vibrio parahaemolyticus*. *Nippon Saikingaku Zasshi.* 55, 539–541. doi: 10.3412/jsb.55.539

Jolley, K. A., Chan, M.-S., and Maiden, M. C. (2004). mlstdbNet–distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics* 5:86. doi: 10.1186/1471-2105-5-86

Jones, J. L., Lüdeke, C. H. M., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B., et al. (2012). Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *J. Clinl. Microbiol.* 50, 2343–2352. doi: 10.1128/JCM.00196-12

Kang, C.-H., Shin, Y., Kim, W., Kim, Y., Song, K., Oh, E.-G., et al. (2016). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from oysters in Korea. *Environ.Sci. Pollut. Res.* 23, 918–926. doi: 10.1007/s11356-015-5650-9

Kaysner, C. A., and Depaola, A. J. (2004). *U. S. Food and Drug Administration; Bacteriological Analytical Manual; Methods for Specific Pathogens; Chapter 9 Vibrio*. Available online at http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070830.htm

Khan, A. A., Mccarthy, S., Wang, R.-F., and Cerniglia, C. E. (2002). Characterization of United States outbreak isolates of *Vibrio parahaemolyticus* using enterobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3: K6 isolates. *FEMS Microbiol. Lett.* 206, 209–214. doi: 10.1111/j.1574-6968.2002.tb11011.x

Khouadja, S., Suffredini, E., Spagnoletti, M., Croci, L., Colombo, M. M., and Amina, B. (2013). Presence of pathogenic *Vibrio Parahaemolyticus* in waters and seafood from the Tunisian Sea. *World J. Microbiol. Biotechnol.* 29, 1341–1348. doi: 10.1007/s11274-013-1297-1

Letchumanan, V., Chan, K.-G., and Lee, L.-H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front. Microbiol.* 5:705. doi: 10.3389/fmicb.2014.00705

Letchumanan, V., Pusparajah, P., Hern, T., Loh, T., Yin, W. F., Lee, L.-H., et al. (2015a). Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* from shellfish in Selangor, Malaysia. *Front. Microbiol.* 6:1417. doi: 10.3389/fmicb.2015.01417

Letchumanan, V., Yin, W.-F., Lee, L.-H., and Chan, K.-G. (2015b). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Front. Microbiol.* 6:33. doi: 10.3389/fmicb.2015.00033

Lopatek, M., Wieczorek, K., and Osek, J. (2015). Prevalence and antimicrobial resistance of *Vibrio parahaemolyticus* Isolated from Raw Shellfish in Poland. *J. Food Protect.* 78, 1029–1033. doi: 10.4315/0362-028X.JFP-14-437

Lüdeke, C., González-Escalona, N., Fischer, M., and Jones, J. L. (2015). Examination of clinical and environmental *Vibrio parahaemolyticus* isolates by multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA). *Front. Microbiol.* 6:564. doi: 10.3389/fmicb.2015.00564

Lynch, T., Livingstone, S., Buenaventura, E., Lutter, E., Fedwick, J., Buret, A. G., et al. (2005). *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production. *Infect. Immun.* 73, 1275–1283. doi: 10.1128/IAI.73.3.1275-1283.2005

Ma, C., Deng, X., Ke, C., He, D., Liang, Z., Li, W., et al. (2014). Epidemiology and etiology characteristics of foodborne outbreaks caused by *Vibrio parahaemolyticus* during 2008–2010 in Guangdong province, China. *Foodborne Pathog. Dis.* 11, 21–29. doi: 10.1089/fpd.2013.1522

Mahoney, J. C., Gerding, M. J., Jones, S. H., and Whistler, C. A. (2010). Comparison of the pathogenic potentials of environmental and clinical *Vibrio parahaemolyticus* strains indicates a role for temperature regulation in virulence. *Appl. Environ. Microbiol.* 76, 7459–7465. doi: 10.1128/AEM.01450-10

Marshall, S., Clark, C. G., Wang, G., Mulvey, M., Kelly, M. T., and Johnson, W. M. (1999). Comparison of Molecular Methods for Typing *Vibrio parahaemolyticus*. *J. Clin. Microbiol.* 37, 2473–2478.

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., et al. (2000). Pandemic spread of an O3: K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. *J. Clin. Microbiol.* 38, 578–585.

Oh, E.-G., Son, K.-T., Yu, H., Lee, T.-S., Lee, H.-J., Shin, S., et al. (2011). Antimicrobial resistance of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* strains isolated from farmed fish in Korea from 2005 through 2007. *J. Food Prot.* 74, 380–386. doi: 10.4315/0362-028X.JFP-10-307

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A. K., et al. (1997). Emergence of a unique O3: K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35, 3150–3155.

Olive, D. M., and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37, 1661–1669.

Pazhani, G. P., Bhowmik, S. K., Ghosh, S., Guin, S., Dutta, S., Rajendran, K., et al. (2014). Trends in the epidemiology of pandemic and non-pandemic strains of *Vibrio parahaemolyticus* isolated from diarrheal patients in Kolkata, India. *PLoS. Negl. Trop. Dis.* 8:2815. doi: 10.1371/journal.pntd.0002815

Qadri, F., Chowdhury, N. R., Takeda, Y., and Nair, G. B. (2005). "*Vibrio parahaemolyticus*—seafood safety and associations with higher organisms," in *Oceans and Health Pathogens in the Marine Environment*, ed S. S. Belkin and R. R. Colwell (New York, NY: Springer), 277–295.

Raghunath, P. (2014). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Front. Microbiol.* 5:805. doi: 10.3389/fmicb.2014.00805

Raghunath, P., Acharya, S., Bhanumathi, A., Karunasagar, I., and Karunasagar, I. (2008). Detection and molecular characterization of *Vibrio parahaemolyticus* isolated from seafood harvested along the southwest coast of India. *Food Microbiol.* 25, 824–830. doi: 10.1016/j.fm.2008.04.002

Raissy, M., Moumeni, M., Ansari, M., and Rahimi, E. (2012). Antibiotic resistance pattern of some Vibrio strains isolated. *Iran. J. Fish. Sci.* 11, 618–626.

Rohlf, F. J. (2000). *NTSYS-pc. Numerical Taxonomy and Multi-Variate Analysis System,* Version 2.1. Setauket, NY: Exeter Software.

Sani, N. A., Ariyawansa, S., Babji, A. S., and Hashim, J. K. (2013). The risk assessment of *Vibrio parahaemolyticus* in cooked black tiger shrimps (Penaeus monodon) in Malaysia. *Food Control* 31, 546–552. doi: 10.1016/j.foodcont.2012.10.018

Shaw, K. S., Rosenberg Goldstein, R. E., He, X., Jacobs, J. M., Crump, B. C., and Sapkota, A. R. (2014). Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. *PLoS ONE* 9:e89616. doi: 10.1371/journal.pone.0089616

Shi, W., Qingping, W., Jumei, Z., Moutong, C., and Zéan, Y. (2015). Prevalence, antibiotic resistance and genetic diversity of Listeria monocytogenes isolated from retail ready-to-eat foods in China. *Food Control* 47, 340–347. doi: 10.1016/j.foodcont.2014.07.028

Shirai, H., Ito, H., Hirayama, T., Nakamoto, Y., Nakabayashi, N., Kumagai, K., et al. (1990). Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *Infect. Immun.* 58, 3568–3573.

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

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

Tan, H., Li, B., Deng, X., Fang, W., Ke, B., and Ke, C. (2010). Molecular characteristics of *Vibrio parahaemolyticus* isolated from food-poisoning events in Guangdong Province, 2008. *South China J. Preven. Med.* 36, 23–27.

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

Tian, M.-S., Zheng, L.-J., Peng, S.-J., Li, J., Zhang, L., Qi, L.-B., et al. (2008). Analysis and measures on collective food poisoning caused by *Vibrio parahaemolyticus* in Shanghai Municipality During 2000-2007 [J]. *China J. Food Hyg.* 6, 017.

West, C. K. G., Klein, S. L., and Lovell, C. R. (2013). High frequency of virulence factor genes tdh, trh, and tlh in *Vibrio parahaemolyticus* strains isolated from a pristine estuary. *Appl. Environ. Microbiol.* 79, 2247–2252. doi: 10.1128/AEM.03792-12

Wong, H.-C., and Lin, C.-H. (2001). Evaluation of typing of *Vibrio parahaemolyticus* by three PCR methods using specific primers. *J. Clin. Microbiol.* 39, 4233–4240. doi: 10.1128/JCM.39.12.4233-4240.2001

Xie, T., Wu, Q., Xu, X., Zhang, J., and Guo, W. (2015). Prevalence and population analysis of *Vibrio parahaemolyticus* in aquatic products from South China markets. *FEMS Microbiol. Lett.* 362:fnv178. doi: 10.1093/femsle/fnv178

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

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

Yano, Y., Hamano, K., Satomi, M., Tsutsui, I., Ban, M., and Aue-Umneoy, D. (2014). Prevalence and antimicrobial susceptibility of Vibrio species related to food safety isolated from shrimp cultured at inland ponds in Thailand. *Food Control* 38, 30–36. doi: 10.1016/j.foodcont.2013.09.019

Zarei, M., Maktabi, S., and Ghorbanpour, M. (2012). Prevalence of Listeria monocytogenes, *Vibrio parahaemolyticus*, Staphylococcus aureus, and Salmonella spp. in seafood products using multiplex polymerase chain reaction. *Foodborne Pathog. Dis.* 9, 108–112. doi: 10.1089/fpd.2011.0989

Zhang, H., Sun, S., Shi, W., Cui, L., and Gu, Q. (2013). Serotype, virulence, and genetic traits of foodborne and clinical *Vibrio parahaemolyticus* isolates in Shanghai, China. *Foodborne Pathog. Dis.* 10, 796–804. doi: 10.1089/fpd.2012.1378

Zhang, W., Pan, J., Meng, D., and Chen, K. (2006). [Molecular typing on *Vibrio parahaemolyticus* isolates from Hangzhou, China, during 2000-2002]. *Zhonghua Liu Xing Bing Xue Za Zhi* 27, 343–346.

Zhao, F., Zhou, D.-Q., Cao, H.-H., Ma, L.-P., and Jiang, Y.-H. (2011). Distribution, serological and molecular characterization of *Vibrio parahaemolyticus* from shellfish in the eastern coast of China. *Food Control* 22, 1095–1100. doi: 10.1016/j.foodcont.2010.12.017

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 April 2016
doi: 10.3389/fmicb.2016.00464

Use of *Lactobacillus plantarum* Strains as a Bio-Control Strategy against Food-Borne Pathogenic Microorganisms

Mattia Pia Arena[1], Amandine Silvain[2], Giovanni Normanno[1], Francesco Grieco[3], Djamel Drider[2], Giuseppe Spano[1] and Daniela Fiocco[4]*

[1] Department of Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy, [2] Laboratoire Régional de Recherche en Agroalimentaire et Biotechnologies, Institut Charles Viollette-Université Lille 1, Université de Lille, Lille, France, [3] Institute of Sciences of Food Production (ISPA), Lecce Section, Consiglio Nazionale delle Ricerche, Lecce, Italy, [4] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

Lactobacillus plantarum is one of the most versatile species extensively used in the food industry both as microbial starters and probiotic microorganisms. Several *L. plantarum* strains have been shown to produce different antimicrobial compounds such as organic acids, hydrogen peroxide, diacetyl, and also bacteriocins and antimicrobial peptides, both denoted by a variable spectrum of action. In recent decades, the selection of microbial molecules and/or bacterial strains able to produce antagonistic molecules to be used as antimicrobials and preservatives has been attracting scientific interest, in order to eliminate or reduce chemical additives, because of the growing attention of consumers for healthy and natural food products. The aim of this work was to investigate the antimicrobial activity of several food-isolated *L. plantarum* strains, analyzed against the pathogenic bacteria *Listeria monocytogenes*, *Salmonella Enteritidis*, *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Antagonistic activity was assayed by agar spot test and revealed that strain *L. plantarum* 105 had the strongest ability to contrast the growth of *L. monocytogenes*, while strains *L. plantarum* 106 and 107 were the most active microorganisms against *E. coli* O157:H7. The antimicrobial ability was also screened by well diffusion assay and broth micro-dilution method using cell-free supernatants (CFS) from each *Lactobacillus* strain. Moreover, the chemical nature of the molecules released in the CFS, and possibly underlying the antagonistic activity, was preliminary characterized by exposure to different constraints such as pH neutralization, heating, catalase, and proteinase treatments. Our data suggest that the ability of *L. plantarum* cultures to contrast pathogens growth *in vitro* depends, at least in part, on a pH-lowering effect of supernatants and/or on the presence of organic acids. Cluster analysis was performed in order to group *L. plantarum* strains according to their antimicrobial effect. This study emphasizes the tempting use of the tested *L. plantarum* strains and/or their CFS as antimicrobial agents against food-borne pathogens.

Keywords: *Lactobacillus plantarum*, antimicrobial compounds, inhibiting activity, cell-free supernatant (CFS), organic acid, pathogens

OPEN ACCESS

Edited by:
Rosanna Tofalo,
University of Teramo, Italy

Reviewed by:
Ebrahim Hadavi,
Islamic Azad University, Iran
Learn-Han Lee,
Monash University Malaysia, Malaysia

****Correspondence:***
Giuseppe Spano
giuseppe.spano@unifg.it

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

Received: *27 December 2015*
Accepted: *21 March 2016*
Published: *13 April 2016*

Citation:
Arena MP, Silvain A, Normanno G, Grieco F, Drider D, Spano G and Fiocco D (2016) Use of Lactobacillus plantarum Strains as a Bio-Control Strategy against Food-Borne Pathogenic Microorganisms. Front. Microbiol. 7:464. doi: 10.3389/fmicb.2016.00464

INTRODUCTION

Lactobacilli are widespread microorganisms which are extensively used in the food field both as technological starters in the fermented products and as probiotics due to their strain-specific healthy properties (Cebeci and Gürakan, 2003; Georgieva et al., 2009; Altay et al., 2013). Among Lactobacilli, *Lactobacillus plantarum* is one of the most versatile species, including strains with valuable technological skills and recognized probiotic features (da Silva Sabo et al., 2014; Guidone et al., 2014). Moreover, a number of probiotic *L. plantarum* strains hold multipurpose features as they can both carry out appreciable fermentative and metabolic processes, e.g., increasing the amount of specific beneficial compounds such as vitamins in the fermented food product, and promote the maintenance of consumers' health, since their capacity to modulate the host immune response and to *de novo* produce vitamins in the human gut (Arena et al., 2014, 2015). Concurrently, the increasing attention of consumers for healthy and natural food prompts food industry and scientific research to investigate the application of natural compounds for the processing of food products, in order to eliminate or reduce chemical additives used as antimicrobial agents. Thus, in recent decades, several lines of research have tried to find "the natural solution" to "the chemical problem." Among these, the selection of microbial molecules, and/or bacterial strains able to produce such compounds, to be used as antimicrobials and preservatives, proved that Lactic Acid Bacteria (LAB) could be suitable candidates for such "natural purpose" (Šušković et al., 2010; da Silva Sabo et al., 2014).

LABs, including several *L. plantarum* strains, have been shown to produce different antimicrobial agents such as organic acids, hydrogen peroxide, diacetyl, bacteriocins, and antimicrobial peptides, with a variable spectrum of action (Herreros et al., 2005; Tharmaraj and Shah, 2009; Cortés-Zavaleta et al., 2014). Several lactobacilli, including *L. plantarum*, exhibit antagonistic activity against pathogenic and spoilage microorganisms. Such antimicrobial effect has been often ascribed to the production of organic acids, including lactic and phenyllactic acids (Tharmaraj and Shah, 2009; Neal-McKinney et al., 2012; Tejero-Sariñena et al., 2012; Rodríguez-Pazo et al., 2013). However, also the synthesis of bacteriocins and/or bacteriocin like substances has been reported to account for the antagonistic activity exerted by probiotic lactobacilli (Kos et al., 2011; Al Kassaa et al., 2014).

The antagonistic activity of selected microorganisms and/or their extracellular antibacterial agents included in the cell free supernatants (CFS) offer valuable opportunities for food preservation (Kecerová et al., 2004) as well as feed supplements or in veterinary medicine (Nousiainen et al., 2004; Bilkova et al., 2011; Cortés-Zavaleta et al., 2014). Because of their widespread association with foods and their generally recognized as safe (GRAS) status, the use of LAB and/or their metabolites as natural drugs has attracted considerable interest in recent years (Reis et al., 2012). In the food industry, the use of the bacteriocins nisin and pediocin has allowed to reduce the addition of chemical preservatives and the intense thermal treatments, thus enhancing sensory and nutritional properties without impairing safety (De Vuyst and Leroy, 2007; Sobrino-Lopez and Martin-Belloso, 2008). Moreover, several other antimicrobial peptides produced by probiotic LABs have been characterized and suggested for potential and relevant applications in food preservation and safety (Reis et al., 2012; Gupta and Srivastava, 2014).

As the antimicrobial activity of LAB bacteriocins is usually restricted to Gram-positive bacteria, organic acids and organic acid-producing LAB could have even wider applications in food safety (De Vuyst and Leroy, 2007; Mu et al., 2012). In this regard, the use of probiotics which produce antimicrobial metabolites, including organic acids, has been proposed as part of effective bio-control strategies to contrast the contamination of animal feed by spoilage and pathogenic microorganisms, and to reduce pathogen loads in livestock (Gerbaldo et al., 2012; Neal-McKinney et al., 2012), consequently decreasing food-borne illness in humans. Recently, culture supernatants from probiotic LAB, with *in vitro* inhibitory action on *Clostridium difficile* (CD), were suggested as a basis for alternative therapies to treat CD infections in humans (Joong-Su et al., 2013). Accordingly, cell-free probiotic extracts were proposed as alternative ingredients to probiotic live cells for nutritional and medicinal applications (Saadatzadeh et al., 2013).

Our main objective was to understand whether *Lactobacillus* spp. could represent a natural alternative to the chemical antimicrobials commonly used in the food preparation. Therefore, this study evaluated the antimicrobial activity of 79 wine-derived *L. plantarum* strains against seven pathogenic bacteria, generally involved in foodborne poisoning and infections. The pathogens used in this work were *Listeria monocytogenes*, which can cause abortions and/or gastrointestinal diseases leading to death (Sip et al., 2012), *Escherichia coli* O157:H7, which provokes haemorrhagic colitis and haemolytic uremic syndrome (Mead and Griffin, 1998), *Salmonella Enteritidis,* which determines abdominal pain, nausea, vomiting, and diarrhea (Liu et al., 2011), and methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus,* which is involved in harmful gastroenteritis (Gutiérrez-Larraínzar et al., 2012). Additionally, we investigated on the chemical nature of the molecules possibly accounting for the observed antimicrobial activity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

This study was carried out on 79 *L. plantarum* strains deposited into the culture collection of Foggia University (Italy; UNIFG) and previously isolated from wine and must (**Table 1**). All *L. plantarum* strains were growth on de Man–Rogosa–Sharpe (MRS; Sigma-Aldrich, St. Louis, MO, USA) at 30°C.

The pathogenic bacteria used were: *L. monocytogenes* CECT 4032; *S. Enteritidis* CECT 409, *E. coli* O157:H7 CECT 4267, two methicillin-resistant strains of *S. aureus* MSSA1220, and *S. aureus* MRSA1209, two methicillin-susceptible strains of *S. aureus* MRSA1208 and *S. aureus* MRSA1070. All pathogens were grown in tryptone soy broth (TBS, Oxoid) and incubated at 37°C, with the exception of *S. aureus* strains that were grown in Brain Heart Infusion broth (BHI, Oxoid).

TABLE 1 | *Lactobacillus plantarum* strains used in this work.

	No. collection	Name	Isolation source		No. collection	Name	Isolation source
1	UNIFG 6	*Lactobacillus plantarum*	wine	41	UNIFG 74	*Lactobacillus plantarum*	wine
2	UNIFG 9	*Lactobacillus plantarum*	wine	42	UNIFG 75	*Lactobacillus plantarum*	wine
3	UNIFG 10	*Lactobacillus plantarum*	wine	43	UNIFG 79	*Lactobacillus plantarum*	wine
4	UNIFG 22	*Lactobacillus plantarum*	wine	44	UNIFG 80	*Lactobacillus plantarum*	wine
5	UNIFG 30	*Lactobacillus plantarum*	wine	45	UNIFG 81	*Lactobacillus plantarum*	wine
6	UNIFG 31	*Lactobacillus plantarum*	wine	46	UNIFG 82	*Lactobacillus plantarum*	wine
7	UNIFG 32	*Lactobacillus plantarum*	wine	47	UNIFG 83	*Lactobacillus plantarum*	wine
8	UNIFG 33	*Lactobacillus plantarum*	wine	48	UNIFG 84	*Lactobacillus plantarum*	wine
9	UNIFG 35	*Lactobacillus plantarum*	wine	49	UNIFG 85	*Lactobacillus plantarum*	wine
10	UNIFG 36	*Lactobacillus plantarum*	wine	50	UNIFG 86	*Lactobacillus plantarum*	wine
11	UNIFG 37	*Lactobacillus plantarum*	wine	51	UNIFG 87	*Lactobacillus plantarum*	must
12	UNIFG 38	*Lactobacillus plantarum*	wine	52	UNIFG 88	*Lactobacillus plantarum*	wine
13	UNIFG 44	*Lactobacillus plantarum*	must	53	UNIFG 89	*Lactobacillus plantarum*	must
14	UNIFG 45	*Lactobacillus plantarum*	wine	54	UNIFG 90	*Lactobacillus plantarum*	must
15	UNIFG 46	*Lactobacillus plantarum*	wine	55	UNIFG 91	*Lactobacillus plantarum*	must
16	UNIFG 47	*Lactobacillus plantarum*	must	56	UNIFG 92	*Lactobacillus plantarum*	wine
17	UNIFG 48	*Lactobacillus plantarum*	must	57	UNIFG 93	*Lactobacillus plantarum*	wine
18	UNIFG 49	*Lactobacillus plantarum*	wine	58	UNIFG 94	*Lactobacillus plantarum*	must
19	UNIFG 50	*Lactobacillus plantarum*	must	59	UNIFG 95	*Lactobacillus plantarum*	must
20	UNIFG 51	*Lactobacillus plantarum*	wine	60	UNIFG 96	*Lactobacillus plantarum*	must
21	UNIFG 52	*Lactobacillus plantarum*	must	61	UNIFG 97	*Lactobacillus plantarum*	wine
22	UNIFG 53	*Lactobacillus plantarum*	wine	62	UNIFG 98	*Lactobacillus plantarum*	wine
23	UNIFG 54	*Lactobacillus plantarum*	must	63	UNIFG 99	*Lactobacillus plantarum*	wine
24	UNIFG 55	*Lactobacillus plantarum*	wine	65	UNIFG 103	*Lactobacillus plantarum*	wine
25	UNIFG 56	*Lactobacillus plantarum*	wine	66	UNIFG 104	*Lactobacillus plantarum*	wine
26	UNIFG 57	*Lactobacillus plantarum*	wine	67	UNIFG 105	*Lactobacillus plantarum*	wine
27	UNIFG 58	*Lactobacillus plantarum*	wine	68	UNIFG 106	*Lactobacillus plantarum*	wine
28	UNIFG 59	*Lactobacillus plantarum*	must	69	UNIFG 107	*Lactobacillus plantarum*	wine
29	UNIFG 60	*Lactobacillus plantarum*	must	70	UNIFG 108	*Lactobacillus plantarum*	wine
30	UNIFG 61	*Lactobacillus plantarum*	must	71	UNIFG 109	*Lactobacillus plantarum*	wine
31	UNIFG 62	*Lactobacillus plantarum*	must	72	UNIFG 115	*Lactobacillus plantarum*	wine
32	UNIFG 63	*Lactobacillus plantarum*	wine	73	UNIFG 117	*Lactobacillus plantarum*	wine
33	UNIFG 66	*Lactobacillus plantarum*	wine	74	UNIFG 118	*Lactobacillus plantarum*	wine
34	UNIFG 67	*Lactobacillus plantarum*	wine	75	UNIFG 119	*Lactobacillus plantarum*	wine
35	UNIFG 68	*Lactobacillus plantarum*	wine	76	UNIFG 120	*Lactobacillus plantarum*	wine
36	UNIFG 69	*Lactobacillus plantarum*	wine	77	UNIFG 121	*Lactobacillus plantarum*	wine
37	UNIFG 70	*Lactobacillus plantarum*	must	78	UNIFG 122	*Lactobacillus plantarum*	wine
38	UNIFG 71	*Lactobacillus plantarum*	wine	79	UNIFG 134	*Lactobacillus plantarum*	wine
39	UNIFG 72	*Lactobacillus plantarum*	wine				
40	UNIFG 73	*Lactobacillus plantarum*	must				

Antimicrobial Activity

The antimicrobial activity was evaluated by (i) agar spot test, (ii) well-diffusion method, and (iii) broth microdilution method. The agar spot test was carried out according to Gaudana et al. (2010). Briefly, overnight cultures of lactobacilli were spotted (5 μL) on MRS agar and incubated for 24 h at 37°C. Pathogen overnight cultures were mixed 1:100 with TSB or BHI soft agar (containing 0.6% agar, w/v) and poured over MRS agar plates containing the developed colonies of lactobacilli. Plates were incubated for 24 h and the radii of the inhibition zones were measured.

For the well diffusion assays, cultures of lactobacilli were grown in MRS broth (pH 6.5) for 18 h and, then, centrifuged (8000 × g for 20 min, 4°C). The cell-free supernatant (CFS) was recovered and sterilized by filtration through Millex-GV 0.22 μm hydrophilic Durapore PVDF membrane (Millipore, Billerica, MA, USA). To investigate on the chemical nature of the potentially inhibitory substances secreted by each *L. plantarum* strain, showing antagonistic effects, filtered CFSs (CFS-A) were submitted to different treatments. An aliquot of filtered CFS was sequentially treated according to Herreros et al. (2005). First of

all, CFSs (CFS-A) were heated at 80°C for 10 min (CFS-B) and neutralized with 2 M NaOH (to pH 6.5; CFS-C), in order to rule out inhibiting effects due to organic acids. The neutralized CFSs (CFS-C) were subjected to the following treatments: (i) catalase digestion (1 mg/ml; Sigma-Aldrich Corporation, USA) at 37°C for 1 h, in order to eliminate the possible inhibitory action of hydrogen peroxide (CFS-D); (ii) separate digestion at 37°C for 2 h with different proteases, i.e., proteinase K (CFS-E; 1 mg/ml), trypsin (CFS-F, 1 mg/ml), α-chemotrypsin (CFS-G, 1 mg/ml), and papain (CFS-H, 1 mg/ml; all purchased from from Sigma, USA); (iii) heating at 80°C for 60 (CFS-I) and 90 min (CFS-L), 100°C for 60 (CFS-M) and 90 min (CFS-N), and at 121°C for 15 min (CFS-O).

Another aliquot of filtered CFS was separately treated according to Cortés-Zavaleta et al. (2014). A part of this aliquot (CFS-A*) was heated at 80°C for 10 min (CFS-B*) and then neutralized with 2 M NaOH (to pH 6.5) (CFS-C*), as mentioned above. Another portion of CFS-A* was exposed to catalase (1 mg/ml; 37°C for 1 h; CFS-D*), or to different proteases, i.e., proteinase K (CFS-E*; 1 mg/ml, 37°C for 2 h), trypsin (CFS-F*, 1 mg/ml, 37°C for 2 h), α-chemotrypsin (CFS-G*, 1 mg/ml, 37°C for 2 h), and papain (CFS-H*, 1 mg/ml, 37°C for 2 h), or to different thermal treatments, i.e., 80°C for 60 (CFS-I*) and 90 min (CFS-L*), 100°C for 60 (CFS-M*) and 90 min (CFS-N*), and 121°C for 15 min (CFS-O*).

All treated CFSs were collected and 100 μl of each were used to fill 6 mm diameter wells previously punched on MRS agar plates. The plates were incubated for 2 h at 4°C in order to permit CFSs diffusion onto MRS agar. Overnight cultures of pathogenic bacteria were inoculated (1% v/v) into fresh TSB or BHI soft agar (0.6% agar, w/v) and poured over MRS agar plate containing CFSs. All plates were incubated at 37°C for 24 h and, then, the inhibition zones around the wells were measured.

Broth microdilution assays were assessed as described by Mayrhofer et al. (2008). Overnight cultures of pathogenic bacteria were inoculated (1% v/v) into fresh medium, i.e., TSB or BHI, and seeded in 96-well plates (Costar-Corning Incorporated, Corning, NY, USA). 200 μl of test solution consisting of 100 μl of pathogenic culture and 100 μl of CFS were mixed into the wells. Untreated CFSs (CFS-A) were diluted in physiological solution (NaCl 8.5 g/L) and used at different percentages, i.e., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50% in the final volume (200 μl), in order to determine the minimum percentage of CFS able to inhibit the growth of target pathogens. Plates were incubated at 37°C for 24 h and growth of pathogenic bacteria was monitored by measuring optical density (OD_{600} nm). Furthermore, after identifying the minimum inhibiting percentage, treated CFSs were also used against pathogenic bacteria. The antimicrobial activity was expressed as inhibition (%) of pathogen growth relative to the control (i.e., pathogen grown in optimal conditions, without CFS).

Lyophilization of Cell-Free Supernatant

The supernatants of lactobacilli were collected by centrifugation and 10-fold concentrated by lyophilization as reported by Bermudez-Brito et al. (2013).

Statistical Analysis

Three independent experiments were conducted for all trials. Cluster analysis was used to determine the grouping of lactobacilli according to their antimicrobial activity against target pathogens. Statistical comparisons were performed by one-way ANOVA test ($p < 0.005$ was considered as statistically significant). All statistical study was performed using IBM SPSS Statistics 21.0 software program (IBM, Armonk, NY, USA).

RESULTS

Agar Spot Test

In this study, 79 *L. plantarum* strains were investigated for their possible antimicrobial activity against seven pathogenic bacteria, i.e., *L. monocytogenes, S. Enteritidis*, *E. coli* O157:H7, and four strains of *S. aureus*. The preliminary screening of all *Lactobacillus* strains, carried out by agar spot test, revealed a different range of antimicrobial activity, depending both on *L. plantarum* strain tested and on pathogen considered. **Figure 1** reports data obtained with the best inhibiting *L. plantarum* strains, i.e. which determined overall inhibition halos of more than 5 radius mm, according to the classification proposed by Gaudana et al. (2010). As shown in **Figure 1**, some strains exhibited a very strong ability to inhibit the growth of food pathogens. In particular, *L. plantarum* 105 exhibited the major ability to inhibit *L. monocytogenes*, while both *L. plantarum* 106 and *L. plantarum* 107 presented the highest antagonistic effect on growth of *E. coli* O157:H7. *S. Enteritidis*, *S. aureus* R1070, R1208, S1209, and S1220 were mainly inhibited by *L. plantarum* 119, *L. plantarum* 32, *L. plantarum* 106, and *L. plantarum* 108, respectively. Contrariwise, *L. plantarum* 118 and 119 did not show any inhibition effects on the growth of *S. aureus* R1208. Similarly, *L. plantarum* 30 was not able to affect the development of *S. aureus* S1209.

Well-Diffusion Assays

The antagonistic effect of those *L. plantarum* strains which exhibited appreciable antimicrobial activity, as determined by agar spot test (**Figure 1**), was further assessed by well diffusion assay using CSFs. In contrast to the results obtained with the agar spot assays, no CFS was able to contrast the growth of pathogenic bacteria (data not shown). In order to ascertain if the concentration of the inhibiting substance could be not adequate to sustain antagonistic action, the CFSs were concentrated by lyophilization, prior to their use in well diffusion tests. The 10x concentrated CFSs exhibited inhibition activities that were similar to that previously obtained by agar spot test (**Table 2**). The concentrated and differently treated CFSs were also tested, however, the inhibiting effect was lost after pH neutralization, while enzymatic and heat treatment had no impact on the inhibitory effect (data not shown).

Broth Microdilution Assays

CFSs were further tested against pathogens by broth microdilution method. Interestingly, untreated CFSs from all selected *L. plantarum* strains determined significant inhibition of pathogen growth when used at ≥25% (25:75,

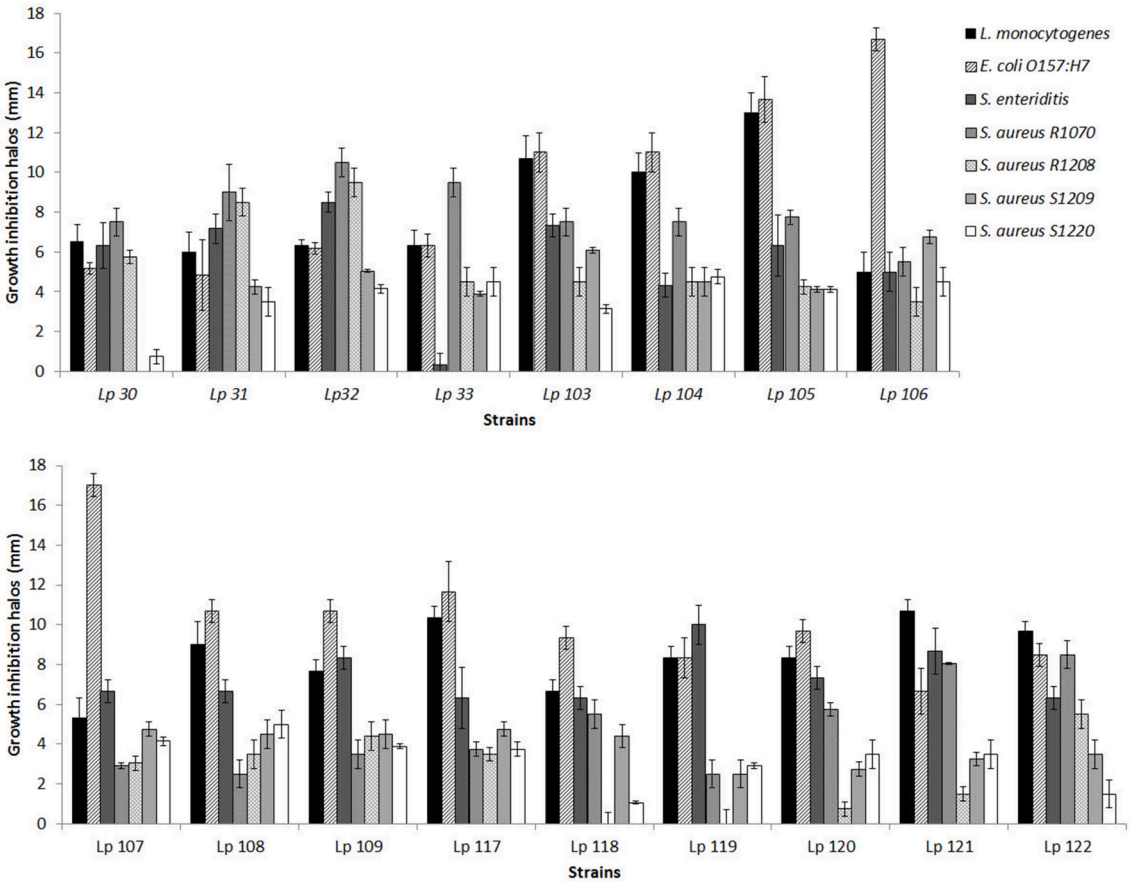


FIGURE 1 | Antimicrobial ability of selected *L. plantarum* strains against pathogenic bacteria as measured by agar spot assay. Data are the mean ± SD of at least three independent experiments.

CFS:growth medium), i.e., 30, 40, 50, 60, 70, 80, 90% (data not shown). **Figure 2** shows the antagonism resulted by untreated CFSs of *Lactobacillus* strains using 25% of CFSs-A. *E. coli* O157:H7 growth was reduced by around 70 and 93% by CFS of *L. plantarum* 108 and 104, respectively. *L. monocytogenes* was inhibited up to 90% by *L. plantarum* 116, while the growth of *S. Enteritidis* was reduced by 96 % in presence of *L. plantarum* 30 CFS. The growth of all strains of *S. aureus* was significantly contrasted by all CFSs and, the highest reductions were around 90% (*S. aureus* R1070), 99% (*S. aureus* R1208), 85% (*S. aureus* S1209), and 86% (*S. aureus* S1220). Based on these findings, an aliquot consisting of 100 μl of untreated, 2-fold diluted CFSs was determined as the amount of CFSs showing more than 50% of inhibition ability for 98% of cases analyzed, and chosen for the further assays. The only two-fold diluted CFS causing an inhibition lower than 50% was that from *L. plantarum* 104 against *L. monocytogens* and *S. Enteritidis*.

In order to investigate on the nature of the inhibitory substances secreted by each *L. plantarum* strain showing antagonistic effects, CFS-As were submitted to different treatments. As a result, the potential inhibiting molecules lost their antagonistic ability after pH neutralization (data not shown).

Statistical Relationship among Lactobacilli for their Antimicrobial Activity

Cluster analysis was performed on the inhibition halos data obtained by agar spot test, in order to group *Lactobacillus* strains showing similar antimicrobial activity. Euclidean distance was used to measure the proximity between two data and average linkage clustering was used as linkage criteria. As a result, four clusters of lactobacilli were distinguished (**Figure 3**). Group A contained 10 strains of *L. plantarum* (103, 104, 105, 108, 109, 117, 119, 120, 121, 122); group B was constituted of strain 118, group C consisted of *L. plantarum* 106 and 107; group D comprised four strains of *L. plantarum* (30, 31, 32, 33). Furthermore, ANOVA was elected as a method to study the statistical differences ($p < 0.005$) among the four groups (data not shown). Overall, the results indicated that the strains included in group A were able to contrast the growth of *L. monocytogenes* significantly better than strains in groups B, C, and D. *L. plantarum* 118, within group B, showed no or very low activity against *S. aureus* R1208 and *S. aureus* S1220, while *L. plantarum* 106 and *L. plantarum* 107, belonging to group C, were mostly active against *E. coli* O157:H7. Group D strains, including *L. plantarum* 30, 31, 32, and 33, were able to inhibit the growth of *S. aureus* R1208 significantly better than lactobacilli of group A, B, and C.

TABLE 2 | Antimicrobial activity of CFS of *L. plantarum* strains determined by well-diffusion assay and expressed as the size of inhibition zones around the wells (mm).

	CFS-A	CFS-B	CFS-A	CFS-B	CFS-A	CFS-B	CFS-A	CFS-B	CFS-A	CFS-B	CFS-A	CFS-B	CFS-A	CFS-B
	E. coli O157:H7		*L. monocytogenes*		*S. Enteritidis*		*S. aureus R1070*		*S. aureus R1208*		*S. aureus S1209*		*S. aureus S1220*	
L. plantarum 30	5.0	4.4	5.0	4.9	6.0	5.5	7.0	6.5	5.0	5.0	0.0	0.0	0.5	0.0
L. plantarum 31	3.3	3.1	5.0	5.0	7.0	6.4	8.0	6.3	6.0	5.5	4.0	4.0	2.5	2.0
L. plantarum 32	4.1	4.0	5.5	5.0	7.7	6.9	8.8	6.4	8.0	7.8	4.0	3.8	3.0	2.8
L. plantarum 33	4.3	4.3	5.4	5.0	1.0	1.0	7.6	7.0	3.0	2.0	4.0	3.8	3.0	3.0
L. plantarum 103	6.0	6.0	8.0	8.0	6.8	6.3	7.0	6.3	3.5	2.0	5.5	4.0	3.0	3.0
L. plantarum 104	5.5	5.4	8.7	8.5	4.0	3.6	7.0	6.2	3.0	2.0	4.0	4.0	4.0	3.5
L. plantarum 105	7.2	7.1	10.2	10.0	5.2	5.0	6.5	6.0	3.1	2.0	4.0	4.0	4.0	3.5
L. plantarum 106	11.0	11.0	9.2	8.8	5.0	5.0	5.0	5.0	3.0	2.0	5.5	5.0	4.0	3.5
L. plantarum 107	10.8	1.5	9.3	9.0	5.1	5.0	3.0	2.1	3.0	2.5	4.5	4.0	4.0	4.0
L. plantarum 108	6.0	6.0	4.3	4.3	4.8	4.7	2.0	1.9	3.1	3.0	4.5	4.0	4.8	4.0
L. plantarum 109	6.3	6.0	4.3	4.5	7.0	7.2	3.0	2.5	4.0	3.2	4.5	4.5	4.0	3.6
L. plantarum 117	4.2	4.3	7.2	7.0	6.0	5.5	3.1	2.6	3.0	3.0	4.5	4.0	4.0	3.6
L. plantarum 118	4.6	4.5	5.5	5.3	5.9	5.8	5.0	4.9	1.0	1.0	4.0	4.0	1.0	1.0
L. plantarum 119	4.1	4.0	6.9	6.8	7.6	7.6	2.0	2.0	0.0	0.0	2.5	2.0	3.0	2.0
L. plantarum 120	3.9	3.8	6.8	6.8	8.0	7.9	5.0	4.9	0.0	0.0	2.5	2.0	3.0	2.5
L. plantarum 121	5.2	5.2	8.8	8.8	7.0	7.0	7.4	7.0	1.0	0.0	3.0	3.0	3.0	2.5
L. plantarum 122	6.9	6.5	8.9	8.8	5.4	5.0	7.1	7.0	4.0	4.0	3.0	3.0	1.0	1.0

CFS-A, untreated and 10x concentrated cell free supernatant; CFS-B, 10x concentrated CFS heated at 80°C for 10 min.

DISCUSSION

All analyzed *Lactobacillus* strains were shown to inhibit the growth of pathogens in a lactobacillus strain- and pathogen strain-depending manner. Using the agar spot method, 17 *L. plantarum* strains were identified as very strong inhibitors, according to the classification made by Gaudana et al. (2010), as they showed inhibition halos of more than 5 mm against the majority of the food pathogens tested. Cluster analysis was useful to group the *Lactobacillus* strains in four clusters, each of them denoted by a different antimicrobial activity. Within each group, peculiar abilities to contrast the growth of target pathogens were underlined such as a higher inhibitory activity against Gram-positive bacteria *L. monocytogenes* and *S. aureus* R1208 and Gram-negative bacteria *E. coli* O157:H7 by group A, group D, and group C, respectively.

The antimicrobial activity of the tested *L. plantarum* strains was mostly observed when they were grown on solid media and then brought into contact with pathogenic bacteria. This could be a considerable feature to be sought in the choice of starter or probiotic microorganisms. Indeed, live microorganisms carry out antimicrobial and preservative activity in the food when used as starters. Moreover, as probiotics, they can provide a protective benefit for the consumer when, following ingestion, can activate their metabolism in the intestine (De Vuyst and Leroy, 2007; Tejero-Sariñena et al., 2012; Arena et al., 2014).

The antimicrobial capability was also confirmed when 10x concentrated CFSs from *L. plantarum* strains were used in agar well diffusion assay, thus indicating that a minimal concentration of antimicrobial compounds is necessary to sustain that antagonism. CSF may include also other molecules, besides those effectively secreted by bacteria (i.e., medium components and/or intracellular compounds which may be accidentally released during CFS preparation). However CFS are routinely used to preliminarily screen the antimicrobial capacity of lactobacilli by well diffusion method (Herreros et al., 2005; Guo et al., 2010; Al Kassaa et al., 2014; Wang et al., 2014). For most of the tested *L. plantarum* strains, we found a good correspondence between the antimicrobial activities as assessed by either agar spot test or by well diffusion, using CFS. This indicates that the inhibitory effects mainly depend on exudates (which are included in the CFS) and only in part, if any, on other antagonistic mechanisms which require a more direct interaction, possibly occurring during co-culture on solid media (e.g., metabolic competition).

In addition, 1x CFSs were tested by broth microdilution method in order to understand whether the CFSs components could reduce the growth of pathogens in liquid-medium respect to agar-medium. The results suggested a greater capability of CFSs to contrast pathogenic bacteria in liquid-medium than in agar plates. Minimum amounts of CFS with inhibitory effect on pathogens were determined, indicating that two-fold diluted CFSs could exhibit more than 50% of inhibition for 98% of the cases analyzed. Absent or low activity by not concentrated CFSs in agar well diffusion tests was also previously reported (Saadatzadeh et al., 2013). It was also demonstrated that the antimicrobial effect was improved by CFS lyophililization and, more interestingly, such procedure was praised as an innovative strategy in bacterial products preparation (Saadatzadeh et al., 2013).

In order to investigate on the nature of the inhibitory substances secreted by each *L. plantarum* strain, both 1x CFSs and 10x CFSs were submitted to different treatments and

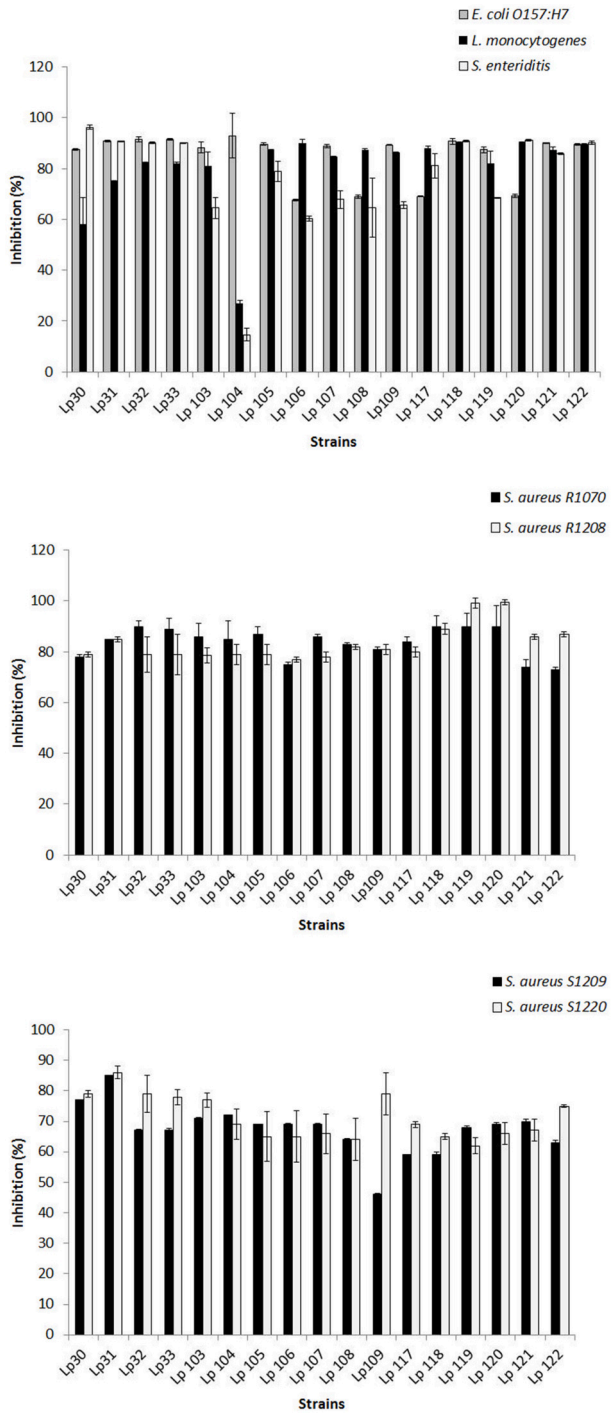


FIGURE 2 | Antimicrobial activity of CFSs-A (25%) of selected *L. plantarum* strains as determined by micro-dilution method. Data are the mean ± SD of at least three independent experiments.

tested against pathogens by broth method and well diffusion assay, respectively. Since pH neutralization eliminated the antimicrobial feature of CSF, while neither protease nor heat treatment had any impact on the inhibitory effect (both in sequential and in separate treatments, see the experimental section), we hypothesize that acidic pH and/or to the presence of organic acids could account for most of the observed antimicrobial activity. Indeed, although CFSs from bacterial cultures may contain many cellular metabolites, organic acids have been indicated as the principal antimicrobial agents when the antimicrobial activity is reduced or eliminated by alkaline neutralization (Bilkova et al., 2011; Zhang et al., 2011; Tejero-Sariñena et al., 2012). However, data obtained by CFS neutralization provide only a preliminary indication of the active compounds. Further experiments, including monitoring the pH of CFS and the acidification of growing cultures, as well as HPLC analysis, could help to better substantiate the potential role of organic acids. Moreover, organic acids, if any, could have enhanced the activity of other antimicrobial metabolites, which might require acidification and/or acid-mediated cell membrane disruption to exert an apparent antagonistic effect (Alakomi et al., 2000).

The antimicrobial effect of organic acids has been observed for several lactobacilli. The growth-inhibiting activity of different LAB, i.e., strains belonging to *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus,* and *Bacillus* genera, against pathogens such as *Salmonella typhimurium*, *E. coli*, *Enterococcus faecalis*, *S. aureus*, and *Clostridium difficile,* was attributed to a pH reduction and/or to the production of organic acids, including lactate and acetic acid (Tejero-Sariñena et al., 2012). Moreover, De Keersmaecker et al. (2006) also found a strong antimicrobial activity of *L. rhamnosus* strains against *S. typhimurium*, which was ascribed to the accumulation of lactic acid.

The organic acids secreted by LAB determine an environmental pH reduction that can be adverse for those microorganisms sharing the same niche (Tharmaraj and Shah, 2009). In their undissociated form, organic acids can penetrate the cytoplasmic membrane of target microorganisms, thus resulting in intracellular acidification and in the collapse of the transmembrane proton motive force. Such mode of action is pH-dependent, because the undissociated forms are prevalent when the pH value is below the pKa of the organic acid (Batish et al., 1997; Dalié et al., 2010; Schillinger and Villareal, 2010; Cortés-Zavaleta et al., 2014). Accordingly, the production of undissociated organic acids was indicated as the main mechanism through which several intestinal lactobacilli contrast the growth of a range of both Gram-negative and Gram-positive pathogenic bacteria in liquid media (Annuk et al., 2003; Topisirovic et al., 2007; Toy et al., 2015). Despite, the neutral pH conditions of the large intestine, probiotic bacteria could produce locally high concentrations of organic acids, thus establishing chemical microenvironments where the antagonistic action can be carried out (Alakomi et al., 2000).

Besides their pH lowering properties, sometimes, the antimicrobial effect of organic acids reflects a specific mode of action which may be relatively pH-independent. For instance, lactate, i.e., the main acid produced by LAB fermentation, was proved to specifically permeabilize the outer membrane of Gram negative species, causing structural alterations in the phospholipid component (Alakomi et al., 2000). Likewise, probiotic lactobacilli inhibited *Campylobacter jejuni* growth by the secretion of lactic acid, which was shown to disrupt

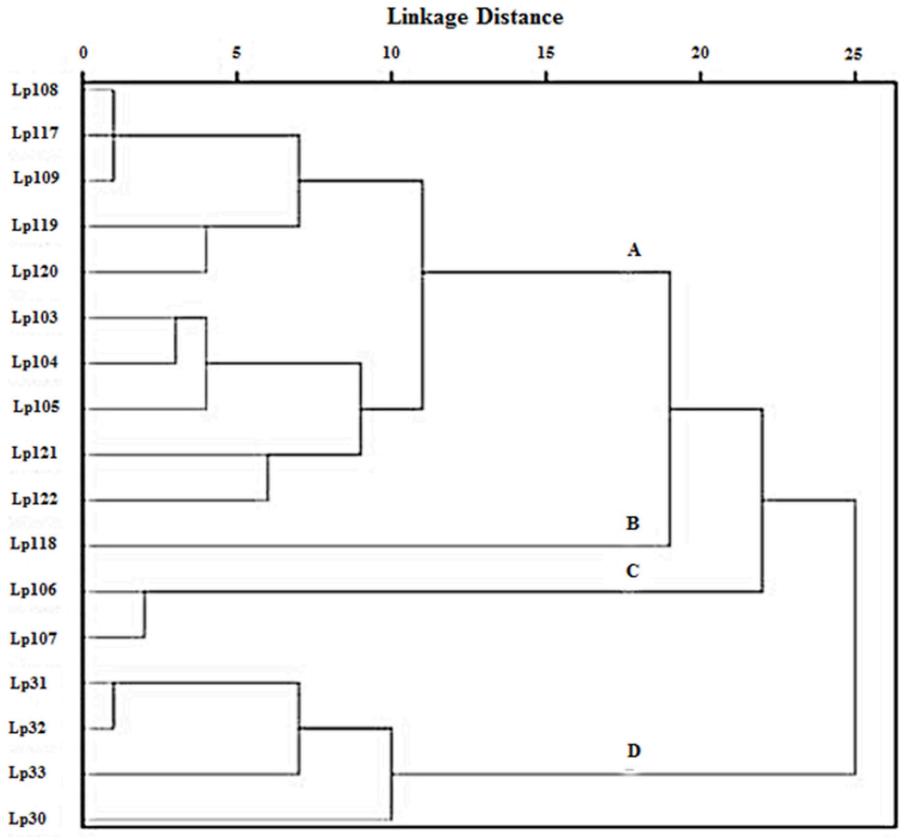


FIGURE 3 | Clustering of *L. plantarum* strains as a function of their antimicrobial activity against pathogens as observed by agar spot test. Statistically significant difference ($p < 0.005$) among subgroups **(A), (B), (C),** and **(D)** was determined by ANOVA test.

membrane integrity through a mechanism which is not solely pH-dependent (Neal-McKinney et al., 2012).

In the last decades, the main molecules which have been extensively studied as antimicrobial agents have been bacteriocins (Adebayo et al., 2014; Gupta and Srivastava, 2014). Bacteriocins are ribosomally-synthesized peptides that can act against bacteria of the same species (narrow spectrum) or of the same genera (broad spectrum). These compounds can be produced directly in fermented food either by bacteriocin-producing starter cultures (fermentative and bioconservative actions) or by protective culture strains (only bioconservative action). Additionally, isolated and purified bacteriocins can be used as food additives or included in the packaging materials (Kos et al., 2011; Fan and Song, 2013). One of the major drawbacks in the use of bacteriocins as natural antimicrobials is that their proteinaceous structure can be easily altered by diverse proteases possibly occurring in the food, being secreted by different bacteria and/or occurring in the human digestive tract (Saavedra et al., 2004). Moreover, the efficacy of bacteriocins in food can be decreased by their adsorption to food components, poor solubility and uneven distribution within the food matrix (Hartmann et al., 2011). Compared to LAB bacteriocins, which are mainly active against Gram-positive bacteria, organic acids exhibit a broader spectrum of antimicrobial action. Besides, organic acids are not sensitive to proteases and may be better solubilized. Therefore, the bioprotective potential of organic acid-producing LAB is high and suited to wide applications in food safety and nutritional medicine (Mu et al., 2012; Pawlowska et al., 2012).

The use of CFS as antimicrobial ingredients could be an interesting strategy in food preparation. CFS produced by selected bacteria could be effective in inhibiting pathogens, especially when the inoculation of live inhibiting microorganisms may not be feasible, e.g., in food subjected to refrigeration in which the psychrotrophic *L. monocytogenes,* but not lactobacilli, could easily grow. Furthermore, the use of CFS rather than purified antimicrobials could determine the advantage to have different biologically active substances, with possible synergistic effects, in one product (Hartmann et al., 2011). It is worthwhile mentioning that some recent *in vitro* studies have suggested potential and intriguing biomedical applications of CFSs. For instance, CFS from LAB, with antimicrobial activity against *C. difficile,* was proposed as a plausible alternative to the therapies for the treatment of CD-associated gut disorders (Joong-Su et al., 2013). Moreover, CFSs from *Lactobacillus* strains were ascribed health beneficial effects, including inhibition of cancer metastatis (Escamilla et al., 2012), positive modulation of the intestinal immune response (Bermudez-Brito et al., 2013) and cholesterol-reducing properties (Kim et al., 2008).

To sum up, this study provides evidence that several of the screened *L. plantarum* strains possess a significant ability to contrast various pathogenic bacteria, including both Gram negative and Gram positive species, which can contaminate food and are responsible for diseases in humans. The biosynthesis of organic acids is proposed as one of the main mechanism through which the antimicrobial activity is exerted. The antagonistic feature could be a distinctive trait to take into account for the selection of starters and or probiotics that could also function as bio-control agents against potentially harmful microorganisms during food processing and storage. In a future perspective of reducing or eliminating the use of chemical compounds, this work contributes to the existing knowledge in a context in which the consumer's attention is increasingly aimed at healthy and natural food products.

AUTHOR CONTRIBUTIONS

GS designed the experimental plan, analyzed the results and read the final paper. MA made the experimental trials and wrote up the paper. AS supported the technical part. GN provided some biological materials, participated to the experimental plan and read the final paper. FG and DD participated to the experimental plan and read the final paper. DF made the experimental trials and read the final paper.

ACKNOWLEDGMENTS

This work was partially supported by a grant from the project PON02_00186_3417512 in the framework of the project named "S.I.Mi.S.A.," (PON02_00186_2937475).

REFERENCES

Adebayo, F. A., Afolabi, O. R., and Akintokun, A. K. (2014). Antimicrobial properties of purified bacteriocins produced from *Lactobacillus casei* and *Lactobacillus fermentum* against selected pathogenic microorganisms. *Br. J. Med. Med. Res.* 4, 3415–3431. doi: 10.9734/BJMMR/2014/8584

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., and Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl. Environ. Microbiol.* 66, 2001–2005. doi: 10.1128/AEM.66.5.2001-2005.2000

Al Kassaa, I., Hamze, M., Hober, D., Chihib, N. E., and Drider, D. (2014). Identification of vaginal lactobacilli with potential probiotic properties isolated from women in North Lebanon. *Microb. Ecol.* 67, 722–734. doi: 10.1007/s00248-014-0384-7

Altay, F., Karbancıoglu-Güler, F., Daskaya-Dikmen, C., and Heperkan, D. (2013). A review on traditional Turkish fermented non-alcoholic beverages: microbiota, fermentation process and quality characteristics. *Int. J. Food Microbiol.* 167, 44–56. doi: 10.1016/j.ijfoodmicro.2013.06.016

Annuk, H., Shchepetova, J., Kullisaar, T., Songisepp, E., Zilmer, M., and Mikelsaar, M. (2003). Characterization of intestinal lactobacilli as putative probiotic candidates. *J. Appl. Microbiol.* 94, 403–412. doi: 10.1046/j.1365-2672.2003.01847.x

Arena, M. P., Caggianiello, G., Russo, P., Albenzio, M., Massa, S., Fiocco, D., et al. (2015). Functional starters for functional yogurt. *Foods* 4, 15–33. doi: 10.3390/foods4010015

Arena, M. P., Fiocco, D., Massa, S., Capozzi, V., and Russo, P. (2014). *Lactobacillus plantarum* as a strategy for an *in situ* production of vitamin B2. *J. Food. Nutr. Disord.* S1, 4. doi: 10.4172/2324-9323

Batish, V. K., Roy, U., Lal, R., and Grower, S. (1997). Antifungal attributes of lactic acid bacteria-a review. *Crit. Rev. Biotechnol.* 17, 209–225. doi: 10.3109/07388559709146614

Bermudez-Brito, M., Muñoz-Quezada, S., Gomez-Llorente, C., Matencio, E., Bernal, M. J., Romero, F., et al. (2013). Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. *PLoS ONE* 8:e59370. doi: 10.1371/journal.pone.0059370

Bilkova, A., Sepova, H. K., Bukovsky, M., and Bezakova, L. (2011). Antibacterial potential of lactobacilli isolated from a lamb. *Vet. Med.* 56, 319–324.

Cebeci, A., and Gürakan, C. (2003). Properties of potential probiotic *Lactobacillus plantarum* strains. *Food Microbiol.* 20, 511–518. doi: 10.1016/S0740-0020(02)00174-0

Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A., and García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int. J. Food Microbiol.* 173, 30–35. doi: 10.1016/j.ijfoodmicro.2013.12.016

Dalié, D. K. D., Deschamps, A. M., and Richard-Forget, F. (2010). Lactic acid bacteria-Potential for control of mould growth and mycotoxins: a review. *Food Control* 21, 370–380. doi: 10.1016/j.foodcont.2009.07.011

da Silva Sabo, S., Vitolo, M., González, J. M. D., and de Souza Oliveira, R. P. (2014). Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. *Food Res. Int.* 64, 527–536. doi: 10.1016/j.foodres.2014.07.041

De Keersmaecker, S. C., Verhoeven, T. L., Desair, J., Marchal, K., Vanderleyden, J., and Nagy, I. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiol. Lett.* 259, 89–96. doi: 10.1111/j.1574-6968.2006.00250.x

De Vuyst, L., and Leroy, F. (2007). Bacteriocins from lactic acid bacteria: production, purification, and food applications. *J. Mol. Microbiol. Biotechnol.* 13, 194–199. doi: 10.1159/000104752

Escamilla, J., Lane, M. A., and Maitin, V. (2012). Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion *in vitro*. *Nutr. Cancer* 64, 871–878. doi: 10.1080/01635581.2012.700758

Fan, L. and Song, J. (2013). "Antimicrobial microbes-bacteriocin producing lactic acid bacteria", in *Microbial pathogens and strategies for combating them: science, technology and education*, ed. A. Méndez-Vilas (Badajoz, ES: Formatex Research Center), 899–909.

Gaudana, S. B., Dhanani, A. S., and Bagchi, T. (2010). Probiotic attributes of *Lactobacillus* strains isolated from food and of human origin. *Br. J. Nutr.* 103, 1620–1628. doi: 10.1017/S0007114509993643

Georgieva, R., Iliev, I., Haertlé, T., Chobert, J.-M., Ivanova, I., and Danova, S. (2009). Technological properties of candidate probiotic *Lactobacillus plantarum* strains. *Int. Dairy J.* 19, 696–702. doi: 10.1016/j.idairyj.2009.06.006

Gerbaldo, G. A., Barberis, C., Pascual, L., Dalcero, A., and Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiol. Lett.* 332, 27–33. doi: 10.1111/j.1574-6968.2012.02570.x

Guidone, A., Zotta, T., Ross, R. P., Stanton, C., Rea, M. C., Parente, E., et al. (2014). Functional properties of *Lactobacillus plantarum* strains: A multivariate screening study. *LWT- Food Sci. Tech.* 56, 69–76. doi: 10.1016/j.lwt.2013.10.036

Guo, X. H., Kim, J. M., Nam, H. M., Park, S. Y., and Kim, J. M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe* 16, 321–326. doi: 10.1016/j.anaerobe.2010.03.006

Gupta, R., and Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from *Lactobacillus plantarum* strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiol.* 42, 1–7. doi: 10.1016/j.fm.2014.02.005

Gutiérrez-Larraínzar, M., Rúa, J., Caro, I., de Castro, C., de Arriaga, D., García-Armesto, M. R., et al. (2012). Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne

pathogens and spoilage bacteria. *Food Control* 26, 555–563. doi: 10.1016/j.foodcont.2012.02.025

Hartmann, H. A., Wilke, T., and Erdmann, R. (2011). Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. *Int. J. Food Microb.* 146, 192–199. doi: 10.1016/j.ijfoodmicro.2011.02.031

Herreros, M. A., Sandoval, H., González, L., Castro, J. M., Fresno, J. M., and Tornadijo, M. E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiol.* 22, 455–459. doi: 10.1016/j.fm.2004.11.007

Joong-Su, L., Myung-Jun, C., and Jae-Gu, S. (2013). *In vitro* evaluation of antimicrobial activity of lactic acid bacteria against *Clostridium difficile*. *Toxicol. Res.* 29, 99–106. doi: 10.5487/TR.2013.29.2.099

Kecerová, K., Pristaš, P., and Javorský, P. (2004). Bacteriocin production and sensitivity. *Folia Microbiol.* 49, 172–174. doi: 10.1007/BF02931395

Kim, Y., Whang, J. Y., Whang, K. Y., Oh, S., and Kim, S. H. (2008). Characterization of the cholesterol-reducing activity in a cell-free supernatant of *Lactobacillus acidophilus* ATCC 43121. *Biosci. Biotechnol. Biochem.* 72, 1483–1490. doi: 10.1271/bbb.70802

Kos, B., Beganović, J., Jurašić, L., Švaďumović, M., Leboš Pavunc, A., Uroić, K., et al. (2011). Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture *Lactococcus lactis*. *Mljekarstvo* 61, 273–282.

Liu, W., Liu, B., Zhu, X., Yu, S., and Shi, X. (2011). Diversity of *Salmonella* isolates using serotyping and multilocus sequence typing. *Food Microbiol.* 28, 1182–1189. doi: 10.1016/j.fm.2011.04.001

Mayrhofer, S., Domig, K. J., Mair, C., Zitz, U., Huys, G., and Kneifel, W. (2008). Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *Lactobacillus acidophilus* group members. *Appl. Environ. Microbiol.* 74, 3745–3748. doi: 10.1128/AEM.02849-07

Mead, P. S., and Griffin, P. M. (1998). *Escherichia coli* O157: H7. *Lancet* 352, 1207–1212. doi: 10.1016/S0140-6736(98)01267-7

Mu, W., Yu, S., Zhu, L., Zhang, T., and Jiang, B. (2012). Recent research on 3-phenyllactic acid, a broad-spectrum antimicrobial compound. *Appl. Microbiol. Biotechnol.* 95, 1155–1163. doi: 10.1007/s00253-012-4269-8

Neal-McKinney, J. M., Lu, X., Duong, T., Larson, C. L., Call, D. R., Shah, D. H., et al. (2012). Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. *PLoS ONE* 9:e43928. doi: 10.1371/journal.pone.0043928

Nousiainen, J., Javanainen, P., Setala, J., and von Wright, A. (2004). "20 Lactic acid bacteria as animal probiotics," in *Lactic Acid Bacteria: Microbial and Functional Aspects, 3rd Edn,* eds S. Salminen, A. Ouwehand, and A. von Wright (New York, NY: Marcel Dekker), 547–580.

Pawlowska, A. M., Zannini, E., Coffey, A., and Arendt, E. K. (2012). "Green preservatives": combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. *Adv. Food Nutr. Res.* 66, 217–238. doi: 10.1016/B978-0-12-394597-6.00005-7

Reis, J. A., Paula, A. T., Casarotti, S. N., and Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Eng. Rev.* 4, 124–140. doi: 10.1007/s12393-012-9051-2

Rodríguez-Pazo, N., Vázquez-Araújo, L., Pérez-Rodríguez, N., Cortés-Diéguez, S., and Domínguez, J. M. (2013). Cell-free supernatants obtained from fermentation of cheese whey hydrolyzates and phenylpyruvic acid by *Lactobacillus plantarum* as a source of antimicrobial compounds, bacteriocins, and natural aromas. *Appl. Biochm. Biotechnol.* 171, 1042–1060. doi: 10.1007/s12010-013-0408-7

Saadatzadeh, A., Fazeli, M. R., Jamalifar, H., and Dinarvand, R. (2013). Probiotic properties of Lyophilized cell free extract of *Lactobacillus casei*. *Jundishapur J. Nat. Pharm. Prod.* 8, 131–137. doi: 10.17795/jjnpp-8564

Saavedra, L., Minahk, C., Holgado, A. P., and Sesma, F. (2004). Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH2-terminal sequence. *Antimicrob. Agents Chemother.* 48, 2778–2781. doi: 10.1128/AAC.48.7.2778-2781.2004

Schillinger, U., and Villareal, J. V. (2010). Inhibition of Penicillium nordicum in MRS medium by lactic acid bacteria isolated from foods. *Food Control* 21, 107–111. doi: 10.1016/j.foodcont.2008.11.010

Sip, A., Więckowicz, M., Olejnik-Schmidt, A., and Grajek, W. (2012). Anti-Listeria activity of lactic acid bacteria isolated from golka, a regional cheese produced in Poland. *Food Control* 26, 117–124. doi: 10.1016/j.foodcont.2012.01.014

Sobrino-Lopez, A., and Martin-Belloso, O. (2008). Review: use of nisin and other bacteriocins for preservation of dairy products. *Int. Dairy J.* 18, 329–343. doi: 10.1016/j.idairyj.2007.11.009

Šušković, J., Kos, B., Beganović, J., Leboš Pavunc, A., Habjanič, K., and Matošić, S. (2010). Antimicrobial activity–the most important property of probiotic and starter lactic acid bacteria. *Food Techn. Biotechnol.* 48, 296–307.

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G. R., and Rowland, I. (2012). *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: evidence for the effects of organic acids. *Anaerobe* 18, 530–538. doi: 10.1016/j.anaerobe.2012.08.004

Tharmaraj, N., and Shah, N. P. (2009). Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips. *Int. Food Res. J.* 16, 261–276.

Topisirovic, L., Veljovic, K., Terzic Vidojevic, A., Strahinic, I., and Kojic, M. (2007). Comparative analysis of antimicrobial and proteolytic activity of lactic acid bacteria isolated from Zlatar cheese. *Genetika* 39, 125–138. doi: 10.2298/GENSR0702125T

Toy, N., Özogul, F., and Özogul, Y. (2015). The influence of the cell free solution of lactic acid bacteria on tyramine production by food borne-pathogens in tyrosine decarboxylase broth. *Food Chem.* 173, 45–53. doi: 10.1016/j.foodchem.2014.10.001

Wang, G., Zhao, Y., Tian, F., Jin, X., Chen, H., Liu, X., et al. (2014). Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*. *Food Control* 44, 49–57. doi: 10.1016/j.foodcont.2014.03.042

Zhang, Y., Zhang, L., Du, M., Yi, H., Guo, C., Tuo, Y., et al. (2011). Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiol. Res.* 167, 27–31. doi: 10.1016/j.micres.2011.02.006

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 30 March 2016
doi: 10.3389/fmicb.2016.00346

Structural Elucidation and Toxicity Assessment of Degraded Products of Aflatoxin B1 and B2 by Aqueous Extracts of *Trachyspermum ammi*

Wajiha Iram[1], Tehmina Anjum[1], Mazhar Iqbal[2], Abdul Ghaffar[3] and Mateen Abbas[4]*

[1] Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan, [2] Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan, [3] Department of Chemistry, University of Engineering and Technology, Lahore, Pakistan, [4] Department of Toxicology, Quality Operating Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan

In this study aqueous extract of seeds and leaves of *Trachyspermum ammi* were evaluated for their ability to detoxify aflatoxin B1 and B2 (AFB1; 100 μg L^{-1} and AFB2; 50 μg L^{-1}) by *in vitro* and *in vivo* assays. Results indicated that *T. ammi* seeds extract was found to be significant ($P < 0.05$) in degrading AFB1 and AFB2 i.e., 92.8 and 91.9% respectively. However, *T. ammi* leaves extract proved to be less efficient in degrading these aflatoxins, under optimized conditions i.e., pH 8, temperature 30°C and incubation period of 72 h. The structural elucidation of degraded toxin products by LCMS/MS analysis showed that eight degraded products of AFB1 and AFB2 were formed. MS/MS spectra showed that most of the products were formed by the removal of double bond in the terminal furan ring and modification of lactone group indicating less toxicity as compared to parent compounds. Brine shrimps bioassay further confirmed the low toxicity of degraded products, showing that *T. ammi* seeds extract can be used as an effective tool for the detoxification of aflatoxins.

Keywords: aflatoxin, degradation, plant extract, LCMS/MS, toxicity

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Issmat Kassem,
The Ohio State University, USA
Robert Lawrence Brown,
Agricultural Research Service, USA

****Correspondence:***
Tehmina Anjum
tehminaanjum@yahoo.com

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

Received: *24 November 2015*
Accepted: *04 March 2016*
Published: *30 March 2016*

Citation:
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

INTRODUCTION

Mycotoxins are chemically and biologically active secondary metabolites produced by fungi in cereals, nuts, fruits and vegetables (Sinha and Sinha, 1991; Aly, 2002). About 25% of the world cereals are contaminated with known mycotoxins produced by variety of toxigenic fungi. The food and agriculture organization (FAO) estimates that about 1000 million metric tons of foodstuffs could be contaminated with mycotoxins each year (Bhat et al., 2010). Currently, more than 400 mycotoxins are identified, among them, aflatoxins are the most serious carcinogenic, hepatotoxic, teratogenic, and mutagenic secondary metabolites which adversely affect humans and animal health. They are classified as group-1 carcinogens by International Agency for Research on cancer (IARC, 2002). Aflatoxin contamination can occur at any stage of food production from pre-harvest to storage (Wilson and Payne, 1994). Factors that affect aflatoxin contamination include the climate, genotype of the crop planted, soil type, temperature fluxes, intercropping with infected grains, early and delayed harvesting, improper drying and meager storage conditions (Folayan, 2013).

Aflatoxins are biologically active polyketide derived secondary metabolites which consist of a group of closely related highly oxygenated bisfurano-coumarin heterocyclic compounds, mostly

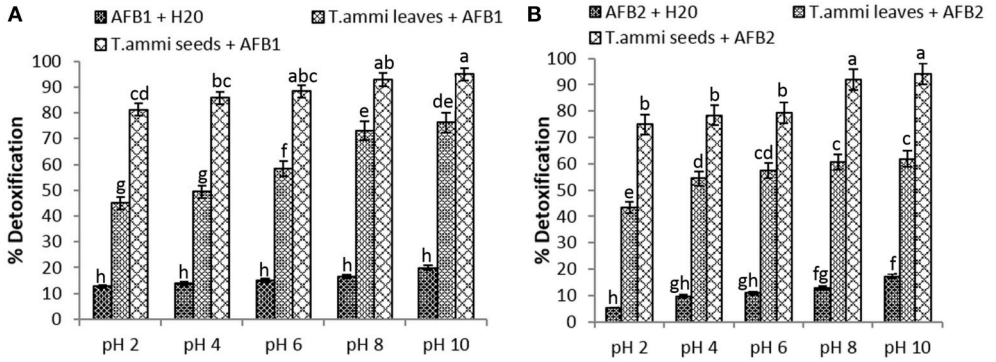


FIGURE 1 | Effect of pH on detoxification of aflatoxin by aqueous extracts of *T. ammi*. Whereas **(A)** AFB1; **(B)** AFB2.

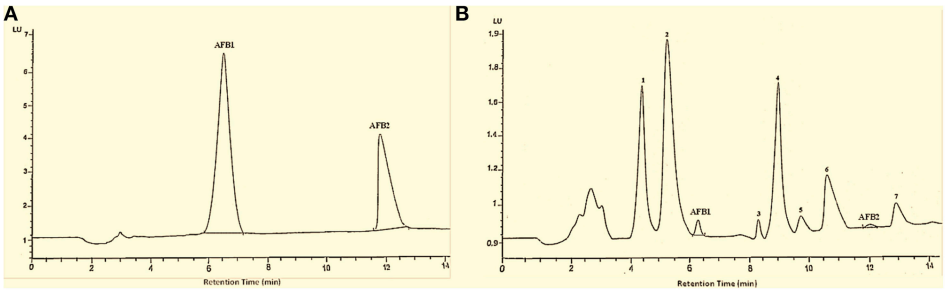


FIGURE 2 | HPLC chromatogram of AFB1 and AFB2. Whereas **(A)** untreated toxins; **(B)** toxin treated with *T. ammi* seeds extract at 30°C and pH 8.

produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Ellis et al., 1991; Bhatnagar et al., 1992). The G series of aflatoxins differs chemically from B series by the presence of a β lactone ring, instead of cyclopentenone ring. In AFB1 and AFG1 a double bond undergoes reduction forming vinyl ether at the terminal furan ring but not in AFB2 and AFG2 (Samarajeewa et al., 1990). AFB1 and AFG1 are carcinogenic and considerably more toxic than AFB2 and AFG2 probably due to these small difference in structure (Jaimez et al., 2000).

Various physical, chemical and biological methods have been described for detoxification of mycotoxins. Routinely, physical and chemical methods like roasting, flaking, canning, alkalization, oxidation, reduction and acidification are being frequently used. But these methods have not yet proven to be as effective and desirable because they are considered to be potentially unsafe as some form toxic residues or even alter the nutritional contents and flavor of treated commodity. In addition these methods require sophisticated equipment rendering for their high cost and environmental pollution (Joseph et al., 2005; Shukla et al., 2012). So, health hazards from exposure to such methods and economic consideration make biological control and natural plants extracts ideal alternatives to protect food and feed from fungal and mycotoxin contamination as they are biologically safe, eco-friendly, cheap, easily available, lack residual effects and are easily degradable (Reddy et al., 2007).

For the last decade, the use of herbal food additives has been encouraged (Mirzaei-Aghsaghali, 2012). The intensive efforts have been made by various researches for the clarification of biochemical structures and physiological functions of various food and feed additives like prebiotics, probiotics, organic acids and plant extracts. Numerous aromatic plants have been found to inhibit the microbial growth and thereby traditionally used to extend the shelf life of food (Ahmed et al., 2014). Similarly, several medicinal herbs and spices have been reported to counteract deleterious effects of mycotoxins either by chemical modification or by inclusion into the plant matrix (Wallnofer et al., 1996). Powder and extract of many medicinal herbs and higher plants have been shown inhibitory effect on growth of toxigenic fungi and production of toxins (Solis et al., 1993; Momoh et al., 2012). Based on the literature following plants were found to be effective in detoxifying aflatoxins and growth inhibition of toxigenic fungi: *Withania somnifera* (Linn.), *Camellia sinensis* (Linn.), *Citrus medica* (Linn.), *Syzigium aromaticum* (L.) Merr. Et Perry, *Curcuma longa* (L.), *Allium sativum* L. and *Ocimum sanctum* (Linn.), *Trachyspermum ammi* (L.), *Eucalyptus globolus* (Linn.), *Olea europaea* (Linn.), *Thymus vulgaris*, *Hibiscus sabdariffa* (Linn.), *Boswellia sacra*, *Adhatoda vasica* Nees and *Barleria lupulina* Lindl (Krishnamurthy and Shashikala, 2006; Reddy et al., 2009; Velazhahan et al., 2010; Al-Rahmah et al., 2011; El-Nagerabi et al., 2012, 2013; Kannan and Velazhahan, 2014; Vijayanandraj et al., 2014).

In the present investigation, *Trachyspermum ammi* (Family: Apiaceae, Common name: Ajwain) was used to evaluate its aflatoxin detoxifying potential. *T. ammi* has been known

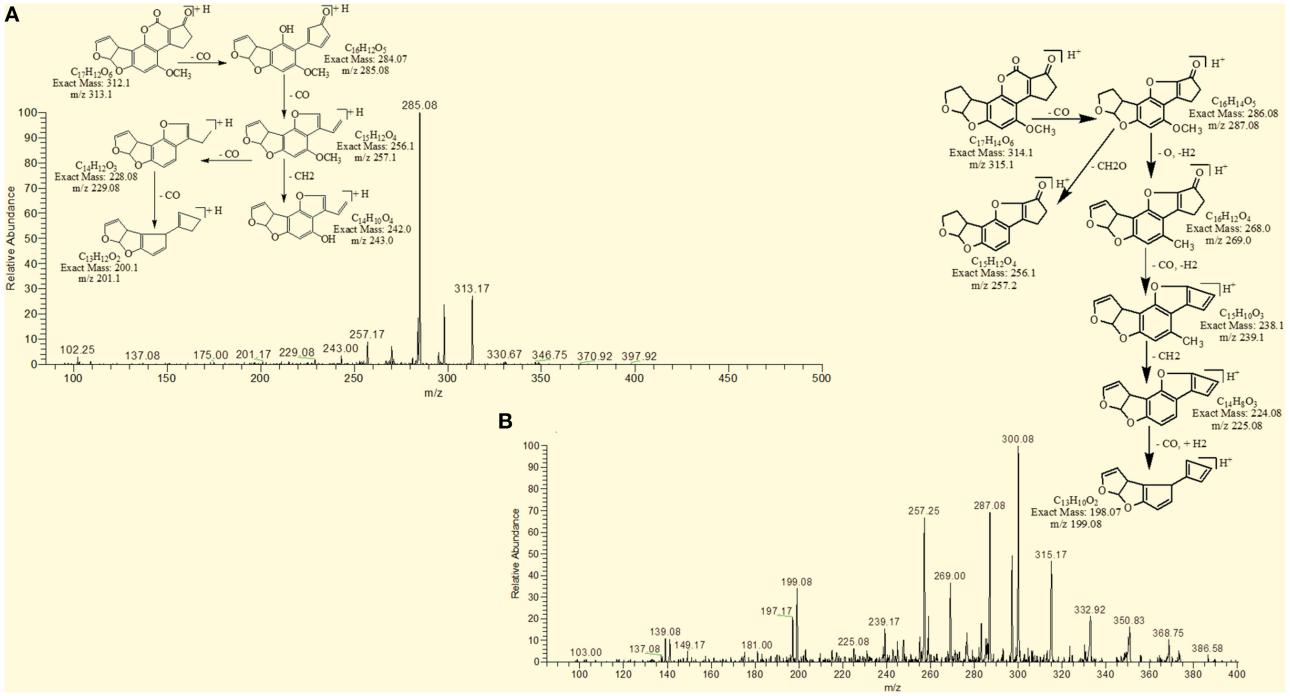


FIGURE 3 | MS/MS Spectra and fragmentation pathway. (A) AFB1 and **(B)** AFB2.

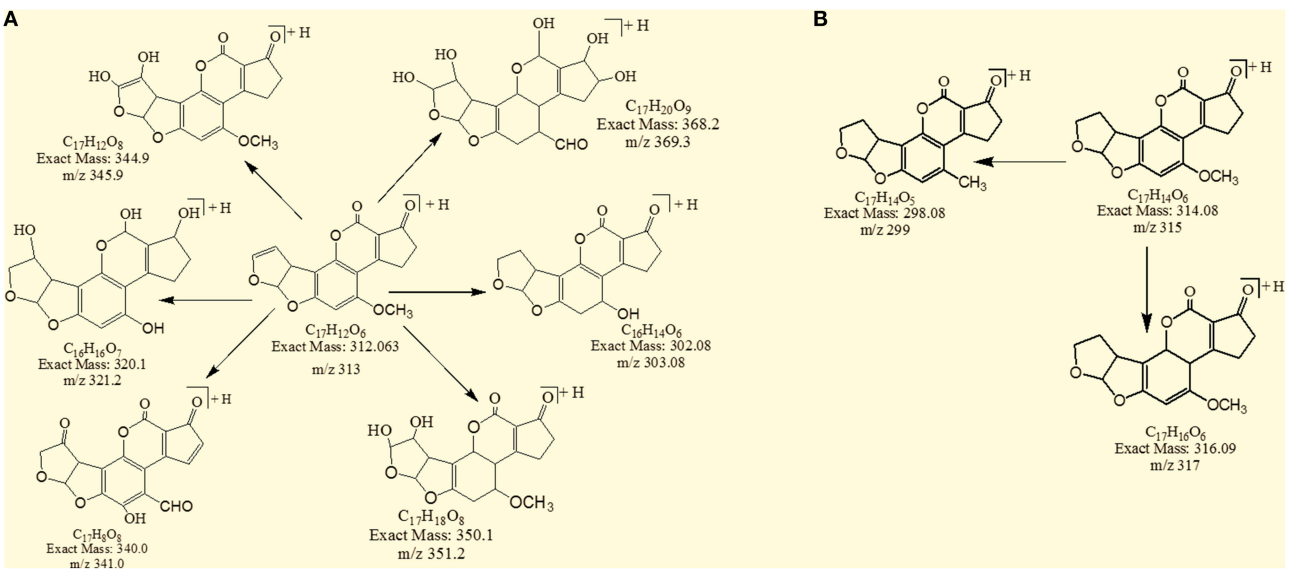


FIGURE 4 | Possible degraded products of (A) AFB1 and (B) AFB2 after treatment with *T. ammi* seeds extracts at 30°C and pH 8.

to possess known antimicrobial, antispasmodic, antiflatulent, antioxidant and antirheumatic effect due to the presence of several active compounds (Bairwa et al., 2012). Phytochemical studies on *T. ammi* revealed the presence of alkaloids, phenolics, steroids, fixed oils, glycosides, tannins, saponin and flavonoids, cumene, thymene, amino acids and thymol (Asifa et al., 2014). Literature showed that phenols, thymol and carvacol, are responsible for its antimicrobial, anti mycotoxigenic, antiseptic, antitussive and expectorant properties (Pathak et al., 2010). Ajwain is generally regarded as safe when taken in the recommended doses, however, in rare cases, it can cause nausea and headache (Grossberg and Fox, 2008). Although previous

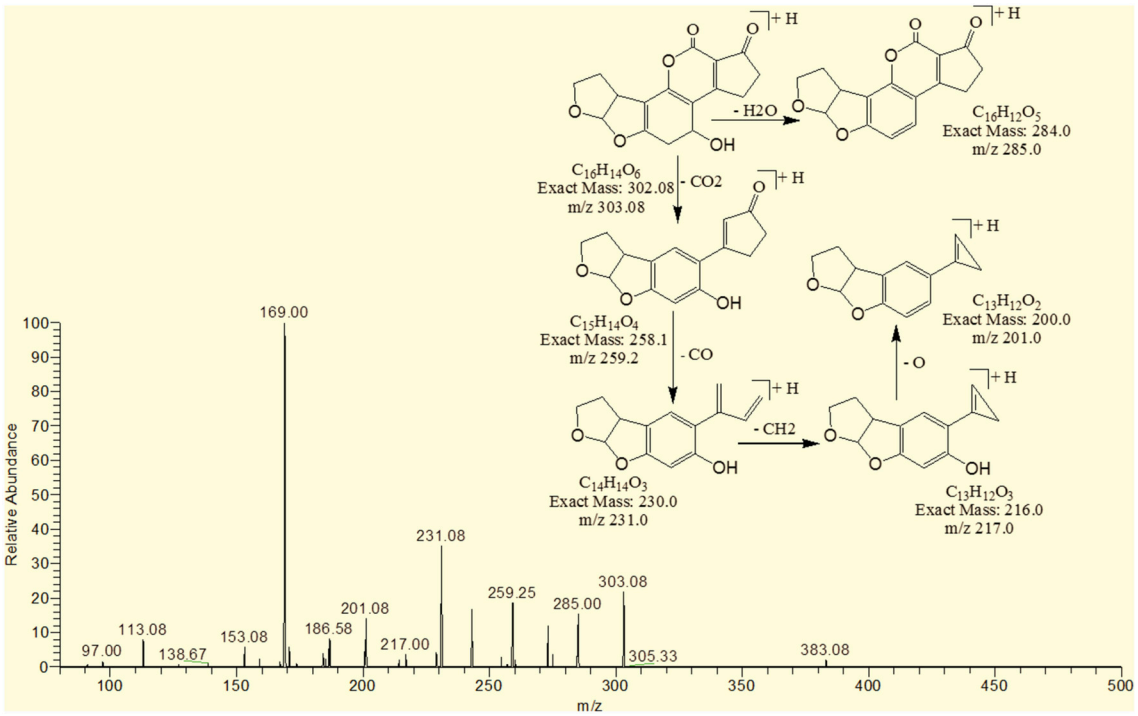


FIGURE 5 | MS/MS spectra and fragmentation pathway of degradation product with 303.08 m/z.

studies have been conducted with *T. ammi* seeds extract to detoxify aflatoxins but their resulting degradation products are not described in detail. However, in the present manuscript structural identification and fragmentation patterns of proposed degradation products were included along with their toxicity assessment. Various parameters were optimized for *in vitro* and *in vivo* detoxification of aflatoxins.

MATERIALS AND METHODS

Extraction and Purification of Aflatoxins

Aflatoxin B1 and B2 were extracted from a toxigenic isolate of *Aspergillus flavus* (isolated from stored maize samples) grown on coconut cream media by solvent extraction method as described by Yazdani et al. (2010) with some modifications. For extraction, colony margins were scraped together with surrounding zones into 250 mL Erlenmeyer flask containing 10 mL chloroform: acetone (85:15 v/v) and shacked for 30 min at 200 rpm. The crude extracts were filtered through gauze, and then through Whatman No.1 filter paper. Then, the filtrate was passed through the immunoaffinity column (Aflatest column, VICAM, Waters, USA) in solid phase extraction assembly for the separation of aflatoxins, according to the method described by Stroka et al. (2000) with some modifications. The column was rinsed with HPLC grade water and 10 ml of the sample was passed through it. Based on the highly specific antibody antigen reaction, the aflatoxins present in the sample form a conjugate with the antibody and the remaining impurities are separated out. Finally aflatoxins bound in the column are cleaved from their respective antibodies using methanol and compared with standard AFB1 and AFB2 purchased from (Sigma-Aldrich, St. Louis, MO, USA) through High Performance Liquid Chromatography. Stock solutions of AFB1 (1000 $\mu g\ L^{-1}$) and AFB2 (500 $\mu g\ L^{-1}$) were prepared in methanol and stored at 4°C. The working solutions of AFB1 (100 $\mu g\ L^{-}$) and AFB2 (50 $\mu g\ L^{-1}$) were prepared by diluting the stock solution.

Preparation of Plant Extract

Plant extracts were prepared according to the method described by Velazhahan et al. (2010) with some modifications. Samples were surface-sterilized using 1% sodium hypochlorite for 10 min and washed several times with sterile distilled water. After that aqueous extract of *Trachyspermum ammi* leaves and seeds was prepared by homogenizing 10 g of leaves/seeds with 10 mL of sterile distilled water. Homogenate was filtered through muslin cloth and centrifuged at 14,000 rpm for 20 min. Supernatant was sterilized using syringe filter assembly and used for further detoxification studies.

In vitro Toxin Inactivation Assay

For detoxification studies, 50 µL of working solution containing (100 $\mu g\ L^{-1}$) AFB1 and (50 $\mu g\ L^{-1}$) AFB2 was mixed with 250 µL of *T. ammi* plant extracts and incubated for various intervals of time. After incubation, 250 µL of Chloroform was added to above mixture and mixed well by vortexing. After that, the mixture was centrifuged at 13,000 rpm for 10 min in a centrifuge (Eppendorf, 5424C) to separate the chloroform fraction. After centrifugation, the chloroform fraction was

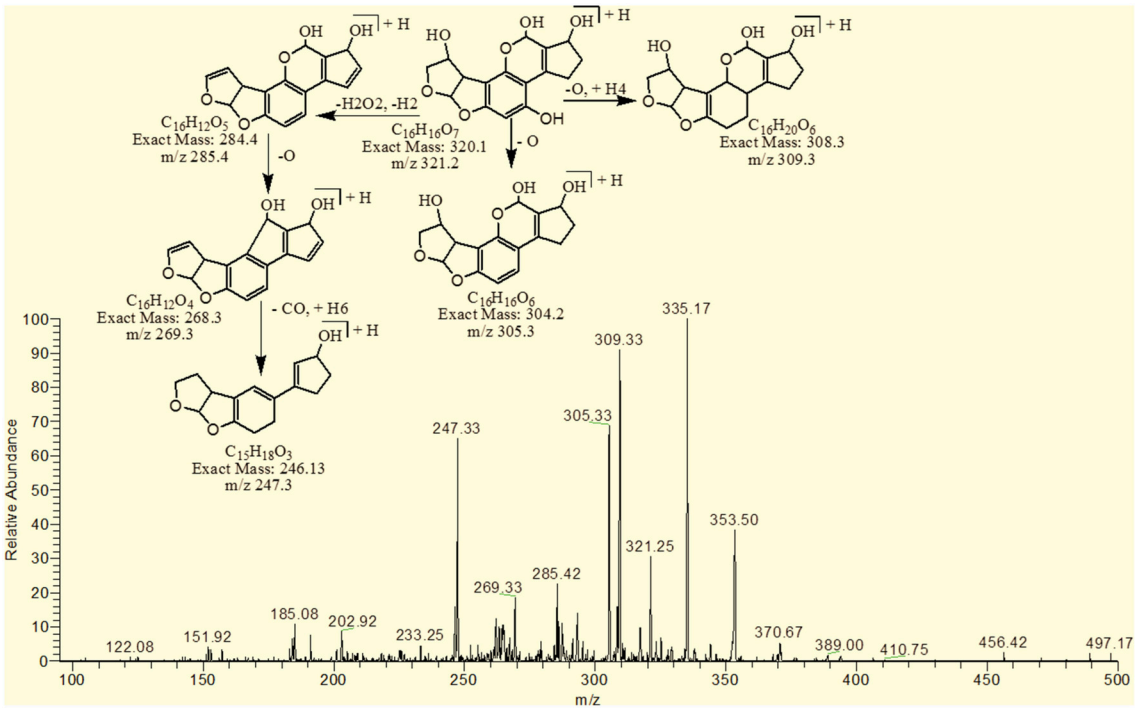


FIGURE 6 | MS/MS spectra and fragmentation pathway of degradation product with 321.25 m/z.

transferred to another glass tube, evaporated to dryness under gentle stream of nitrogen and re-dissolved in methanol. Control consisted of 50 μL of toxin in 250 μL of water and was incubated under same conditions. All experiments were conducted in triplicate.

In vitro Optimization of Parameters for Toxin Detoxification

pH

The optimal pH was determined by modifying the original pH of the *T. ammi* aqueous extracts in the range of 2.0–10.0 (adjusted using either 1 N HCl or 1 N NaOH) and then assayed for toxin detoxification activity. Distilled water with same pH range as well as untreated extract was used as control.

Temperature and Incubation Time

For assessing optimum temperature and incubation period, *T. ammi* extracts were incubated with toxins at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C for 3, 6, 12, 24, 48, and 72 h respectively. After incubation, the toxin content in the reaction mixture was determined as described above.

Effect of Boiling on Toxin Detoxification Properties of Plants Extracts

In order to study the effect of boiling on toxin detoxification properties of plants extracts, 1mL of aqueous plant extract was added in 1.5 mL eppendorf tube and placed in a boiling water bath for 5–10 min, cooled to room temperature and then tested for toxin detoxification activity.

Detoxification of Maize Samples Using Plant Extracts (*In vivo* Studies)

In *In vivo* studies, ten grams of maize seeds were kept in each 250 ml Erlenmeyer flask and spiked with 3ml of aflatoxins (with concentration B1 100 μg L^{-1} and B2 50 μg L^{-1}) according to the method described by Das and Mishra (2000) with some modification. These samples were then incubated with 10 ml of *T. ammi* seeds and leaves aqueous extract at 30°C for 72 h.

After incubation, aflatoxins extraction was performed according to the method described by Stroka et al. (2000) with some modifications. Maize samples were extracted with water–acetonitrile (15: 85 v/v) and incubated on shaking water bath for 2 h. After incubation, the extracts were filtered through filter paper (Whatman, Inc., Clifton, NJ, USA). Immunoaffinity columns were conditioned with double distilled water. Then, the filtrate was passed through the column in a solid phase extraction assembly. Toxins were slowly eluted from the column with 1mL methanol in a glass vial. The residual toxin was qualitatively and quantitatively analyzed by TLC and HPLC respectively. Controls consist of untreated maize sample, sample with toxin without *T. ammi* extract, sample with *T. ammi* extract without toxin. Experiments were done in triplicate.

Detection and Quantification of Treated Toxin

The detection and qualification of residual toxin was determined by thin layer chromatography (TLC) according to the method described by Ramesh et al. (2013) with some modifications.

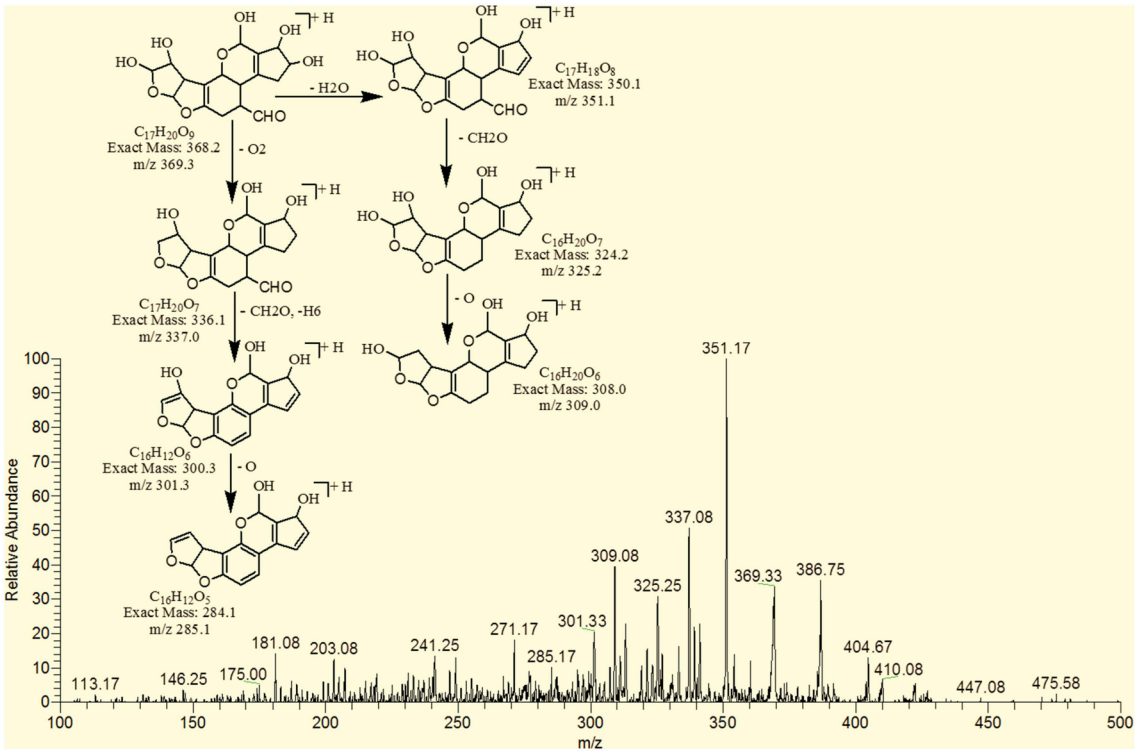


FIGURE 7 | MS/MS spectra and fragmentation pathway of degradation product with 369.33 m/z.

Twenty microliters of chloroform methanol fraction of treated and control samples were spotted on 0.25 mm silica gel $60F_{254}$ (20 × 20 cm, Merck) TLC plate and developed in chloroform: acetone (92:8 v/v). The developed plates were viewed under UV light at 365 nm.

Quantitative analysis of treated and untreated toxin was done by using High Performance Liquid Chromatography (HPLC) after derivatization. Derivatization was carried out as described by Hernandez-Hierro et al. (2008) with some modifications. For this purpose, elute was evaporated to dryness with gentle stream of nitrogen, redissolved in 200 µL of n-Haxane, vortexed for 30 s. Next, 50 µL of trifluroacetic acid (TFA) was added to it. Finally, 950 µL of acetonitrile-water (1:9) was added to above solution and filtered by using syringe filter assembly. The filtrate was analyzed by HPLC.

A HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) with a reversed- phase C18 column (Merck, Darmstadt, Germany) and a fluorescence detector was used for quantification. Mobile phase consisting of water: methanol: acetonitrile in the volume ratio 60:20:20 at a flow rate of 1 mL/min was applied and aflatoxin was detected at excitation and emission wavelengths of 360 and 440 nm respectively. For HPLC method validation, calibration curves were drawn using a series of calibration solutions in methanol. Each standard solution was chromatographed in duplicate. Further, identification of degraded toxin metabolites was carried out by mass spectral studies.

LCMS Analysis of Degraded Toxin

Toxin products were analyzed by using surveyor LC system equipped with mass spectrophotometer and PDA plus detectors (Thermo Fisher Scientific). The system was validated with known standards individually and in mixture form. All analysis were performed in triplicate using luna phenomenex C_{18} column (150 × 4.6 mm, 3 µm), in isocratic mode. Following are the LC-MS conditions for Aflatoxins. Injection volume was 10 µL. The mobile phase consisted of Methanol: Acetonitrile: Water (22.5: 22.5: 55.0 v/v). Column temperature was maintained at 30°C. The total operation time was 25 min with the flow rate of 0.5 mL min^{-1}. MS conditions were as follows: capillary temperature was 335°C, sheath gas flow and Auxiliary gas flow was 20 L min^{-1} and 4 L min^{-1} respectively. Source voltage, capillary voltage and tube lens voltage was 5 KV, 49 V, and 120 V respectively. Toxins incubated with water instead of plant extracts, under optimized conditions of pH (8.0) and temperature (30°C) were run as control in LCMS analysis.

ESI—MS/MS Conditions for Aflatoxins through Direct Insertion Pump

Samples were further analyzed by mass spectrometer with electrospray ionization (ESI) to predict the molecular formulae as well as elemental composition of degraded products of AFB1 and AFB2. Mass spectrometery/Mass spectrometery was performed on a Thermo Scientific LTQ XL System fitted with electrospray ionization (ESI) source operating in positive ionization mode

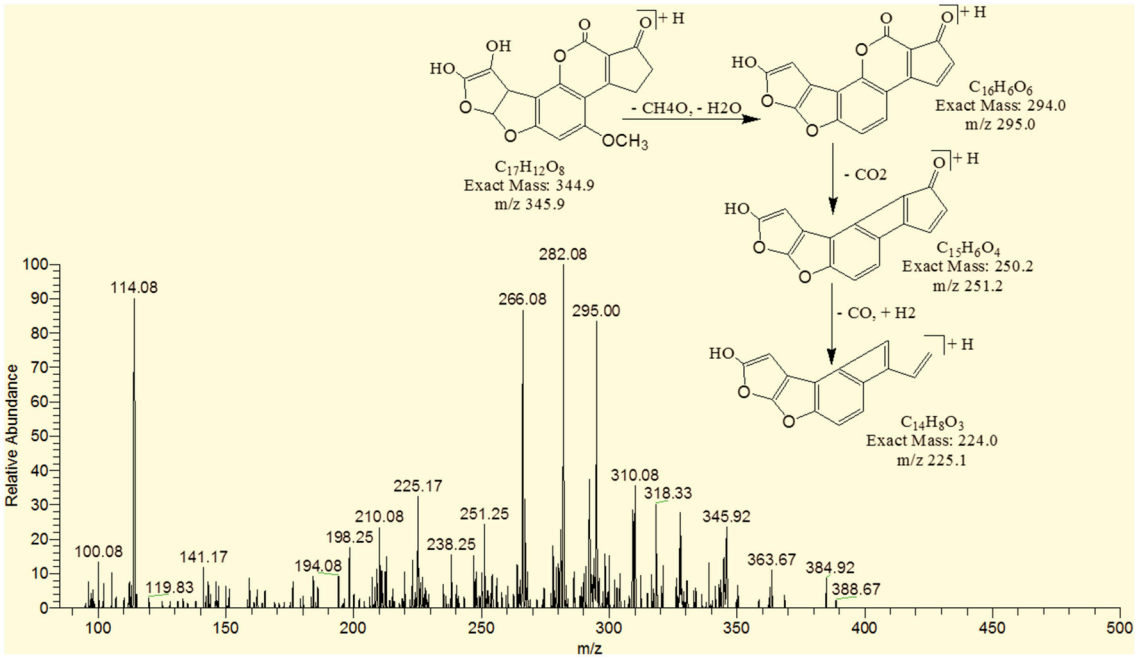


FIGURE 8 | MS/MS spectra and fragmentation pathway of degradation product with 345.92 m/z.

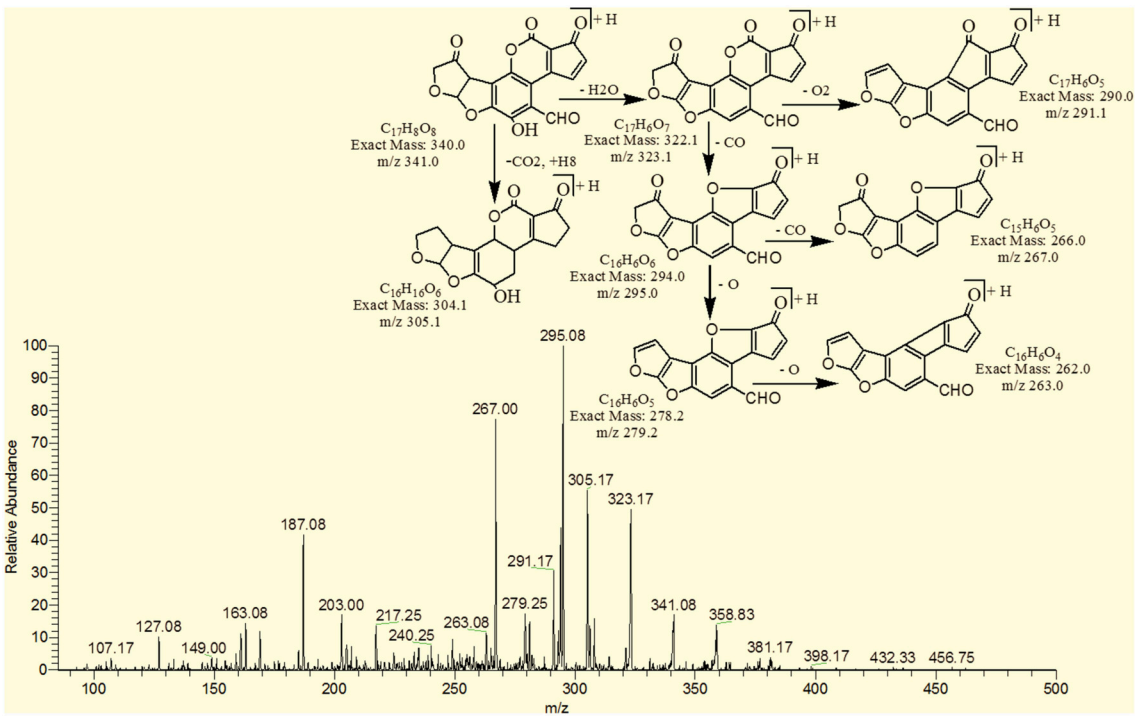


FIGURE 9 | MS/MS spectra and fragmentation pathway of degradation product with 341.08 m/z.

with optimum conditions set as follows: capillary voltage to 49.0 V, source voltage to 5.0 KV, Tube lens voltage to 110 V, and capillary temperature to 275°C. Sheath and auxiliary gas flow were adjusted to get stable spray i.e., 3 L min^{-1} and 0.4 L min^{-1} respectively. Data were collected in positive mode within the range of 100–500 m/z. The final identification of an unknown compound was based on the accurate mass measurement of parent and fragments ions, as well as other useful MS/MS spectrum information (Wang et al., 2011). Untreated toxins (AFB1 and AFB2) and water treated toxins were run as control in MS/MS experiments.

Testing Biological Toxicity of Degraded Products

The biological toxicity of degraded toxin products was tested using brine shrimps (*Artemia salina*) bioassay. The procedure for the bioassay generally followed the method developed by Solis et al. (1993) with some modifications. Brine shrimps dry eggs were procured from local market. 100–200 mg of shrimps eggs were hatched in artificial sea water (34 g sea salt/L of deionized water) by incubation under 60 W lamp, providing direct light and warmth (26°C). After an incubation period, the hatched nauplii were separated from shells and transferred to fresh sea water.

300 µl of treated and untreated AFB1 (100 µg L^{-1}) and AFB2 (50 µg L^{-1}) solution was added to 96 well plate separately and dried overnight. After complete evaporation of solvent, toxins were re-dissolved in 200 µL of sea water. 200 µL of sea water containing 40–45 organisms were pipetted into each well, resulting in a final volume of 400 µL and incubated for 24–96 h at 26°C. Mortality was determined by counting the immobile (dead) larvae under stereoscope microscope. Toxicity of each solution was evaluated in triplicate.

Statistical Analysis

Results obtained in various experiments were subjected to statistical analysis by using DSSTAT software. Data were analyzed by analysis of Variance (ANOVA) and differences among the means were determined for significance at $P \leq 0.05$ using Tukey's multiple range test.

RESULTS

Effect of Temperature and Incubation Period on Toxin Detoxification by *T. ammi* Extracts (*In vitro*)

Aqueous extracts of *Trachyspermum ammi* leaves and seeds were evaluated for their ability to detoxify aflatoxin B1 and B2 at different temperatures and incubation time. The extent of detoxification was compared with that of control under same conditions. Time course study of toxin degradation showed that detoxification of AFB1 and AFB2 started within 3 h of incubation and percentage of degradation increased with increase in incubation time (**Table 1**). Results indicated that at lowest tested temperature of 25°C, aqueous extract of *T. ammi* seeds showed higher detoxification of AFB1 and AFB2 after 3 h of incubation i.e., 52.4 and 69.3% respectively as compared to *T. ammi* leaves extract. This percentage of detoxification increased with increase in incubation time to 72 h that detoxified AFB1 to 79.8% and AFB2 up to 78.9%.

It is evidenced from the results that percentage detoxification of AFB1 and AFB2 by *T. ammi* extracts was progressively increased with the consequent increase in temperature from 30 to 55°C. The highest inactivation was observed at 60°C. At this temperature, respective control (water) showed 10.4 and 8.6% detoxification of AFB1 and AFB2 after 72 h of incubation. However, toxin treated with *T. ammi* leaves and seeds extract showed 79.46 and 94.7% detoxification of aflatoxin B1 while detoxification of aflatoxin B2 was 66.6 and 90.1% respectively, under same conditions. This detoxification may be due to synergistic effect of heat and moisture (**Table 1**).

In the present investigation, it was found that *T. ammi* seeds extract was effective in detoxifying aflatoxin B1 and B2 at all tested temperatures and incubation periods. However, for further studies, 30°C was selected as it is more or less near to room temperature and moreover was found close to be the existing temperature of storehouses in Punjab especially in summer. Therefore, by selecting this temperature cost of maintaining temperature in storehouses can be greatly reduced.

Effect of pH on Toxin Detoxification by *T. ammi* Extracts (In vitro)

The comparative assessment of *T. ammi* leaves and seeds extract to detoxify AFB1 and AFB2 at different pH values revealed that least significant detoxification occurred at pH 2. Results indicated that at pH 2, percentage reduction of AFB1 and AFB2 was 81.2 and 74.9% respectively in samples treated with *T. ammi* seeds extract after 72 h of incubation at 30°C.The efficacy of *T. ammi* seeds extract to detoxify AFB1 and AFB2 was significantly ($P < 0.05$) increased with increase in pH from 4 to 10 (**Figure 1**). Distilled water with pH adjusted to 2, 4, 6, 8, and 10 was used as a control. Control data showed that at pH 10, 19.94% of AFB1 and 17.45% of AFB2 was degraded after 72 h of incubation at 30°C while 15.88 and 13.05% degradation of AFB1 and AFB2 was observed at pH 8 under same conditions. The percentage of degradation decreases as the pH decreases to neutral or acidic range.

Maximum degradation of AFB1 and AFB2 was observed at pH 10 after treatment with *T. ammi* seeds extract i.e., 94.9 and 94.1% as compared to *T. ammi* leaves extract with degradation percentage of 76.2 and 61.9% respectively.

However, at high basic pH conditions aflatoxins are known to become unstable and sensitive, therefore to avoid this in further experimentations pH 8 was selected which is 100 times less alkaline than pH 10. It was also evidenced from the results that *T. ammi* seeds extract significantly ($P < 0.05$) detoxified AFB1 and AFB2 at slightly alkaline pH 8 and their results were closely comparable with the results obtained at pH 10 (**Figure 1**).

Effect of Boiling on Toxin Detoxification Properties of *T. ammi* Extracts

The aflatoxins detoxification efficacy of *T. ammi* leaves and seeds extract was significantly ($P < 0.05$) decreased upon boiling

TABLE 1 | Effect of Temperature and Incubation Period on AFB1 and AFB2 Detoxification by Plant Extracts.

Control	Temp (°C)	AFB1 percentage reduction						AFB2 percentage reduction					
		3 h	6 h	12 h	24 h	48 h	72 h	3 h	6 h	12 h	24 h	48 h	72 h
Aflatoxin	25	$0.25^{d\ I}$(1.64)	$0.71^{cd\ H}$(1.86)	$1.45^{bc\ J}$(0.84)	$1.71^{b\ H}$(1.65)	$1.94^{b\ H}$(0.42)	$2.86^{a\ I}$(1.54)	$0.12^{c\ M}$(0.14)	$0.53^{bc\ M}$(1.63)	0.72^{abcN}(1.38)	0.84^{abcL}(1.89)	$1.02^{ab\ M}$(1.72)	$1.44^{a\ N}$(1.34)
	30	$0.80^{d\ I}$(1.98)	$0.80^{d\ H}$(1.98)	$1.88^{cd\ J}$(1.44)	$2.30^{bc\ H}$(1.32)	$3.07^{ab\ GH}$(1.32)	$3.81^{a\ I}$(1.04)	$0.17^{c\ M}$(1.23)	$0.68^{bc\ M}$(1.73)	$0.79^{bc\ N}$(1.62)	$0.99^{ab\ L}$(0.73)	$1.16^{ab\ M}$(1.11)	$1.66^{a\ N}$(1.20)
	35	$1.21^{c\ I}$(0.96)	$2.54^{bc\ H}$(1.65)	$2.54^{bc\ J}$(1.53)	3.01^{abcGH}(1.08)	$3.80^{ab\ GH}$(1.81)	$4.49^{a\ HI}$(1.76)	$0.23^{c\ M}$(1.16)	$0.82^{bc\ M}$(0.81)	$0.87^{bc\ N}$(1.37)	$1.14^{b\ L}$(1.08)	$1.31^{ab\ M}$(1.21)	$1.88^{a\ N}$(1.15)
	40	$2.21^{b\ I}$(1.85)	$3.53^{ab\ H}$(1.24)	$3.87^{ab\ IJ}$(1.39)	$4.33^{a\ GH}$(1.73)	$4.46^{a\ FGH}$(1.93)	$5.15^{a\ GHI}$(1.34)	$0.28^{c\ M}$(1.87)	$0.94^{bc\ M}$(1.71)	$0.97^{bc\ N}$(1.78)	$1.29^{b\ L}$(1.87)	$1.46^{ab\ M}$(0.77)	$2.11^{a\ MN}$(1.52)
	45	$3.20^{b\ I}$(1.31)	$4.53^{ab\ H}$(1.98)	$5.13^{a\ IJ}$(1.88)	$5.19^{a\ GH}$(0.49)	$5.66^{a\ FGH}$(1.47)	$5.82^{a\ GHI}$(1.72)	$0.33^{c\ M}$(1.28)	$1.02^{bc\ M}$(1.38)	$1.12^{b\ N}$(1.24)	$1.44^{b\ L}$(0.96)	$1.61^{b\ M}$(1.81)	$2.33^{a\ MN}$(0.85)
	50	$4.19^{b\ I}$(1.57)	$5.52^{ab\ H}$(0.74)	$5.79^{ab\ IJ}$(1.97)	$6.48^{a\ GH}$(1.24)	$6.51^{a\ FGH}$(0.48)	$6.98^{a\ GHI}$(0.27)	$0.38^{c\ M}$(1.68)	$1.09^{bc\ M}$(0.28)	$1.27^{b\ N}$(1.56)	$1.59^{b\ L}$(1.26)	$1.76^{b\ M}$(0.56)	$2.55^{a\ MN}$(1.28)
	55	$5.18^{c\ I}$(1.76)	$6.45^{bc\ H}$(1.92)	$6.51^{bc\ IJ}$(1.31)	$7.14^{ab\ GH}$(1.21)	$7.84^{ab\ FGH}$(1.25)	$8.30^{a\ GHI}$(1.64)	$0.43^{d\ M}$(0.92)	$1.16^{cd\ M}$(1.42)	$1.42^{bc\ N}$(1.07)	$1.73^{bc\ L}$(1.37)	$1.91^{b\ M}$(1.29)	$2.78^{a\ MN}$(1.17)
	60	$6.18^{c\ I}$(1.65)	$7.11^{c\ H}$(1.34)	$7.50^{c\ IJ}$(1.14)	$7.80^{bc\ GH}$(1.30)	$9.16^{ab\ FGH}$(1.64)	$9.63^{a\ GHI}$(1.03)	$0.49^{d\ M}$(1.72)	$1.24^{cd\ M}$(1.87)	$1.57^{bc\ N}$(1.81)	$1.88^{bc\ L}$(1.28)	$2.06^{b\ M}$(1.54)	$3.00^{a\ MN}$(1.89)
Aflatoxin + Water	25	$0.28^{b\ I}$(0.62)	$1.48^{b\ H}$(1.46)	$2.72^{ab\ J}$(1.12)	$3.35^{a\ GH}$(1.78)	$3.36^{a\ GH}$(1.54)	$3.38^{a\ I}$(1.17)	$0.29^{c\ M}$(1.78)	$0.47^{c\ M}$(1.21)	$1.32^{bc\ N}$(0.47)	1.47^{abcL}(1.62)	$2.25^{ab\ M}$(1.74)	$2.46^{a\ MN}$(1.12)
	30	$1.10^{c\ I}$(1.35)	$2.64^{bc\ H}$(1.82)	$3.23^{ab\ J}$(0.89)	$3.56^{ab\ GH}$(1.85)	$3.97^{ab\ FGH}$(1.34)	$4.19^{a\ HI}$(0.81)	$0.36^{c\ M}$(1.19)	$1.14^{bc\ M}$(1.91)	$1.56^{bc\ N}$(1.18)	$2.04^{ab\ L}$(1.27)	$2.41^{ab\ M}$(0.37)	$3.41^{a\ MN}$(1.23)
	35	$2.44^{c\ I}$(1.54)	$3.44^{bc\ H}$(0.99)	$4.80^{ab\ IJ}$(1.56)	$5.09^{a\ GH}$(0.65)	$5.49^{a\ FGH}$(0.37)	$5.85^{a\ GHI}$(0.53)	$1.20^{c\ M}$(1.33)	$1.23^{c\ M}$(1.02)	$2.26^{b\ MN}$(1.72)	$2.73^{ab\ KL}$(0.18)	$2.87^{ab\ M}$(1.29)	$3.34^{a\ MN}$(0.62)
	40	$3.76^{c\ I}$(1.98)	$4.76^{bc\ H}$(1.02)	$6.13^{ab\ IJ}$(1.44)	$6.48^{a\ GH}$(1.21)	$6.74^{a\ FGH}$(1.72)	$6.84^{a\ GHI}$(1.71)	$1.98^{c\ LM}$(1.27)	$2.69^{bc\ M}$(1.75)	$3.37^{ab\ MN}$(1.62)	$3.40^{ab\ KL}$(1.29)	$3.71^{a\ M}$(1.13)	$3.84^{a\ MN}$(1.91)
	45	$5.08^{c\ I}$(0.87)	$6.08^{bc\ H}$(1.16)	$7.45^{ab\ IJ}$(0.81)	$7.47^{ab\ GH}$(0.45)	$7.83^{a\ FGH}$(1.11)	$8.40^{a\ GHI}$(0.67)	$2.72^{c\ KLM}$(1.54)	$3.92^{b\ M}$(1.87)	$4.09^{ab\ MN}$(0.31)	$4.18^{ab\ KL}$(1.89)	$4.49^{ab\ M}$(1.82)	$4.96^{a\ MN}$(0.17)
	50	$6.41^{c\ I}$(1.43)	$7.41^{bc\ H}$(1.29)	$8.46^{ab\ IJ}$(1.01)	$8.77^{ab\ GH}$(1.26)	$8.82^{ab\ FGH}$(1.28)	$10.05^{a\ GHI}$(0.45)	$3.46^{b\ KLM}$(0.51)	$4.44^{b\ M}$(1.32)	$4.46^{b\ MN}$(1.85)	$5.60^{a\ KL}$(0.61)	$5.67^{a\ M}$(1.21)	$6.07^{a\ MN}$(1.62)
	55	$7.73^{c\ I}$(1.58)	$8.73^{bc\ H}$(1.73)	$9.46^{bc\ IJ}$(1.72)	$9.82^{b\ GH}$(0.28)	$10.10^{b\ FG}$(1.32)	$11.71^{a\ GH}$(1.49)	$4.21^{b\ KL}$(1.08)	$4.83^{b\ M}$(1.64)	$4.96^{b\ MN}$(0.11)	$6.72^{a\ KL}$(0.12)	$7.15^{a\ M}$(0.60)	$7.19^{a\ MN}$(1.51)
	60	$9.05^{c\ I}$(1.41)	$10.05^{bc\ H}$(0.86)	$10.45^{bc\ I}$(0.26)	$10.81^{bc\ G}$(0.17)	$11.42^{b\ F}$(1.19)	$13.36^{a\ G}$(1.23)	$4.95^{b\ K}$(1.43)	$5.20^{b\ M}$(1.30)	$5.48^{b\ M}$(1.28)	$7.84^{a\ K}$(0.84)	$8.31^{a\ M}$(1.35)	$8.64^{a\ M}$(1.11)
Toxin + *T. ammi* leaves extract	25	$28.81^{c\ H}$(1.98)	$33.64^{c\ G}$(1.54)	$36.25^{bc\ H}$(1.1)	$43.17^{bc\ F}$(2.03)	$52.63^{ab\ E}$(2.96)	$62.16^{a\ F}$(1.21)	$34.60^{e\ J}$(1.72)	39.06^{deL}(1.21)	$44.27^{cd\ L}$(0.70)	$47.99^{bc\ J}$(0.19)	$51.71^{ab\ L}$(1.52)	$55.80^{a\ L}$(1.08)
	30	$34.17^{c\ GH}$(1.84)	$37.51^{c\ G}$(1.65)	$38.34^{bc\ H}$(1.27)	$43.91^{bc\ F}$(1.46)	$55.02^{ab\ E}$(1.08)	$63.35^{a\ F}$(0.89)	$36.09^{d\ IJ}$(1.87)	$40.80^{cd\ L}$(1.61)	$46.50^{bc\ KL}$(1.17)	$50.22^{b\ IJ}$(0.85)	$53.45^{ab\ KL}$(0.59)	$57.66^{a\ KL}$(0.15)
	35	$38.34^{c\ FG}$(1.56)	$41.97^{c\ FG}$(1.87)	$42.50^{bc\ GH}$(1.18)	$47.48^{bc\ EF}$(1.49)	$58.29^{ab\ E}$(1.83)	$66.03^{a\ F}$(1.17)	$37.57^{d\ HIJ}$(1.65)	$43.03^{cd\ KL}$(1.98)	$48.73^{bc\ JKL}$(1.06)	$51.71^{b\ HIJ}$(0.97)	$54.93^{ab\ JKL}$(1.17)	$59.15^{a\ JKL}$(1.39)
	40	$45.53^{c\ EF}$(1.32)	$49.42^{c\ EF}$(1.21)	$49.35^{c\ FG}$(1.57)	$54.63^{bc\ DE}$(1.62)	$65.74^{ab\ D}$(1.74)	$73.18^{a\ E}$(1.87)	$39.06^{d\ G-J}$(1.97)	$45.26^{cd\ JK}$(1.53)	$50.97^{bc\ IJK}$(1.21)	$53.20^{b\ G-J}$(0.81)	$56.42^{ab\ IJK}$(0.43)	$60.64^{a\ IJK}$(1.17)
	45	$47.02^{c\ EF}$(1.70)	$52.99^{c\ DE}$(1.34)	$52.63^{c\ F}$(1.56)	$55.82^{bc\ D}$(1.39)	$66.33^{ab\ D}$(1.32)	$76.75^{a\ DE}$(1.25)	$40.55^{d\ F-I}$(1.78)	$47.49^{cd\ IJ}$(1.42)	$53.20^{bc\ HIJ}$(1.15)	$54.69^{b\ F-I}$(0.82)	$57.91^{ab\ HIJ}$(0.55)	$62.13^{a\ HIJ}$(1.19)
	50	$47.22^{c\ EF}$(1.65)	$52.18^{c\ DE}$(1.42)	$53.22^{c\ EF}$(1.19)	$56.71^{bc\ D}$(0.96)	$66.63^{b\ D}$(0.73)	$77.94^{a\ D}$(1.59)	$42.04^{d\ FGH}$(1.31)	$49.73^{c\ HI}$(1.87)	$55.43^{bc\ GHI}$(1.20)	$56.17^{bc\ FGH}$(1.28)	$59.40^{ab\ GHI}$(1.32)	$63.62^{a\ GHI}$(1.36)
	55	$49.70^{c\ DE}$(1.29)	$55.08^{c\ CDE}$(1.37)	$56.20^{bc\ DEF}$(1.17)	$59.99^{bc\ D}$(1.16)	$67.25^{ab\ C}$(1.1)	$78.24^{a\ D}$(1.04)	$43.53^{d\ FG}$(1.56)	$51.96^{c\ GH}$(1.42)	$57.66^{bc\ GH}$(0.86)	$57.66^{bc\ FG}$(1.14)	$60.89^{ab\ GH}$(1.07)	$65.10^{a\ GH}$(1.28)
	60	$51.79^{c\ DE}$(1.37)	$56.56^{c\ CDE}$(1.45)	$56.50^{c\ DEF}$(1.62)	$60.58^{bc\ D}$(1.79)	$68.51^{ab\ CD}$(2.13)	$79.43^{a\ CD}$(1.96)	$45.01^{d\ F}$(1.78)	$54.19^{c\ G}$(1.12)	59.90^{abcG}(1.58)	$59.15^{bc\ F}$(1.29)	$62.38^{ab\ G}$(1.19)	$66.59^{a\ G}$(1.09)
Toxin + *T. ammi* leaves extract	25	$52.39^{d\ DE}$(1.21)	$55.39^{d\ CDE}$(1.08)	$61.86^{cd\ CDE}$(1.19)	$68.48^{bc\ C}$(1.23)	$73.21^{ab\ BC}$(1.37)	$79.77^{a\ CD}$(1.28)	$69.26^{b\ E}$(1.56)	$72.24^{ab\ F}$(1.67)	$75.22^{ab\ F}$(0.58)	$76.70^{a\ E}$(1.29)	$78.19^{a\ F}$(1.44)	$78.94^{a\ F}$(0.96)
	30	$57.75^{d\ CD}$(1.38)	$59.26^{cd\ CD}$(1.55)	$63.94^{cd\ CD}$(1.73)	$69.22^{bc\ C}$(1.90)	$75.59^{ab\ B}$(1.12)	$80.97^{a\ CD}$(1.04)	$70.75^{c\ DE}$(1.45)	$73.98^{bc\ F}$(1.86)	77.45^{abcEF}(1.24)	$78.94^{ab\ DE}$(1.31)	$79.93^{ab\ EF}$(1.20)	$81.17^{a\ EF}$(1.17)
	35	$61.92^{d\ BC}$(1.67)	$63.72^{d\ BC}$(1.23)	$68.11^{cd\ BC}$(1.47)	$72.79^{bc\ BC}$(1.26)	$78.86^{ab\ B}$(1.16)	$83.65^{a\ C}$(1.02)	$72.24^{b\ CDE}$(1.54)	$76.21^{ab\ EF}$(1.38)	$79.68^{b\ DEF}$(1.63)	$80.42^{a\ CDE}$(1.51)	$81.42^{a\ DEF}$(1.65)	$82.66^{a\ DE}$(1.13)
	40	$69.11^{d\ AB}$(1.56)	$69.98^{d\ AB}$(1.87)	$74.96^{cd\ AB}$(1.23)	$79.94^{bc\ AB}$(1.22)	$86.31^{ab\ A}$(1.06)	$88.41^{a\ B}$(0.89)	$73.73^{b\ B-E}$(1.09)	$78.44^{ab\ DE}$(1.34)	$81.88^{a\ CDE}$(1.78)	$81.91^{a\ B-E}$(1.81)	$82.90^{a\ CDE}$(1.35)	$84.14^{a\ CDE}$(1.79)
	45	$70.60^{e\ AB}$(0.61)	74.44^{deA}(1.78)	$78.23^{cd\ A}$(0.99)	$81.13^{bc\ A}$(1.12)	$86.90^{ab\ A}$(1.34)	$91.98^{a\ AB}$(1.42)	$75.22^{b\ A-D}$(1.21)	$80.67^{ab\ CD}$(1.85)	$84.14^{a\ BCD}$(1.56)	$83.40^{a\ A-D}$(1.07)	$84.39^{a\ BCD}$(1.54)	$85.63^{a\ BCD}$(0.66)
	50	$70.44^{d\ AB}$(1.42)	$73.65^{d\ A}$(1.87)	$78.83^{c\ A}$(1.16)	$81.43^{c\ A}$(1.22)	$87.20^{b\ A}$(1.33)	$93.17^{a\ A}$(1.07)	$76.70^{b\ ABC}$(1.82)	$82.90^{ab\ BC}$(1.36)	$86.38^{a\ ABC}$(1.13)	$84.89^{a\ ABC}$(1.07)	$85.88^{a\ ABC}$(0.84)	$87.12^{a\ ABC}$(1.18)
	55	$72.09^{d\ A}$(1.66)	$75.78^{d\ A}$(1.32)	$81.81^{c\ A}$(0.85)	$84.70^{bc\ A}$(1.87)	$88.58^{ab\ A}$(1.34)	$93.47^{a\ A}$(1.19)	$78.19^{b\ AB}$(1.36)	$85.14^{ab\ AB}$(1.24)	$88.61^{a\ AB}$(1.02)	$86.38^{a\ AB}$(0.87)	$87.37^{a\ AB}$(1.72)	$88.61^{a\ AB}$(1.92)
	60	$72.69^{e\ A}$(1.09)	77.27^{deA}(1.75)	$82.11^{cd\ A}$(1.65)	$85.30^{bc\ A}$(1.82)	$89.09^{b\ A}$(1.73)	$94.66^{a\ A}$(1.22)	$79.68^{b\ A}$(1.52)	$87.37^{a\ A}$(1.31)	$90.84^{a\ A}$(1.82)	$87.86^{a\ A}$(1.30)	$88.86^{a\ A}$(1.67)	$90.10^{a\ A}$(1.17)

Values are mean of three replicates.

Small alphabetic letters with different values indicate significant differences ($p < 0.05$) in toxin reduction at different temperature and incubation periods in each row.

Capital alphabetic letters with different values indicate significant differences ($p < 0.05$) among controls and tested plant extracts at different temperature and incubation period in each column. Standard deviation values are shown in parenthesis.

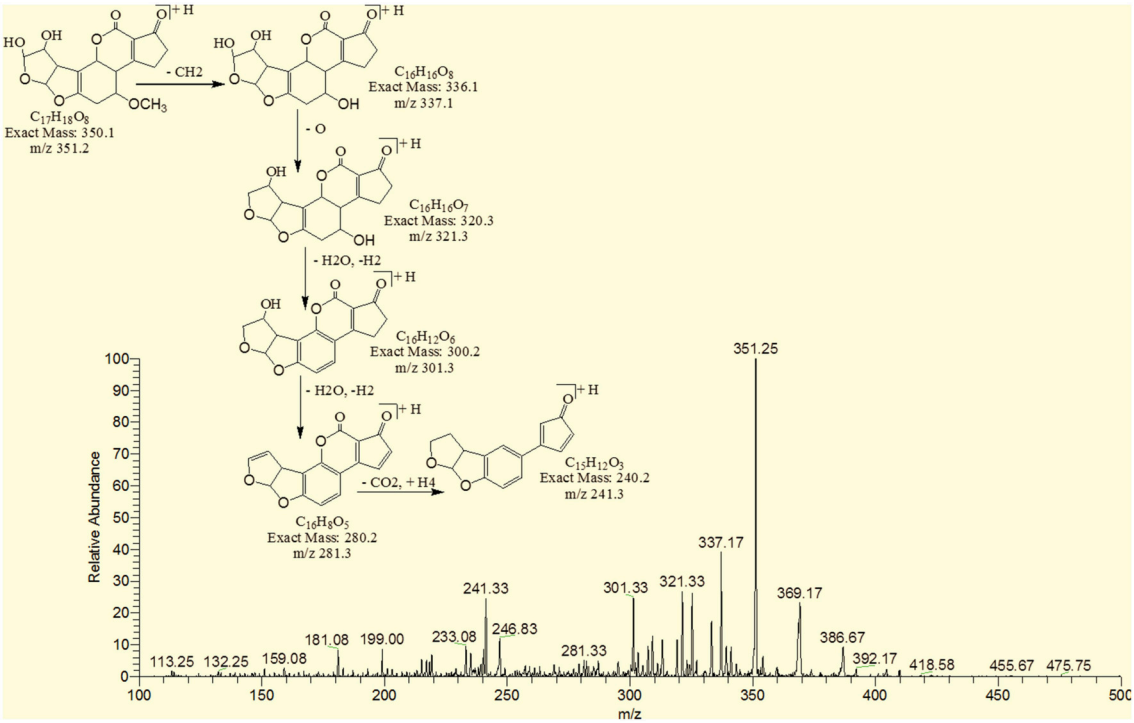


FIGURE 10 | MS/MS spectra and fragmentation pathway of degradation product with 351.25 m/z.

at 100°C for 10 min. The pH of treated (boiled) and untreated (Unboiled) extracts was adjusted to 8. Unboiled *T. ammi* leaves extract showed 73.0 and 60.6% detoxification of AFB1 and AFB2 as compared to boiled extracts i.e., 54.4 and 45.7% respectively. Similarly 92.8 and 91.9% detoxification of AFB1 and AFB2 was recorded after treatment with unboiled *T. ammi* seeds extract in comparison with boiled seeds extract with 69.9 and 74.4% detoxification. Results indicated that upon boiling, AFB1 detoxifying activity of *T. ammi* seeds and leaves extract were decreased up to 23 and 18% respectively. While in case of AFB2, 15 and 17.4% decrease in detoxification was recorded after treatment with boiled extracts of *T. ammi* leaves and seeds respectively. So, these results clearly depicted that unboiled plants extracts are more efficient in degrading aflatoxins as compared to boiled extracts.

In vivo Detoxification of Aflatoxins in Maize Samples

In vivo analysis followed a similar trend as that was recorded in *in vitro* studies. These studies were carried out under conditions optimized in previous *in vitro* assays i.e., pH 8, temperature 30°C and incubation time 72 h. Data obtained from *in vivo* studies showed that maximum detoxification of AFB1 and AFB2 in spiked maize samples was carried out by *T. ammi* seeds extract after 72 h of incubation i.e., 89.6 and 86.5% respectively. As compared to *T. ammi* seeds extract, in *T. ammi* leaves extract 68.8 and 53.7% detoxification of AFB1 and AFB2 was observed in spiked samples (**Table 2**).

HPLC chromatograms confirmed that after *T. ammi* seeds extract treatment, trace amount of aflatoxin was present along with other peaks whose footprints were not found in chromatogram of parent compounds which may be attributed to toxins degradation products (**Figure 2**).

Structural Characterization of AFB1, AFB2, and Their Degradation Products

Both aflatoxin B1 and B2 exhibited good ESI ionization efficiency in the positive ion mode with molecular base ion at m/z 313.17 and m/z 315.17 for protonated adduct $[M+ H]^+$ while m/z 335 and m/z 337 for sodium adduct $[M+ Na]^+$ respectively. Identity of parent compound was validated by its fragmentation into daughter ions. The protonated molecule was chosen as the precursor ion for aflatoxins in the product ion scan mode because the sodium adduct did not exhibit specific fragmentation for any compound.

MS/MS Analysis of AFB1 and AFB2

MS/MS spectrum of AFB1 showed that continuous loss of carbon monoxide (CO) was the main fragmentation pathway. Methyl and methanol losses occurred on methoxy group located on side chain of benzene. The double bond equivalence (DBE) of AFB1 was 12 (**Figure 3A**). However, MS/MS fragmentation pathway of AFB2 revealed that daughter ions were formed by loss of carbon monoxide, oxygen, hydrogen and methyl group (**Figure 3B**). The DBE of AFB2 was 11. The identification of degradation products

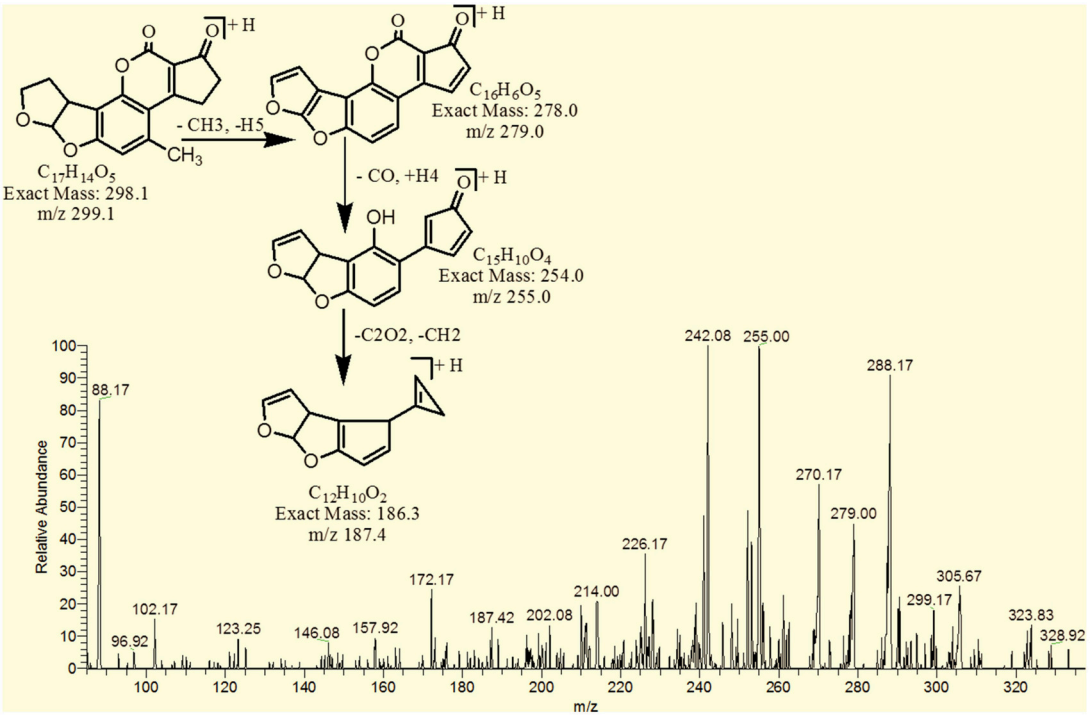


FIGURE 11 | MS/MS spectra and fragmentation pathway of degradation product with 299.17 m/z.

were based on accurate mass measurement of ions and similar fragmentation pathways with AFB1 and AFB2.

Results showed that in most of the degraded products obtained after treatment with *T. ammi* seeds extract additional reactions occurred which leads to the loss of double bond in terminal furan ring responsible for toxicity. Structural formulas of possible hypothesized degraded products of AFB1 and AFB2 are shown in **Figures 4A,B**.

MS/MS Analysis for Confirmation of Degraded Products of AFB1

The degradation product at m/z 303.08 corresponded to molecular formulae $C_{16}H_{14}O_6$ was formed due to the elimination of CH_2 and addition of hydrogen atoms. The DBE of $C_{16}H_{14}O_6$ was less than AFB1 i.e., 10. Loss of H_2O, CO_2, CO and O was the main fragmentation pathway (**Figure 5**).

The degradation product $C_{16}H_{16}O_7$ (with 321.25 m/z) was formed due to the addition of hydroxyl group on the terminal furan ring and replacement of methoxy group on the side chain of benzene ring with hydroxyl group. The DBE of $C_{16}H_{16}O_7$ content was lower than AFB1 i.e., 9. Fragments demonstrated that the loss of oxygen and carbon monoxide was the main fragmentation pathway which was different from that of AFB1 (**Figure 6**).

The degradation product $C_{17}H_{20}O_9$ (with 369.33 m/z) had more H_8O_3 molecules than AFB1. The DBE of $C_{17}H_{20}O_9$ was 7, which was lower than AFB1 implying that additional reactions occurred on the furan rings. Fragmentation pathway was different from that of AFB1. The precursor ion yielded a series of product ions which were represented by 351.17 $[M\text{-}H_2O]^+$, 337.08 $[M\text{-}O_2]^+$, 325.25 $[M\text{-}CO_2]^+$, 309.08 $[M\text{-}CO_3]^+$, 301.33 $[M\text{-}CH_8O_3]^+$ and 285.17 $[M\text{-}CH_8O_4]^+$ (**Figure 7**).

The degradation product 345.92 corresponded to molecular formula $C_{17}H_{12}O_8$ was formed due to the addition of hydroxyl groups on the terminal furan ring. The DBE of $C_{17}H_{12}O_8$ was same as that of AFB1 i.e., 12. The fragments of $C_{17}H_{12}O_8$ showed losses of CH_4O, H_2O, CO_2 and CO. More details on the fragmentation pathway are shown in **Figure 8**.

TABLE 2 | *In vivo* detoxification of AFB1 and AFB2 by aqueous extracts of *T. ammi*.

	Toxin recovery (μg L^{-1})	
	AFB1	**AFB2**
CONTROL		
Unspiked maize	0.49^{a}	0.33^{a}
Unspiked maize + *T. ammi* leaf extract(s)	0.00^{a}	0.00^{a}
Unspiked maize + *T. ammi* branch extract	0.00^{a}	0.00^{a}
Spiked maize with AFB1(100ng/ml) and AFB2 (50 ng/ml)	97.30^{i}	47.65^{n}
TREATMENTS		
Spiked maize with toxin + *T. ammi* leaf extract (s)	31.2^{d}	23.1^{ef}
Detoxification (%)	68.8	53.7
Spiked maize with toxin + *T. ammi* seeds extract	10.4^{ab}	6.8^{bc}
Detoxification (%)	89.6	86.5

Values are mean of three replicates.
Data were analyzed by analysis of Variance (ANOVA).
Values with different letters show significant difference ($P = 0.05$) as determined by Tukey's Multiple Range test.

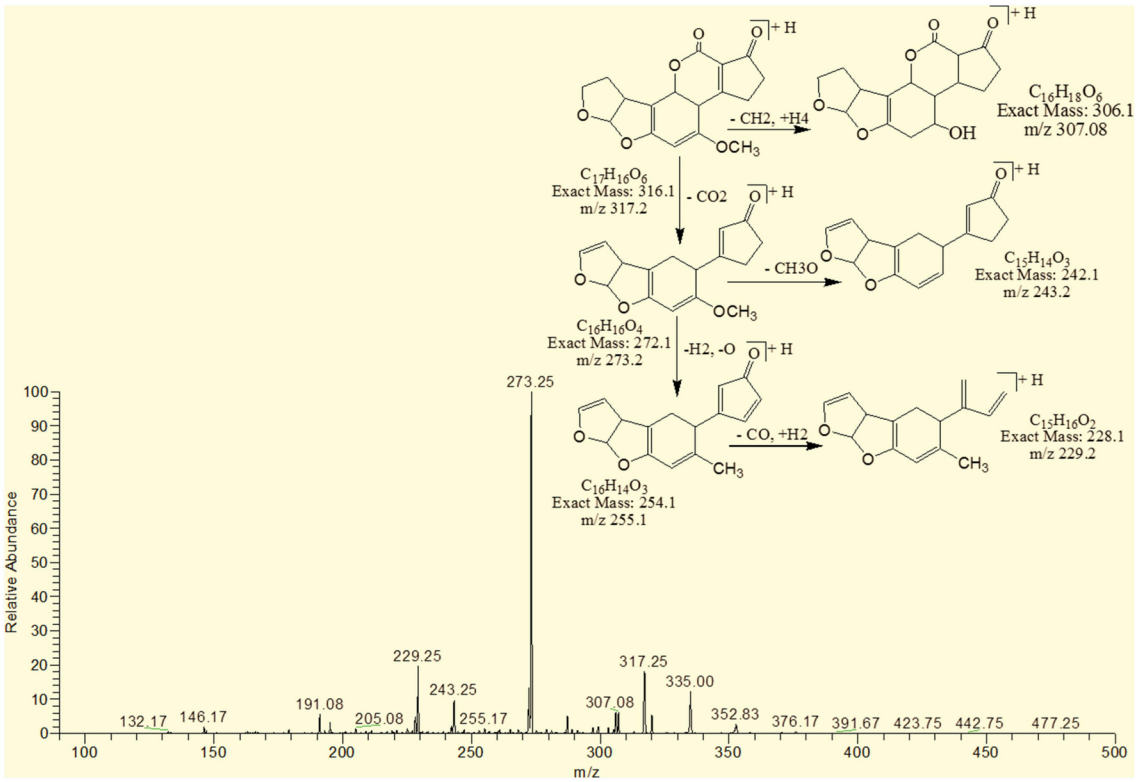


FIGURE 12 | MS/MS spectra and fragmentation pathway of degradation product with 317.25 m/z.

The degradation product $C_{17}H_8O_8$ (with 341.08 m/z) had two more oxygen and four less hydrogen atoms. Addition of oxygen occurred on the terminal furan ring and benzene ring. The DBE of $C_{17}H_8O_8$ was one more than AFB1 i.e., 13. Series of product ions formed by the precursor ion were represented by 323.17 $[M\text{-}H_2O]^+$, 295.08 $[M\text{-}CH_2O_2]^+$, 291.17 $[M\text{-}H_2O_3]^+$, 279.25 $[M\text{-}CH_2O_3]^+$, 267.0 $[M\text{-}C_2H_2O_3]^+$, 263.08 $[M\text{-}CH_2O_4]^+$ (**Figure 9**).

The degradation product $C_{17}H_{18}O_8$ (with 351.25 m/z) was formed by addition of two hydroxyl groups on the double bond of terminal furan ring. The DBE of $C_{17}H_{18}O_8$ was less than AFB1 i.e., 9 with different fragmentation pathway from that of AFB1. Product ions formed from parent ion $C_{17}H_{18}O_8$ was represented by 337.17 $[M\text{-}CH_2]^+$, 321.33$[M\text{-}CH_2O]^+$, 301.33$[M\text{-}CH_6O_2]^+$, 281.33$[M\text{-}CH_{10}O_3]^+$ and 241.33$[M\text{-}C_2H_6O_5]^+$ (**Figure 10**).

MS/MS Analysis for Confirmation of Degraded Products of AFB2

The degradation product $C_{17}H_{14}O_5$ (with m/z 299.17) was formed by the loss of oxygen atom from the side chain of benzene ring. The DBE of $C_{17}H_{14}O_5$ was same as that of AFB2. Fragments of $C_{17}H_{14}O_5$ showed losses of CH_3, C_2O_2 and CO. More detail on fragmentation pathway are shown in **Figure 11**.

However, the degradation product $C_{17}H_{16}O_6$ at m/z 317.25 had two more hydrogen atoms and one less DBE than AFB2. Loss of CO_2, CO, CH_3O and O was the main fragmentation pathway (**Figure 12**).

Assessment of Biological Toxicity of Degraded Products

Biological toxicity of degraded toxin products were tested using brine shrimps (*Artemia salina*) bioassay. The brine shrimps assay actually proved to be a convenient system for monitoring biological activity (Hartl and Humpf, 2000). In this study, the degraded toxin products were incubated with brine shrimp larvae at 26°C for 24–96 h. The percentage of mortality was compared with that of control (**Table 3**).

Results indicated that only 11.7–23.3% mortality in brine shrimps larvae was observed after treatment with degraded toxin products. However, mortality level was 83.0–91.7% when larvae were incubated with untreated AFB1 (100 $\mu g\ L^{-1}$) and AFB2 (50 $\mu g\ L^{-1}$), under same conditions. Percentage of mortality was increased with increase in incubation period.

DISCUSSION

Various food and feed additives like phenolic compounds and plant extracts can be used to reduce toxic effects of mycotoxins (Nahm, 1995; Dvorska et al., 2007). According to the literature, essential oils and extracts of various spices and herbs like cinnamon, peppermint, basil, lemongrass may be

TABLE 3 | Percent mortality of brine shrimps (*Artemia salina*) larvae at 26°C after treatment with toxin (AFB1 and AFB2) detoxified with *T. ammi* seeds extract at various incubation periods.

Treatments	Incubation period (h)	No. of living shrimps	No. of dead shrimps	% Mortality
CONTROL				
Sea water + shrimps	24	40	0	0
	48	40	0	0
	72	40	0	0
	96	39	1	2.5
Methanol + shrimps	24	38	1	2.5
	48	38	2	5
	72	37	3	7.5
	96	36	3	7.5
Untreated toxins + shrimps	24	7	33	83.0
	48	5	35	86.7
	72	4	36	89.2
	96	3	37	91.7
TREATMENT				
Treated toxin with *T. ammi* seeds extract + shrimps	24	35	5	11.7
	48	34	6	15.0
	72	32	8	20.0
	96	31	9	23.3

Sea water was taken as a control. Other Controls consist of Methanol and untreated toxin AFB1 (100 ng/ml) and AFB2 (50 ng/ml) dried and redissolved in sea water.
Values are mean of three replicates.
Data were analyzed by analysis of Variance (ANOVA).

recommended as a plant based safe food additive in protecting the food from deteriorating fungi as well as from aflatoxin contamination (Montes-Belmont and Carvajall, 1998; Burt, 2004; Yang et al., 2007). Similarly, there are several mycotoxins binding commercial products. Some of them are developed and approved in North America and Western Europe, such as Mycofix® and Mycosorb®. These binders are the combination of various things including herbal and yeast cell wall component extracts (Marroquin-Cardona et al., 2009). They have been used worldwide to neutralize or detoxify the mycotoxins in poultry, pig, ruminant feed as well as fish and shrimp diets. The aforementioned products are being extensively used in Pakistan.

In this present study, both *in vitro* and *in vivo* assays were performed with aqueous extracts of *T. ammi* leaves and seeds to check their aflatoxin B1 and B2 detoxification potential under optimized conditions of temperature, pH and incubation period. The results of *in vitro* assays showed that the percentage of detoxification by plants extracts increased with increase in temperature to 60°C but this detoxification could be due to synergistic action of heat and moisture (Basappa and Shantha, 1996; Rustom, 1997). Similarly, Hajare et al. (2005) worked on aflatoxin inactivation by using Ajwain seeds extract under optimized conditions. According to his findings, highest inactivation was observed at 60°C but further studies were conducted on 45°C to reduce the effect of heat and moisture on toxin inactivation.

The pH of reaction mixture plays an important role in the process of detoxification by using plant extracts. The maximum detoxification was observed at pH 10 followed by pH 8. The percentage of detoxification decreased as the pH changed to neutral or acidic range. Subsequently, Mendez-Albores et al. (2004) also found that aflatoxin florescence, attributed to the coumarin moiety, diminish or even disappear in alkaline treatment. In addition, the similar results were in accordance with the findings of Kannan and Velazhahan (2014) who explored the potential of *Barleria lupulina* leaf extract on detoxification of aflatoxins. Results of the present study showed that *in vivo* decontamination of maize samples followed a similar trend as that was recorded in *in vitro* studies. These studies were carried out under conditions optimized in previous *in vitro* assays i.e., pH 8, temperature 30°C and incubation time of 72 h.

Furthermore, *T. ammi* plant extracts showed varied degree of reduction in aflatoxin B1 and B2 detoxification upon boiling. Similar findings were recorded in a study conducted by Velazhahan et al. (2010). The reason behind is that the activity of certain plant phytochemicals like phenolics and alkaloids highly reduces upon boiling as described by Momoh et al. (2012), which is might be responsible for the alteration and breakage of the molecular structure of phytochemicals.

After detoxification, structural changes in aflatoxin molecule have been observed in several studies conducted with micro-organisms, physical and chemical agents, ultraviolet (UV) rays, Gamma rays and plant products (Alberts et al., 2006; Albores et al., 2008; Guan et al., 2010; Velazhahan et al., 2010; Wang et al., 2011; Farzaneh et al., 2012; Inoue et al., 2013; Luo et al., 2013; Samuel et al., 2014; Vijayanandraj et al., 2014). Aflatoxins have been widely researched for their toxicity by various scientists (Guengerich, 2001, 2008; Hussein and Brasel, 2001). Their toxicity data showed that aflatoxins have cyclopentene ring and furan moiety in their chemical structure. In AFB1 the presence of double bond in the terminal furan ring is key factor for its toxic and carcinogenic activities (Wang et al., 2011). In contrast, aflatoxin B2 which have a saturated furan ring is hundreds times less carcinogenic (Dvorackova, 1990). The degraded products of AFB2 may be active but were less potent than that of the parent compound. Thus, removing the double bond of terminal furan ring is a major aim of detoxification. In this present study, among six degraded products of AFB1 acquired after *T. ammi* seeds extract treatment, 50% products (with m/z 303, 341, 351) showed removal of double bond in the terminal furan ring while in products with m/z 369 and 321 both lactone group modification and double bond removal in the terminal furan ring occurred. Therefore, toxicity of most of the degraded products compared with that of aflatoxin was reduced to a much lower level.

Biological toxicity of degraded toxin products were tested using brine shrimps (*Artemia salina*) bioassay. There are several studies on the effects of aflatoxin on the brine shrimp (*Artemia salina*) eggs and larvae (Harwing and Scott, 1971; Schmidt, 1989; Logrieco et al., 1996; Durakovic et al., 2002; Moretti et al., 2007). According to previous findings (Brown, 1969; Hartl and Humpf, 2000; Favilla et al., 2006) the *Artemia salina* larvae appears to be as susceptible as biological indicator of toxicity of some mycotoxins in foods and feeds. The results of present investigation showed significant reduction in larval mortality after incubation with treated toxins as compared to untreated toxins. Similar findings were also observed by Samuel et al. (2014), who worked on detoxification of aflatoxin B1 by

Pseudomonas putida. He compared the toxicity of treated and untreated AFB1 toward HeLa cells and concluded that degraded products are nontoxic (D1) or much less toxic (D2 and D3) than AFB1 to the cells at the tested concentrations.

From the findings of present investigation, *T. ammi* seeds extracts can be used for development of biologically safe herbal additives to food and feed products processed for human consumption to avoid the toxic effects of aflatoxins. Based on previous literature, use of ajwain in food showed no safety issues (Gemeda et al., 2014). Direct spray of aqueous plant extract is convenient for the farmers because these can be easily prepared and its application does not require any technical knowledge. However, there may be some limitations regarding formulations and shelf life of extract and research in this direction is needed.

AUTHOR CONTRIBUTIONS

WI: PhD student who performed all the experimental work. TA: PhD supervisor, who guided and planned this project. MI: Provided expertise and equipment for mass spectroscopy. AG: Helped in data analyses. MA: provided lab facilities for high performance liquid chromatography.

FUNDING

This project did not received any funding from any agency, but was run on indigenous resources of Fungal biotechnology lab, Institute of agricultural sciences, University of the Punjab, Pakistan.

ACKNOWLEDGMENTS

Authors are extremely thankful to Dr. MI (National Institute of Biotechnology and Genetic Engineering), Dr. AG (University of Engineering and Technology) and Dr. MA (University of Veterinary and Animal Sciences) for the provision of specialized apparatus and technical guidance for successful accomplishment of this study.

REFERENCES

Ahmed, A. M. H., El-Sanhoury, M. H. S., and Mohamed, M. E. (2014). "*Mentha piperita* extract as natural growth promoter and its antibacterial activity for broiler performance," in *Proceedings of 7th International Poultry Conference*, November 2014 (Ain Sukhna), 238–254.

Alberts, J. F., Engelbrecht, Y., Steyn, P. S., Holzapfel, W. H., and Zyl, W. H. (2006). Biological degradation of aflatoxin B1 by *Rhodococcus erythropolis* cultures. *Int. J. Food Microbiol.* 109, 121–126. doi: 10.1016/j.ijfoodmicro.2006.01.019

Albores, M. A., Vazquez, N. I., Ruvalcaba, M. R., and Martinez, E. M. (2008). Mass spectrometery/mass spectrometry study on the degradation of B-Aflatoxins in maize with aqueous citric acid. *Am. J. Agric. Biol. Sci.* 3, 482–489. doi: 10.3844/ajabssp.2008.482.489

Al-Rahmah, N., Mostafa, A., and Abdel-Megeed, A. (2011). Antifungal and antiaflatoxigenic activities of some plant extracts. *Afr. J. Microbiol. Res.* 5, 1342–1348. doi: 10.5897/AJMR11.219

Aly, S. E. (2002). Distribution of aflatoxins in product and by-products during glucose production from contaminated corn. *Nahrung* 46, 341–344. doi: 10.1002/1521-3803(20020901)46:5<341::AID-FOOD341>3.0.CO;2-N

Asifa, H. M., Sultanab, S., and Akhtarb, N. (2014). A panoramic view on phytochemical, nutritional, ethanobotanical uses and pharmacological values of *Trachyspermum ammi* Linn. *Asian Pac. J. Trop. Biomed.* 4, 545–553. doi: 10.12980/APJTB.4.2014APJTB-2014-0242

Bairwa, R., Sodha, R. S., and Rajawat, B. S. (2012). *Trachyspermum ammi*. *Pharmacogn Rev.* 6, 56–60. doi: 10.4103/0973-7847.95871

Basappa, S. C., and Shantha, T. (1996). Methods for detoxification of aflatoxin in foods and feeds - a critical appraisal. *J. Food Sci. Technol.* 33, 95–107.

Bhat, R., Rai, R. V., and Karim, A. A. (2010). Mycotoxins in food and feed: present status and future concerns. *Compr. Rev. Food Sci.* 9, 57–81. doi: 10.1111/j.1541-4337.2009.00094.x

Bhatnagar, D., Ehrlich, K. C., and Cleveland, T. E. (1992). "Oxidation-reduction reactions in biosynthesis of secondary metabolites," in *Handbook of Applied Mycology: Mycotoxins in Ecological Systems*, eds D. Bhatnagar, E. B. Lillehoj, and D. K. Arrora (New York, NY: Marcel Dekker), 255–286.

Brown, R. F. (1969). The effect of some mycotoxins on the Brine Shrimp *Artemia salina*. *J. Am. Oil Chem. Soc.* 46, 119–119. doi: 10.1007/BF02541223

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

Das, C., and Mishra, H. N. (2000). *In vitro* degradation of aflatoxin B1 in groundnut (*Arachis hypogea*) meal by horse radish peroxidase. *Lebensm Wiss Technol.* 33, 308–312. doi: 10.1006/fstl.2000.0655

Durakovic, S., Delas, F., and Durakovic, L. (2002). "Microbial indicators of safety and quality of foods," in *Modern Microbiology of Food*, ed S. Durakovic (London: Kugler), 249–282.

Dvorackova, I. (1990). *Aflatoxins and Human Health*. Boca Raton, FL: CRC Press.

Dvorska, J. E., Pappas, A. C., Karadas, F., Speake, B. K., and Surai, P. F. (2007). Protective effect of modified glucomannans and organic selenium against antioxidant depletion in the chicken liver due to T-2 toxin-contaminated feed consumption. *Comp. Biochem. Physiol.* 45, 582–587. doi: 10.1016/j.cbpc.2007.02.005

Ellis, W. O., Smith, J. P., Simpson, B. K., and Oldham, J. H. (1991). Aflatoxins in foods: occurrence, biosynthesis, effects on organisms and methods of detection and control. *Crit. Rev. Food Sci. Nutr.* 30, 404–439. doi: 10.1080/10408399109527551

El-Nagerabi, S. A. F., Al-Bahry, S. N., Elshafie, A. E., and Al-Hilali, S. (2012). Effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil on the growth and aflatoxin B1 production of *Aspergillus flavus* and *Aspergillus parasiticus* strains. *Food Control*. 25, 59–63. doi: 10.1016/j.foodcont.2011.09.033

El-Nagerabi, S. A. F., Elshafie, A. E., Alkhanjari, S. S., Al-Bahry, S. N., and Elamin, M. R. (2013). Biological activities of *Boewellia sacra* extract on the growth and aflatoxins secretion of two aflatoxigenic species of *Aspergillus* species. *Food Control*. 34, 763–769. doi: 10.1016/j.foodcont.2013.06.039

Farzaneh, M., Shi, Z., Ghassempour, A., Sedaghat, N., Ahmadzadeh, M., Mirabolfathy, M., et al. (2012). Aflatoxin B1 degradation by Bacillus subtilis UTBSP1 from pistachio nuts of Iran. *Food Control* 23, 100–106. doi: 10.1016/j.foodcont.2011.06.018

Favilla, M., Macchia, L., Gallo, A., and Altomare, C. (2006). Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) intervertebrate bioassays. *Food. Chem. Toxicol.* 44, 1922–1931. doi: 10.1016/j.fct.2006.06.024

Folayan, J. A. (2013). Determinants of post-harvest losses of maize in Akure north local government area of Ondo State, Nigeria. *J. Sust. Soc.* 2, 12–19. doi: 10.11634/216825851302303

Gemeda, N., Woldeamanuel, Y., Asrat, D., and Debella, A. (2014). Effect of essential oils on *Aspergillus* spore germination, growth and mycotoxin production: a potential source of botanical food preservative. *Asian Pac. J. Trop. Biomed.* 4, 373–381. doi: 10.12980/APJTB.4.2014C857

Grossberg, G. T., and Fox, B. (2008). *The Essential Herb-Drug-Vitamin Interaction Guide: The Safe Way to Use Medications and Supplements Together Random House, 1st Edn.* New York, NY: Broadway Books.

Guan, S., Zhao, L., Qiugang, M., Zhou, T., Wang, N., Xinxu, H., et al. (2010). *In vitro* efficacy of *Myxococcus fulvus* ANSM068 to biotransform aflatoxin B1. *Int. J. Mol. Sci.* 11, 4063–4079. doi: 10.3390/ijms11104063

Guengerich, F. P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83. doi: 10.1021/tx700079z

Guengerich, F. P. (2008). Cytochrome P450 reactions and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83. doi: 10.1021/tx700079z

Hajare, S. S., Hajare, S. N., and Sharma, A. (2005). Aflatoxin inactivation using aqueous extract of ajowan (*Trachyspermum ammi*) seeds. *J. Food Sci.* 70, 29–34. doi: 10.1111/j.1365-2621.2005.tb09016.x

Hartl, M., and Humpf, H. U. (2000). Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. *Food. Chem. Toxicol.* 38, 1097–1102. doi: 10.1016/S0278-6915(00)00112-5

Harwing, J., and Scott, P. M. (1971). Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* 21, 1011–1016.

Hernandez-Hierro, J. M., Garcia-Villanova, R. J., Torrero, P. R., and Fonseca, I. M. T. (2008). Aflatoxins and Ochratoxin A in red paprika for retail sale in Spain: occurrence and evaluation of a simultaneous analytical method. *J. Agric. Food Chem.* 56, 751–756. doi: 10.1021/jf073002c

Hussein, H., and Brasel, J. (2001). Toxicity, metabolism, and impact on humans and animals. *Toxicology* 167, 101–134. doi: 10.1016/S0300-483X(01)00471-1

Inoue, T., Nagatomi, Y., Uyama, A., and Mochizuki, N. (2013). Degradation of aflatoxin B1 during fermentation of alcoholic beverages. *Toxins* 5, 1219–1229. doi: 10.3390/toxins5071219

IARC (2002). Traditional herbal medicine, some mycotoxins, naphthalene and styrene. *IARC Monogr. Eval. Carcinog. Risks Hum.* 82, 171–300.

Jaimez, J., Fente, C. A., Vazquez, B. I., Franco, C. M., Cepeda, A., Mahuzier, G., et al. (2000). Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chrom. A* 882, 1–10. doi: 10.1016/S0021-9673(00)00212-0

Joseph, G. S., Jayaprakasha, G. K., Selvi, A. T., Sena, B. S., and Sakariah, K. K. (2005). Antiaflatoxigenic and antioxidant activities of *Garcinia* extract. *Int. J. Food Microbiol.* 101, 153–160. doi: 10.1016/j.ijfoodmicro.2004.11.001

Kannan, K., and Velazhahan, R. (2014). The potential of leaf extract of *Barleria lupulina* for detoxification of aflatoxins. *Ind. Phytopath.* 67, 298–302.

Krishnamurthy, Y. L., and Shashikala, J. (2006). Inhibition of aflatoxin B1 production of *Aspergillus flavus*, isolated from soybean seeds by certain natural plant products. *Appl. Microbiol.* 43, 469–474. doi: 10.1111/j.1472-765X.2006.02011.x

Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A., Caraffa, M. F., et al. (1996). Fusaproliferin production by *Fusarium subglutinans* and its toxicity to *Artemia salina* SF-9 insect cells, and IARC/LCL 171 human B limphocytes. *Appl. Environ. Microbiol.* 62, 3378–3384.

Luo, X., Wang, R., Wang, L., Wang, Y., and Chen, Z. (2013). Structural elucidation and toxicity analyses of the degradation products of aflatoxin B1 by aqueous ozone. *Food Control.* 31, 331–336. doi: 10.1016/j.foodcont.2012.10.030

Marroquin-Cardona, A., Deng, Y., Taylor, J. F., Hallmark, C. T., Johnson, N. M., and Phillips, T. D. (2009). *In vitro* and *in vivo* characterization of mycotoxin-binding additives used for animal feeds in Mexico. *Food Addit. Contam. A* 26, 733–743. doi: 10.1080/02652030802641872

Mendez-Albores, J. A., Villa, G. A., Rio-Garcia, D. J. C., and Martinez, E. M. (2004). Aflatoxin-detoxification achieved with Mexican traditional nixtamalization process (MTNP) is reversible. *J. Sci. Food. Agric.* 84, 1611–1614. doi: 10.1002/jsfa.1853

Mirzaei-Aghsaghali, A. (2012). Importance of medical herbs in animal feeding: a review. *Ann. Biol Res.* 3, 918–923.

Momoh, A. O., Adebolu, T. T., and Ogundare, A. O. (2012). The effects of different treatments on the phytochemicals, proximate, and mineral contents of beni seeds (*sesamum indicum* linn). *Global Adv. Res J. Biotech.* 1, 8–11.

Montes-Belmont, R., and Carvajall, M. (1998). Control of *Aspergillus flavus* in maize with plant essential oils and their components. *J. Food Protect.* 61, 616–619.

Moretti, A., Mule, G., Ritieni, A., and Logrieco, A. (2007). Further data on the production of beauvericin, enniatins and fusaproliferin and toxicity to *Artemia salina* by *Fusarium* species *Gibberella fujikuroi* species complex. *Inter. J. Food Microbiol.* 118, 158–163. doi: 10.1016/j.ijfoodmicro.2007.07.004

Nahm, K. H. (1995). Possibilities for preventing mycotoxicosis in domestic fowl. *World's Poult. Sci.* 51, 177–185. doi: 10.1079/WPS19950012

Pathak, A. K., Nainwal, N., Goyal, B. M., Singh, R., Mishra, V., and Nayak, S. (2010). Pharmacological activity of *Trachyspermum ammi*: a review. *J. Pharm. Res.* 3, 895–899.

Ramesh, J., Sarathchandra, G., and Sureshkumar, V. (2013). Analysis of feed samples for aflatoxin B1 contamination by HPTLC - a validated method. *Int. J. Curr. Microbiol. Appl. Sci.* 2, 373–377.

Reddy, C. S., Reddy, K. R. N., Prameela, M., Mangala, U. N., and Muralidharan, K. (2007). Identification of antifungal component in clove that inhibits *Aspergillus* spp. colonizing rice grains. *J. Mycol. Plant Pathol.* 37, 87–94.

Reddy, K. R. N., Reddy, C. S., and Muralidharan, K. (2009). Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. *Food Control* 20, 173–178. doi: 10.1016/j.foodcont.2008.03.009

Rustom, I. Y. S. (1997). Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chem.* 59, 57–67. doi: 10.1016/S0308-8146(96)00096-9

Samarajeewa, U., Sen, A. C., Cohen, M. D., and Wei, C. I. (1990). Detoxification of aflatoxins in foods and feeds by physical and chemical methods. *J. Food Prot.* 53, 489–501.

Samuel, M. S., Sivaramakrishna, A., and Mehta, A. (2014). Degradation and detoxification of aflatoxin B1 by *Pseudomonas putida*. *Int. Biodeter. Biodegr.* 86, 202–209. doi: 10.1016/j.ibiod.2013.08.026

Schmidt, R. (1989). "The application of *Artemia salina* L. bioassay for screening of aflatoxins," in *Aspergillus: Mycotoxins Taxonomy and Pathogenicity*, ed J. Chelkowski (Amsterdam: Elsevier), 121–130.

Shukla, R., Singh, P., Prakash, B., and Anuradha, D. N. K. (2012). Antifungal, aflatoxin inhibitory and free radical scavenging activities of some medicinal plants extracts. *J. Food Qual.* 35, 182–189. doi: 10.1111/j.1745-4557.2012.00441.x

Sinha, K. K., and Sinha, A. K. (1991). Monitoring and identification of aflatoxins in wheat, grain and maize flours in Bihar state (India). *Food Addit. Contam.* 8, 453–457. doi: 10.1080/02652039109373995

Solis, P. N., Wright, C. W., Anderson, M. M., Gupta, M. P., and Phillipson, J. D. (1993). A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). *Planta Med.* 59, 250–252. doi: 10.1055/s-2006-959661

Stroka, J., Otterdijk, R., and Anklam, E. (2000). Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices. *J. Chroma A* 904, 251–256. doi: 10.1016/S0021-9673(00)00930-4

Velazhahan, R., Vijayanandraj, S., Vijayasamundeeswari, A., Paranidharan, V., Samiyappan, R., Iwamoto, T., et al. (2010). Detoxification of aflatoxins by seed extracts of the medicinal plant, *Trachyspermum ammi* (L.) Sprague ex Turrill – structural analysis and biological toxicity of degradation product of aflatoxin G1. *Food Control* 21, 719–725. doi: 10.1016/j.foodcont.2009.10.014

Vijayanandraj, S., Brinda, R., Kannan, K., Adhithya, R., Vinothini, S., Senthil, K., et al. (2014). Detoxification of aflatoxin B1 by an aqueous extract from leaves of *Adhatoda vasica* Nees. *Microbiol. Res.* 169, 294–300. doi: 10.1016/j.micres.2013.07.008

Wallnofer, P. R., Preiss, U., Ziegler, W., and Engelhardt, G. (1996). Conjugate organic pollutants in Pfl ora. *Umweltchem. Őkotox.* 8, 43-46.

Wang, F., Xie, F., Xue, X., Wang, Z., Fan, B., and Yiming, H. A. (2011). Structure elucidation and toxicity analysis of the radiolytic produts of aflatoxin B1 in methanol-water solution. *J. Hazard Mater.* 192, 1192–1202. doi: 10.1016/j.jhazmat.2011.06.027

Wilson, D. M., and Payne, G. A. (1994). "Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of the crops," in *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance*, eds D. L. Eaton and J. D. Groopman (San Diego, CA: Academic Press, Inc.), 309–325.

Yang, W. Z., Benchaar, C., Ametaj, B. N., Chaves, A. V., He, M. L., and McAllister, T. A. (2007). Effects of garlic and juniper berry essential oils on ruminal fermentation, site and extent of digestion in lactating cows. *J. Dairy Sci.* 90, 5671–5681. doi: 10.3168/jds.2007-0369

Yazdani, D., Zainal-Abidin, M. A., Tan, Y. H., and Kamaruzaman, S. (2010). Evaluation of the detection techniques of toxigenic Aspergillus isolates. *Afr. J. Biotechnol.* 9, 7654–7659. doi: 10.5897/AJB10.1128

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 February 2016
doi: 10.3389/fmicb.2016.00168

Analysis of Multilocus Sequence Typing and Virulence Characterization of *Listeria monocytogenes* Isolates from Chinese Retail Ready-to-Eat Food

Shi Wu[1,2], *Qingping Wu*[1]*, *Jumei Zhang*[1], *Moutong Chen*[1] *and Weipeng Guo*[1]

[1] *Guangdong Institute of Microbiology, State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangzhou, China,* [2] *School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China*

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Luca Settanni,
Università degli Studi di Palermo, Italy
Fatih Ozogul,
Çukurova Üniversity, Turkey
Rosanna Tofalo,
Università degli Studi di Teramo, 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: *20 October 2015*
Accepted: *01 February 2016*
Published: *16 February 2016*

Citation:
Wu S, Wu Q, Zhang J, Chen M and Guo W (2016) Analysis of Multilocus Sequence Typing and Virulence Characterization of Listeria monocytogenes Isolates from Chinese Retail Ready-to-Eat Food. Front. Microbiol. 7:168. doi: 10.3389/fmicb.2016.00168

Eighty *Listeria monocytogenes* isolates were obtained from Chinese retail ready-to-eat (RTE) food and were previously characterized with serotyping and antibiotic susceptibility tests. The aim of this study was to characterize the subtype and virulence potential of these *L. monocytogenes* isolates by multilocus sequence typing (MLST), virulence-associate genes, epidemic clones (ECs), and sequence analysis of the important virulence factor: internalin A (inlA). The result of MLST revealed that these *L. monocytogenes* isolates belonged to 14 different sequence types (STs). With the exception of four new STs (ST804, ST805, ST806, and ST807), all other STs observed in this study have been associated with human listeriosis and outbreaks to varying extents. Six virulence-associate genes (*inlA*, *inlB*, *inlC*, *inlJ*, *hly,* and *llsX*) were selected and their presence was investigated using PCR. All strains carried *inlA*, *inlB*, *inlC*, *inlJ*, and *hly*, whereas 38.8% (31/80) of strains harbored the listeriolysin S genes (*llsX*). A multiplex PCR assay was used to evaluate the presence of markers specific to epidemic clones of *L. monocytogenes* and identified 26.3% (21/80) of ECI in the 4b-4d-4e strains. Further study of *inlA* sequencing revealed that most strains contained the full-length InlA required for host cell invasion, whereas three mutations lead to premature stop codons (PMSC) within a novel PMSCs at position 326 (GAA→TAA). MLST and *inlA* sequence analysis results were concordant, and different virulence potentials within isolates were observed. These findings suggest that *L. monocytogenes* isolates from RTE food in China could be virulent and be capable of causing human illness. Furthermore, the STs and virulence profiles of *L. monocytogenes* isolates have significant implications for epidemiological and public health studies of this pathogen.

Keywords: *Listeria monocytogenes*, MLST, *inlA*, PMSC, virulence genes, epidemic clones

INTRODUCTION

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium that is responsible for listeriosis. Listeriosis can cause meningitis, newborn septicemia, encephalomyelitis, or even death in humans, especially in the elderly, pregnant women, or newborn. Every year, ∼1591 cases of listeriosis in humans are report, with a 19% case-fatality rate in the United States (Scallan et al., 2011). In the European Union, a total of 1763 confirmed human cases of listeriosis (notification rate of 0.44 cases per 100,000 population) were reported in 2013 (EFSA, 2015). As an important foodborne pathogen, it is widespread in nature and lives naturally in plants and soil environments. Its ability to survive and grow over a wide range of environmental conditions, including refrigeration temperatures, high salt concentration and low pH, makes it a potential hazard in foods (Ryser and Marth, 2007).

Differences in virulence between *L. monocytogenes* strains may also influence infection and clinical outcome. Some strains are highly pathogenic and sometimes deadly, whereas others are less virulent or even avirulent and produce little harm in the host (Olier et al., 2002). Several methods have been used to differentiate *L. monocytogenes* strains. Based on somatic (O) and flagellar (H) antigens represents a conventional approach to understanding *L. monocytogenes* isolates ecological distribution and epidemiology. However, there are 13 serotypes and only four serotype (1/2a, 1/2b, 1/2c, and 4b) cause almost all cases of listeriosis in human. To further discriminate these strains, numerous molecular methods have been developed for epidemiological investigations and of help for the surveillance and control of listeriosis. Multilocus sequence typing (MLST) method is one of the most robust tools for investigating the global epidemiology of microbial populations (Sullivan et al., 2005). Based on the sequencing of seven housekeeping genes, it is highly discriminatory and provides unambiguous results that can be comparable directly among laboratories via the internet. In recent years, MLST has evolved to the reference method for global epidemiology and population biology (Maiden, 2006; Ragon et al., 2008).

Certain virulence and virulence-associated genes also play very important roles in intracellular survival, cell-to-cell spread, and virulence of *L. monocytogenes*. The presence of a number of virulence factors such as surface-associated internalins, listeriolysin O, and listeriolysin S (LLS) in *L. monocytogenes* significantly regulates its pathogenicity (Cotter et al., 2008; Shen et al., 2013). Of which, internalin A (InlA), encode by *inlA*, is responsible for facilitating the entry of *L. monocytogenes* into nonprofessional phagocytic cells expressing the human isoform of E-cadherin (Lecuit et al., 2001). It has been shown that mutations in *inlA* leading to a premature stop codon (PMSC) significantly reduce the invasion of the strain to human epithelial cells (Nightingale et al., 2005a). In addition, a small number of closely related *L. monocytogenes* clones have caused multiple outbreaks worldwide. These epidemic clones (EC) are divided into seven groups: ECI, ECII and ECIV within serotype 4b, ECIII, ECV and ECVII in serotype 1/2a, and ECVI within serotype 1/2b (Kathariou, 2003; Knabel et al., 2012; Lomonaco et al., 2013).

Since the main food linked to listeriosis outbreaks was ready-to-eat (RTE) food, the consumer had limit opportunities to destroy the pathogen before the food consumed. Therefore, the risk associated with strains isolated from RTE food may be more severe. In our previous study, 80 RTE *L. monocytogenes* isolates were examined (Wu et al., 2015a). In order to determine the potential pathogenic profile and relative risk of these RTE *L. monocytogenes* isolates, this study provide a phylogenetic framework based on MLST analysis of *L. monocytogenes* isolates and evaluate their virulence-genes and EC-specific markers. In addition, we analyzed the full-length sequences of important virulence factor (inlA) to investigate correlations among the MLST types, amino acid sequence of inlA, and virulence potential.

MATERIALS AND METHODS

Bacterial Isolates

A total of 80 *L. monocytogenes* isolates were collected from retail ready-to-eat food samples in 24 Chinese cities were analyzed, comprising 27 isolates from cold vegetable dishes in sauce, 7 isolates from cold noodles dishes in sauce, 17 isolates from roast chicken, 12 isolates from roast duck, 11 isolates from roast pork, and 5 isolates from pasteurized milk (**Table 1**). The isolates were obtained between September 2012 and January 2014 according to the GB 4789.30-2010 of food microbiological examination of *L. monocytogenes* (National Food Safety Standards of China) with slight modifications and the most probable number (MPN) method (Gombas et al., 2003). They were identified by Gram stain, catalase, and oxidase tests and Micro ID *Listeria* identification system (Microgen, Camberley, UK). Serovars and antibiotic susceptibility were confirmed in previous study (Wu et al., 2015a). Additionally, 35 isolates were selected for the sequencing analysis of *inlA*, based on MLST profile, molecular serogroup, food origin, and antimicrobial resistance. Each isolate was incubated at 37°C overnight on TSB-YE (Tryptic soy agar with yeast extract). Genomic DNA was extracted using a Genomic DNA Extraction kit (Dongsheng Biotech, Guangzhou, China) according to the manufacturer's instructions. The concentration of genomic DNA was determined at 260 nm using a NanoDrop-ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA).

Multilocus Sequence Typing

The MLST scheme used to characterize *L. monocytogenes* isolates is based on the sequence analysis of the following seven housekeeping genes: *abcZ* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhkA* (histidine kinase) (Salcedo et al., 2003). The PCR amplification conditions were as follow: an initial cycle of 94°C for 4 min; 35 cycles of 94°C for 30 s, 52°C for 30 s (45°C for *bglA*), 72°C for 2 min, and a final extension at 72°C for 10 min. The DNA fragments were purified using a PCR purification kit (Qiagen, Genman) and were sequenced in each direction with Big Dye fluorescent terminators on an ABI3730XL sequencer (Applied BioSystems).

TABLE 1 | Characteristics of 80 *L. monocytogenes* strains isolated from retail ready-to-eat food in this study.

Strain/isolate	Strain orgin	Sample city	Year	Serovar[a]	Antibiotic susceptibility[a]	ST	*hly*	*inlB*	*inlA*	*inlC*	*inlJ*	*llsX*	EC
859-1LM	Cold vegetable dish in sauce	Hefei	2012	1/2a-3a	AMP-CIP-DA	ST-8	+	+	+	+	+	−	−
859-2LM	Cold vegetable dish in sauce	Hefei	2012	1/2b-3b-7	CIP-DA-E-K-RD-S-TE	ST-87	+	+	+	+	+	−	−
859-3LM	Cold vegetable dish in sauce	Hefei	2012	1/2a-3a	DA-RD	ST-8	+	+	+	+	+	−	−
859-4LM	Cold vegetable dish in sauce	Hefei	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
860-1LM	Cold noodles dishes in sauce	Hefei	2012	1/2b-3b-7	AMP-CIP-DA	ST-87	+	+	+	+	+	−	−
860-2LM	Cold noodles dishes in sauce	Hefei	2012	1/2a-3a	AMP-CIP-DA	ST-8	+	+	+	+	+	−	−
860-3LM	Cold noodles dishes in sauce	Hefei	2012	1/2b-3b-7	CIP-DA	ST-5	+	+	+	+	+	−	−
860-4LM	Cold noodles dishes in sauce	Hefei	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
959-1LM	Cold vegetable dish in sauce	Wuhan	2012	1/2b-3b-7	DA	ST-87	+	+	+	+	+	−	−
959-2LM	Cold vegetable dish in sauce	Wuhan	2012	1/2a-3a	AMP-KF-C-DA-E-CN-K-RD-S-TE-VA	ST-8	+	+	+	+	+	−	−
959-3LM	Cold vegetable dish in sauce	Wuhan	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
959-4LM	Cold vegetable dish in sauce	Wuhan	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
1009-1LM	Cold vegetable dish in sauce	Chengdu	2012	1/2b-3b-7	DA	ST-224	+	+	+	+	+	+	−
1009-2LM	Cold vegetable dish in sauce	Chengdu	2012	1/2b-3b-7	DA	ST-224	+	+	+	+	+	+	−
1009-3LM	Cold vegetable dish in sauce	Chengdu	2012	1/2b-3b-7	E	ST-224	+	+	+	+	+	+	−
1009-4LM	Cold vegetable dish in sauce	Chengdu	2012	1/2b-3b-7	DA	ST-224	+	+	+	+	+	+	−
1059-1LM	Cold vegetable dish in sauce	Kunming	2012	1/2b-3b-7	DA	ST-87	+	+	+	+	+	−	−
1059-2LM	Cold vegetable dish in sauce	Kunming	2012	1/2b-3b-7	SUS	ST-87	+	+	+	+	+	−	−
1059-3LM	Cold vegetable dish in sauce	Kunming	2012	1/2c-3c	SUS	ST-87	+	+	+	+	+	−	−
1059-4LM	Cold vegetable dish in sauce	Kunming	2012	1/2c-3c	SUS	ST-87	+	+	+	+	+	−	−
1111-1LM	Roast chicken	Lanzhou	2012	1/2a-3a	DA	ST-7	+	+	+	+	+	−	−
1111-2LM	Roast chicken	Lanzhou	2012	1/2a-3a	SUS	ST-7	+	+	+	+	+	−	−
1111-3LM	Roast chicken	Lanzhou	2012	1/2a-3a	SUS	ST-7	+	+	+	+	+	−	−
1111-4LM	Roast chicken	Lanzhou	2012	1/2a-3a	SUS	ST-7	+	+	+	+	+	−	−
1159-1LM	Cold vegetable dish in sauce	Haerbin	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
1159-2LM	Cold vegetable dish in sauce	Haerbin	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
1159-3LM	Cold vegetable dish in sauce	Haerbin	2012	1/2c-3c	DA	ST-9	+	+	+	+	+	−	−
1159-4LM	Cold vegetable dish in sauce	Haerbin	2012	1/2c-3c	SUS	ST-9	+	+	+	+	+	−	−
1194-1LM	Roast chicken	Haerbin	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
1194-2LM	Roast chicken	Haerbin	2012	1/2a-3a	DA	ST-8	+	+	+	+	+	−	−
1194-3LM	Roast chicken	Haerbin	2012	1/2b-3b-7	DA-TE	ST-804	+	+	+	+	+	+	−
1194-4LM	Roast chicken	Haerbin	2012	1/2b-3b-7	AMP-KF-C-DA-E-K-RD-S-TE-VA	ST-805	+	+	+	+	+	+	−
1244-1LM	Cold vegetable dish in sauce	Xi'an	2012	1/2b-3b-7	SUS	ST-3	+	+	+	+	+	+	−
1244-2LM	Cold vegetable dish in sauce	Xi'an	2012	1/2b-3b-7	SUS	ST-3	+	+	+	+	+	+	−
1244-3LM	Cold vegetable dish in sauce	Xi'an	2012	1/2b-3b-7	SUS	ST-804	+	+	+	+	+	+	−
1244-4LM	Cold vegetable dish in sauce	Xi'an	2012	1/2b-3b-7	DA	ST-3	+	+	+	+	+	+	−
1309-1LM	Cold vegetable dish in sauce	Beijing	2013	1/2a-3a	SUS	ST-121	+	+	+	+	+	−	−
1309-2LM	Cold vegetable dish in sauce	Beijing	2013	1/2a-3a	SUS	ST-121	+	+	+	+	+	−	−
1329-1LM	Roast pork	Beijing	2013	1/2a-3a	DA-TE	ST-155	+	+	+	+	+	−	−
1329-2LM	Roast pork	Beijing	2013	1/2a-3a	AMP-KF-C-DA-E-CN-K-RD-S-TE-VA	ST-155	+	+	+	+	+	−	−
1330-1LM	Cold vegetable dish in sauce	Beijing	2013	1/2a-3a	DA-TE	ST-155	+	+	+	+	+	−	−
1330-2LM	Cold vegetable dish in sauce	Beijing	2013	1/2a-3a	AMP-KF-C-DA-E-CN-K-RD-S-TE-VA	ST-806	+	+	+	+	+	−	−
1342-1LM	Pasteurized milk	Beijing	2013	1/2a-3a	AMP-KF-C-DA-E-CN-K-RD-S-TE-VA	ST-806	+	+	+	+	+	−	−
1342-2LM	Pasteurized milk	Beijing	2013	1/2a-3a	AMP-KF-C-DA-E-CN-K-RD-S-TE-VA	ST-155	+	+	+	+	+	−	−
1584-1LM	Roast duck	Guangzhou	2013	4b-4d-4e	DA	ST-807	+	+	+	+	+	+	ECI

(Continued)

TABLE 1 | Continued

Strain/isolate	Strain orgin	Sample city	Year	Serovar[a]	Antibiotic susceptibility[a]	ST	*hly*	*inlB*	*inlA*	*inlC*	*inlJ*	*llsX*	EC
1584-2LM	Roast duck	Guangzhou	2013	4b-4d-4e	CIP	ST-807	+	+	+	+	+	+	ECI
1584-3LM	Roast duck	Guangzhou	2013	4b-4d-4e	C-CIP	ST-807	+	+	+	+	+	+	ECI
1586-1LM	Roast pork	Guangzhou	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1586-2LM	Roast pork	Guangzhou	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1586-3LM	Roast pork	Guangzhou	2013	4b-4d-4e	DA	ST-1	+	+	+	+	+	+	ECI
1588-1LM	Roast chicken	Guangzhou	2013	4b-4d-4e	DA	ST-1	+	+	+	+	+	+	ECI
1588-2LM	Roast chicken	Guangzhou	2013	4b-4d-4e	CIP	ST-807	+	+	+	+	+	+	ECI
1588-3LM	Roast chicken	Guangzhou	2013	4b-4d-4e	CIP	ST-807	+	+	+	+	+	+	ECI
1634-1LM	Roast duck	Guangzhou	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1634-2LM	Roast duck	Guangzhou	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1634-3LM	Roast duck	Guangzhou	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1760-1LM	Cold noodles dishes in sauce	Zhanjiang	2013	1/2a-3a	CIP	ST-8	+	+	+	+	+	–	–
1760-2LM	Cold noodles dishes in sauce	Zhanjiang	2013	1/2a-3a	CIP-DA	ST-8	+	+	+	+	+	–	–
1760-3LM	Cold noodles dishes in sauce	Zhanjiang	2013	1/2a-3a	SUS	ST-8	+	+	+	+	+	–	–
1834-1LM	Roast duck	Shaoguan	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1834-2LM	Roast duck	Shaoguan	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1834-3LM	Roast duck	Shaoguan	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1836-1LM	Roast chicken	Shaoguan	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1836-2LM	Roast chicken	Shaoguan	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1836-3LM	Roast chicken	Shaoguan	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1838-1LM	Roast pork	Shaoguan	2013	4b-4d-4e	SUS	ST-1	+	+	+	+	+	+	ECI
1838-2LM	Roast pork	Shaoguan	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
1838-3LM	Roast pork	Shaoguan	2013	4b-4d-4e	CIP	ST-1	+	+	+	+	+	+	ECI
2311-1LM	Roast pork	Xiamen	2013	1/2c-3c	SUS	ST-9	+	+	+	+	+	–	–
2311-2LM	Roast pork	Xiamen	2013	1/2c-3c	SUS	ST-9	+	+	+	+	+	–	–
2311-3LM	Roast pork	Xiamen	2013	1/2c-3c	SUS	ST-9	+	+	+	+	+	–	–
2339-1LM	Roast duck	Xiamen	2013	1/2a-3a	DA	ST-8	+	+	+	+	+	–	–
2339-2LM	Roast duck	Xiamen	2013	1/2a-3a	DA	ST-8	+	+	+	+	+	–	–
2339-3LM	Roast duck	Xiamen	2013	1/2a-3a	DA	ST-8	+	+	+	+	+	–	–
2408-1LM	Pasteurized milk	Haikou	2014	1/2a-3a	DA	ST-8	+	+	+	+	+	–	–
2408-2LM	Pasteurized milk	Haikou	2014	1/2a-3a	SUS	ST-8	+	+	+	+	+	–	–
2408-3LM	Pasteurized milk	Haikou	2014	1/2a-3a	C	ST-8	+	+	+	+	+	–	–
2436-1LM	Roast chicken	Haikou	2014	1/2a-3a	DA	ST-8	+	+	+	+	+	–	–
2436-2LM	Roast chicken	Haikou	2014	1/2a-3a	SUS	ST-8	+	+	+	+	+	–	–
2436-3LM	Roast chicken	Haikou	2014	1/2a-3a	SUS	ST-8	+	+	+	+	+	–	–

[a]*SUS, susceptibility; P, penicillin G (5U); AMP, ampicillin (10 μg); KF, Cephalothin (30 μg); C, chloramphenicol (30 μg); CIP, Ciprofloxacin (5 μg); DA, Clindamycin (2 μg); E, Erythromycin (15 μg); CN, gentamicin (10 μg); K, Kanamycin (30 μg); MEZ, Mezlocillin (30 μg); RD, Rifampicin (5 μg); S, Streptomycin (25 μg); TE, tetracycline (30 μg); VA, Vancomycin (30 μg). The serovars and antibiotic susceptibility of L. monocytogenes isolates were detected in previous study by Wu et al. (2015a).*

Determination of Virulence Genes and Epidemic Clone

The isolates were identified using duplex PCR detection containing *hly* (707 bp) and *inlB* (367 bp) genes, which are specific to *L. monocytogenes*, as previously described (Xu et al., 2009). Multiplex PCR (Liu et al., 2007) was used to determine the presence of the virulence genes *inlA*, *inlC,* and *inlJ*. The *llsX* gene was detected by PCR assays to identify the LLS-positive *L. monocytogenes* strains (Clayton et al., 2011). Presumptive major ECs (ECI, ECII, and ECIII) were found in the isolates as described previously (Chen and Knabel, 2007). All primers and PCR conditions are presented in Supplementary Table 1.

inlA Gene Sequencing

The 2400 bp long *inlA* gene was sequenced in 35 isolates representing the clonal diversity of *L. monocytogenes*. External primers were used to amplification covering the whole *inlA* ORF and internal primers for sequencing (Supplementary Table 2). The *inlA* sequences were assembled using Seqman (DNASTAR, Lasergene). Mutation types were determined according to the site of mutation that leads to PMSC in *inlA* (Nightingale et al., 2005a)

and by comparing the obtained *inlA* sequence data to that of the *L. monocytogenes* EGDe reference strain (Glaser et al., 2001).

Data Analysis

For each MLST locus, an allele number was given to each distinct sequence variant, and a distinct sequence type (ST) number was attributed to each distinct combination of alleles among the seven genes. Sequence types (STs) were determined by using the *L. monocytogenes* MLST database (http://bigsdb.web.pasteur.fr/listeria/listeria.html). Sequence Type Analysis and Recombinational Tests software (S.T.A.R.T. ver.2; http://pubmlst.org/software/analysis/start2) was used to analyze the data of MLST.

Nucleotide diversity (π, average pairwise nucleotide difference/site; and k, average pairwise nucleotide difference/sequence), number of polymorphic sites, number of mutations, number of alleles, G+C content, Tajima's D test for neutrality, number of synonymous mutations (*Ks*), number of nonsynonymous mutations (*Ka*), and the *Ks*/*Ka* ratios [the number of nonsynonymous substitutions/nonsynonymous site (*Ks*) to the number of synonymous substitutions/synonymous site (*Ka*)] with a Jukes and Cantor correction were calculated using DnaSP version 5.10.01. Sequence analysis of *inlA* was performed using the ClustalX algorithm (version 1.83) (Thompson et al., 1997), which was followed by phylogenetic analysis using the maximum likelihood algorithm in MEGA 6 (version 6.05) (Tamura et al., 2011).

RESULTS

Multilocus Sequence Typing of *L. monocytogenes* Isolates

MLST detected a total of 14 different sequence types in the 80 isolates, including four new STs (ST804, ST805, ST806, and ST807) (**Table 2**). The most common allelic profile was ST8 (24/80, 30% of isolates) followed by ST1 (16/80, 20% of isolates) independently of the isolates source. With the exception of ST5 and ST805, the remainder of the STs included more than one isolate. Of the STs, ST-87, ST5, ST224, ST804, ST805, and ST3 belonged to serovar II.2 (1/2b-3b-7), ST8, ST7, ST122, ST155, and ST806 belonged to serovar I.1 (1/2a-3a), ST1 and ST807 belonged to serovars II.1 (4b-4d-4e), and ST9 belonged to serovar I.2 (1/2c-3c). Based on each ST matches at least one other ST at $\geq$6 of the 7 loci, three clonal complexes (CCs) were identified, including CC1 (ST1, ST807), CC3 (ST3, ST804), and CC155 (ST155, ST806). A phylogenetic tree based on the seven concatenated MLST sequences (**Figure 1**) shows the relatedness between the RTE strains. STs correlated well with serotype and lineage. Most isolates recovered from same city were clustered into one type. Furthermore, it should be noted that the multidrug resistant strains 1194-4LM, 1330-2LM, and 1342-1LM were belonged to new STs (ST805, ST806) indicating that these isolates were genetically diverse from other isolates.

The evolutionary characteristics of the seven housekeeping genes among the isolates were analyzed using the software dnasp5, which can distinguish between randomly (i.e., neutrally) and non-randomly evolving DNA sequences. Tajima's D test indicated that *abcZ, cat, dapE, dat, ldh,* and *lhkA* evolved randomly, whereas *bglA* evolved non-significantly (0.9151, $P > 0.10$) (**Table 3**). However, seven gene portions harbored a total of 195 polymorphisms (5.93%; range 3.51–12.1% per gene). The average nucleotide diversity π was 2.6%, ranging from 0.9 to 6.2% per gene. The GC% observed in all alleles ranged from 36.4 to 42.9%, which was consistent with the 39% value observed across the entire *L. monocytogenes* EGDs genome (Glaser et al., 2001).

TABLE 2 | Allelic profile (STs) of RTE *L. monocytogenes* isolates for MLST.

STs	Profile							No. of isolates (%)	Serovar
	abcZ	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*		
8	5	6	2	9	5	3	1	24 (30)	1/2a-3a
1	3	1	1	1	3	1	3	16 (20)	4b-4d-4e
87	12	1	4	14	3	39	4	7 (8.75)	1/2b-3b-7
9	6	5	6	4	1	4	1	5 (6.25)	1/2c-3c
807	106	1	1	1	3	1	3	5 (6.25)	4b-4d-4e
7	5	8	5	7	6	2	1	4 (5)	1/2a-3a
155	7	10	16	7	5	2	1	4 (5)	1/2a-3a
224	11	3	12	38	3	94	2	4 (5)	1/2b-3b-7
3	4	4	4	3	2	1	5	3 (3.75)	1/2b-3b-7
804	4	4	4	3	5	1	5	2 (2.5)	1/2b-3b-7
121	7	6	8	8	6	37	1	2 (2.5)	1/2a-3a
806	7	10	16	7	5	1	1	2 (2.5)	1/2a-3a
5	2	1	11	3	3	1	7	1 (1.25)	1/2b-3b-7
805	4	4	4	3	1	1	1	1 (1.25)	1/2b-3b-7

Prevalence of Virulence Associated Genes and EC Markers

Eighty isolates of *L. monocytogenes* from retail RTE food were detected for the presence of virulence genes. The result showed that all isolates harbored listeriolysin O genes (*hly*) and internalin genes (*inlA*, *inlB*, *inlC,* and *inlJ*), and 38.8% (31/80) of isolates harbored listeriolysin S genes (*llsX*) (**Table 4**). The *llsX*-positive isolates including twenty-one 4b-4d-4e isolates, eleven 1/2b-3b-7 isolates. EC markers were identified in 26.3% (21/80) of isolates by multiplex PCR. ECI was the only EC marker identified and was observed in 4b-4d-4e isolates from roast meat (roast chicken/duck/pork) in this study. These EC isolates were also found to be positive for *llsX*.

Analysis of Selected Isolates for *inlA* Sequence

Sequencing the full 2400 bp *inlA* ORF in 35 *L. monocytogenes* isolates yielded 14 different *inlA* allelic types, indicating 88.6% of gene diversity. A total of 126 (5.2%) sites were polymorphic, including 85 sites with synonymous substitutions and 41 sites with non-synonymous substitutions. The average number of nucleotide differences per site between two sequences (π) was 0.01907; the average number of nucleotide differences (k) was 45.834. The GC% observed in *inlA* was 37.1%.

The phylogenetic tree generated using the *inlA* sequences of 35 RTE isolates and the *L. monocytogenes* EGDe reference strain

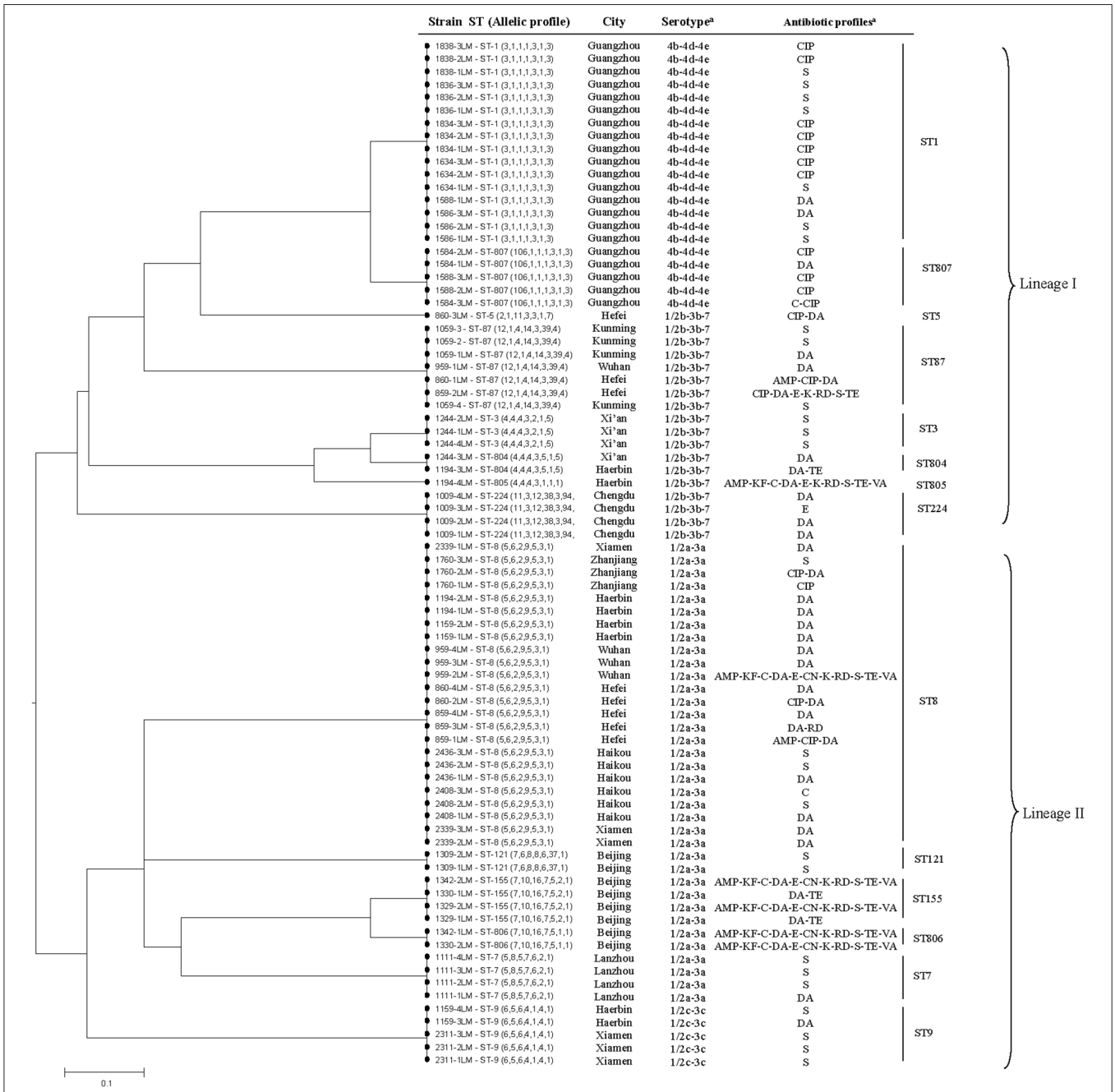


FIGURE 1 | The UPGMA (unweighted pair group method with arithmetic mean) tree of the seven multi-locus sequence typing loci (3288 base pair concatenated length) of RTE *L. monocytogenes* isolates (Supplementary Table 1). This tree was generated using the S.T.A.R.T (version 2).

(NC_003210.1; serotype 1/2a, ST35) revealed a similar grouping based on MLST analysis (**Figure 2**). The same lineage exhibited close relationships. Truncated forms of *InlA* have been described and are associated with reduced virulence (Nightingale et al., 2005a; Van Stelten et al., 2010), three distinct *inlA* alleles were found to harbor PMSC at position 492 (PMSC type 6), carried by ST 121 (1309-1LM, 1/2a-3a), the mutation at position 685 (PMSC type 11) harbored by the serotype 1/2c-3c isolate (2311-1LM, ST9) and a nonsense mutation at position 326 (GAA→TAA) of the *inlA* gene where a change of glutamic acid codon to a stop codon occurs showed a novel mutation type of PMSC (1159-3LM, 1/2c-3c, ST9).

Comparison of the amino-acid sequences of *inlA* between 35 *L. monocytogenes* isolates and the homologous sequence type strain EGDs, 13 internalin A variants were grouped. Each STs showed same variants, excluding CC1 (ST1, ST807), CC3 (ST3, ST804), and ST5 and ST805. Isolates from ST1 divided into two variants. In total, 40 amino acids were substituted (40/800, 5%).

TABLE 3 | Polymorphism of seven housekeeping protein-coding genes among *L. monocytogenes* isolates.

Gene	Template size	No. (%) polymorphic sites	G+C content (%)	Ks	Ka	Ka/ks	π	Tajima's D
abcZ	537	32 (5.96)	37.5	0.09824	0.00145	0.01476	0.02116	2.38645*
bglA	399	14 (3.51)	40.5	0.04069	0.00101	0.02482	0.00938	0.9151
cat	486	22 (4.53)	41.2	0.09135	0.00223	0.02441	0.0201	3.34927***
dapE	462	36 (7.79)	42.9	0.15169	0.0095	0.06263	0.03581	2.71776***
dat	471	57 (12.1)	36.4	0.31833	0.01483	0.04659	0.06114	4.53398***
ldh	453	17 (3.75)	43.4	0.06825	0	0.00000	0.01465	2.71776**
lhkA	480	17 (3.54)	37.1	0.07193	0.00273	0.03795	0.01693	3.98189***
Concatenate	3288	195 (5.93)	39.8	0.1116	0.00456	0.04086	0.02586	3.8258***

Ks, number of synonymous mutations; Ka, number of non-synonymous mutations; π, Nucleotide diversity.
**Indicate statistical differences of $P < 0.05$.*
***Indicate statistical differences of $P < 0.01$.*
****Statistical differences of $P < 0.001$.*

TABLE 4 | Prevalence of virulence markers in 80 RTE *Listeria monocytogenes* strains.

Virulence markers	No. (%) of positive samples	No. (%) of serotype-positive isolates				
		1/2a-3a	1/2c-3c	4b-4d-4e	1/2b-3b-7	4a-4c
hly	80/80 (100)	36 (100)	5 (100)	21 (100)	18 (100)	0 (0)
inlB	80/80 (100)	36 (100)	5 (100)	21 (100)	18 (100)	0 (0)
inlA	80/80 (100)	36 (100)	5 (100)	21 (100)	18 (100)	0 (0)
inlC	80/80 (100)	36 (100)	5 (100)	21 (100)	18 (100)	0 (0)
inlJ	80/80 (100)	36 (100)	5 (100)	21 (100)	18 (100)	0 (0)
llsX	31/80 (38.8)	0 (0)	0 (0)	21 (100)	10 (55.6)	0 (0)
ECI	21/80 (26.3)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)
ECII	0/80 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ECIII	0/80 (0)	10 (8.9)	0 (0)	0 (0)	0 (0)	0 (0)

Most of the substitutions (31/40, 77.5%) occurred in the leucine-rich repeats (LLR), the inter-repeat (IR) domain and the B-repeat domain (**Figure 3**). The most conserved region of InlA among the 35 RTE *L. monocytogenes* isolates was the LRR domain. In parallel, a previous study (Ragon et al., 2008) reported the high constraint of the LRR-region and the moderate constraint of the IR- and B-repeat regions.

DISCUSSION

As an important foodborne pathogen, *L. monocytogenes* remains a signification public health and food safety threat worldwide. In China, many studies of the prevalence of *L. monocytogenes* in food have been reported (Chen et al., 2009, 2014), but the outbreaks and infection of listeriosis in food are very limited. However, not all *L. monocytogenes* isolates have an equal capacity to cause disease. Therefore, it is important to investigate the molecular characteristics and virulence potential of *L. monocytogenes* isolates for designing and implementing more effective prevention strategies. The RTE isolates analyzed in this study were isolated from most of provincial capitals of China (Wu et al., 2015a), which could be better understanding the characterization of this important pathogen in China.

To enable increased characterization, MLST can be complemented by the nucleotide sequence determination of one or more highly diverse genes, such as those encoding antigens or antibiotic resistance determinants (Sullivan et al., 2005). In this study, 80 *L. monocytogenes* RTE isolates belonged to limited number of major clones. Except four new STs (ST804, 805, 806, 807), the other detected STs were already described in the *L. monocytogenes* Institute Pasteur MLST database (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates_public&page=profiles): 327 strains of ST1 (51% from human isolates), 253 strains of ST3 (37% from human and 55% from food and environment), 143 strains of ST7 (63% from animal and feed), 37 strains of ST8 (including 15 human and 10 food), 130 strains of ST9 (35% from human and 21% from food), 52 strains of ST5 (50% from human and 23% from food), 22 strains of ST87 (55% from human and 23% from food), 70 strains of ST155 (50% from human), 6 strains of ST224 (2 isolates from human and 4 from environment), and 74 strains of ST121 (54% from food). Strains of all STs observed in our study have been associated to various extents with human listeriosis and outbreaks, indicating that *L. monocytogenes* strains of these STs have at least a theoretical pathogenic potential. It is worth nothing that ST1, comprising 20% of the isolates in this study, have the same sequences as F2365 (a serotype 4b isolates from a soft cheese outbreak in California in 1985), CLIP12848 (a serotype 4b isolates from outbreak in France in 1989), and CLIP68868 (a serotype 4b isolates from outbreak in Sweden 1995) which should the worthy of the attention of the food hygiene supervision department. Interestingly, novel STs (ST805, ST806) were found in multidrug resistant strains (**Figure 1**), showing some allele numbers had exchanged. Multiple resistance in *L. monocytogenes* was linked to the presence of a self-transferable plasmid that was proposed to originate in *Enterococcus-Streptococcus* (Charpentier and Courvalin, 1999). Therefore, the self-transferable plasmid may also impact the homologous recombination of housekeeping genes. However, further research is needed to determine the reason underlying this correlation.

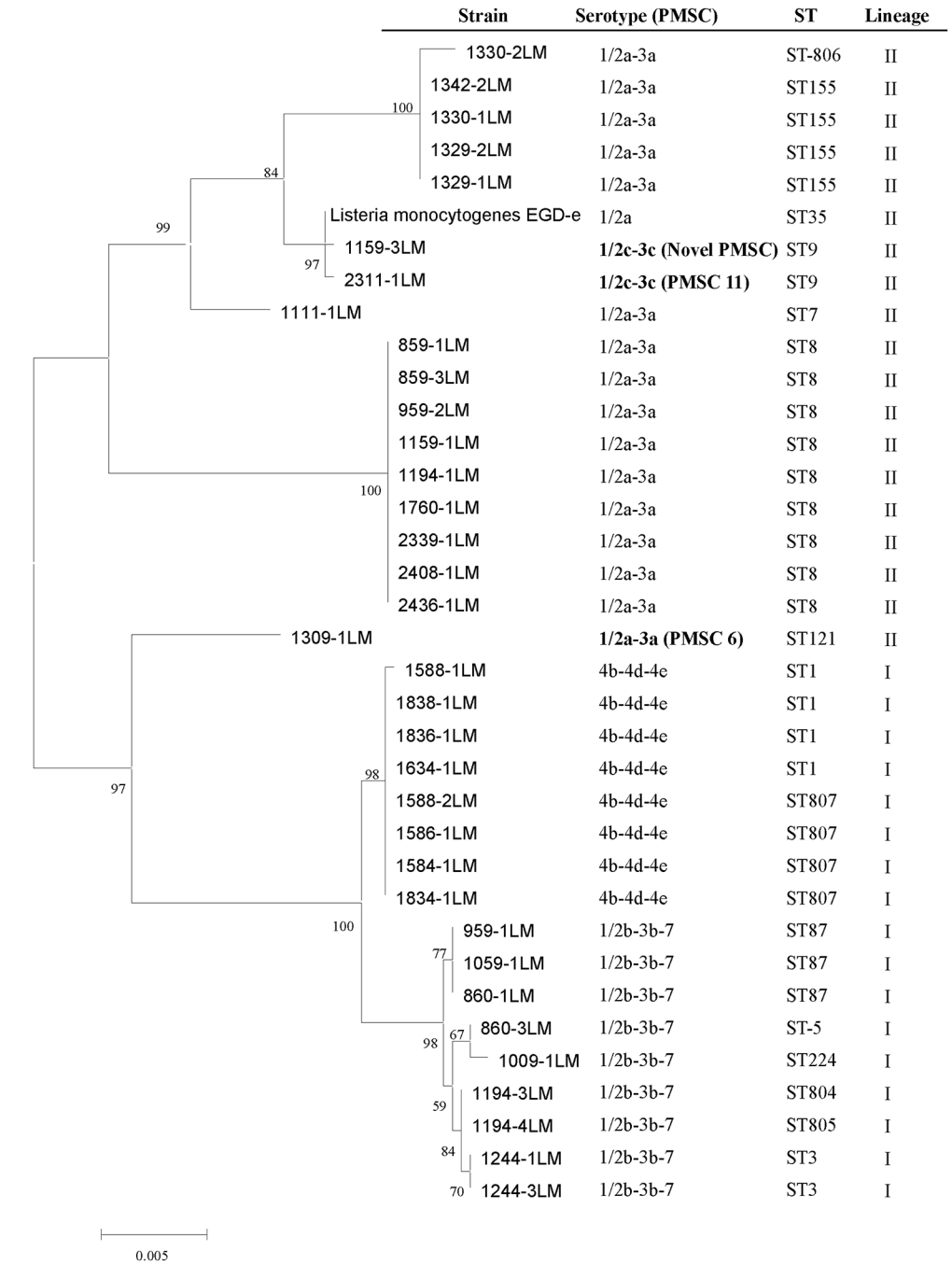


FIGURE 2 | Maximum likelihood tree of selected RTE *L. monocytogenes* isolates based on *inlA* sequence. The *inlA* sequences of 36 isolates are shown, including 35 selected isolates in this study and *Listeria monocytogenes* EGDe.

The presence of *hly*, *inlA*, *inlB*, *inlC*, *inlJ*, and *llsX* was evaluated in *L. monocytogenes* isolates recovered from patients, food and the environment (Kathariou, 2003; Wieczorek et al., 2012; Wu et al., 2015b). In this study, PCR based analysis of the 80 RTE isolates showed that most of these isolates possessed virulence genes similar to those of clinical isolates (Mammina et al., 2009). The *hly* gene is encoding the listeriolysin O (LLO), the determinant that is required for the disruption of the phagocytic vacuole and the release of bacteria into the cytoplasm, a prerequisite for their intracellular proliferation. Therefore, LLO is an essential virulence factor and its absence leads to total avirulence (Portnoy et al., 1992). *L. monocytogenes* adheres to and actively through actions of internalins, a complex family of LRR-containing proteins (Bierne et al., 2007). In this study,

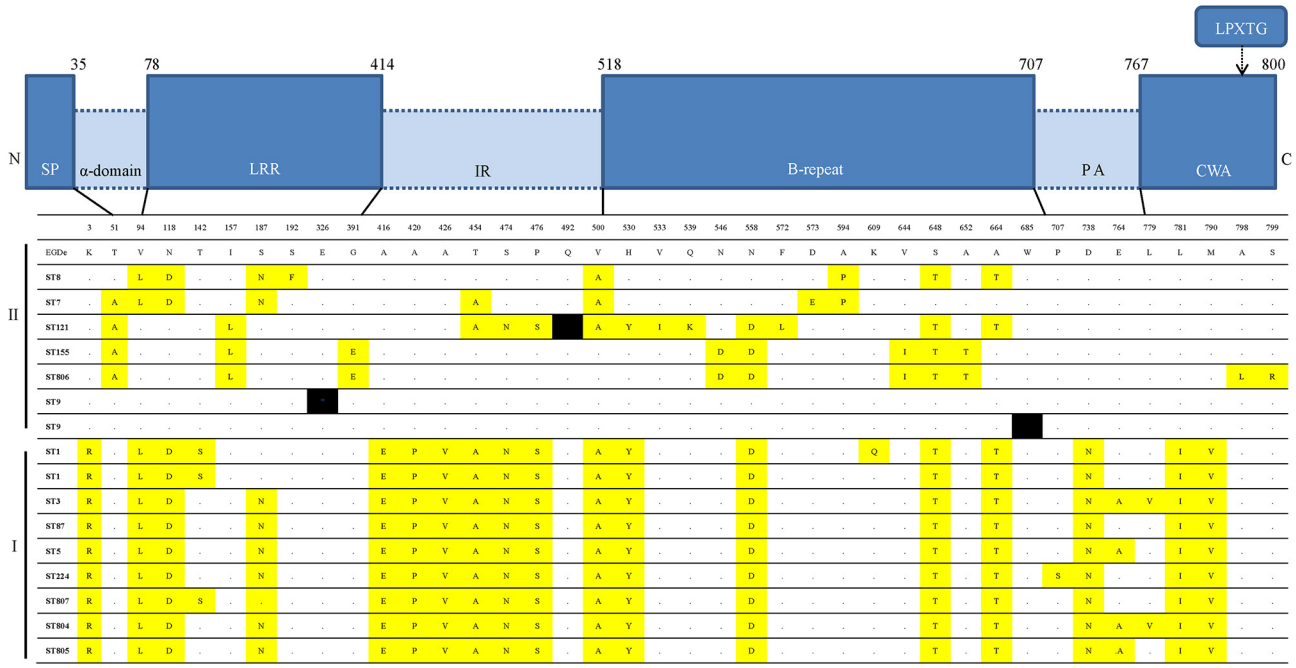


FIGURE 3 | Internalin A variants in the 16 distinct ST and *inlA* alleles encountered. Comparison of the amino acid sequences of inlA of 35 *L. monocytogenes* isolated from Chinese RTE food to the homologous sequence of type strain EGDs. InlA functional domains are represented as distinct blocks: SP, signal peptide, α-domain, alpha-domain linker; LRRs, leucine rich repeats; IR, intragenic repeat, B-repeats, PA, pre-anchor domain; CWA, cell wall anchor; and C, C terminus. Yellow represents replacement with amino-acid and black indicates nonsense mutations leading to a premature stop codon (PMSC).

we selected *inlA*, *inlB*, *inlC*, and *inlJ* for tested internalins in *L. monocytogenes* isolates. These genes are claimed to play a role in pathogenesis of human listeriosis. Besides, the *llsX* gene (encoding LLS) was employed as a genetic marker to detect *L. monocytogenes* with *Listeria* pathogenicity island 3 (LIPI-3) (Cotter et al., 2008), and strains from the present study exhibited 38.8% (31/80) positivity. The LLS, which is present in a subset of strains of lineage I, is induced only under oxidative stress conditions and contributes to murine virulence and in survival in polymorphonuclear neutrophils (Cotter et al., 2008). Overall, our result showed that the positive-*llsX* strains were found exclusively in lineage I (shown **Table 3** and **Figure 1**), the evolutionary lineage of *L. monocytogenes* that contributes to the majority of spontaneous and epidemic outbreaks of listeriosis (Jeffers et al., 2001). Besides, most of positive-*llsX* strains involving ECI (21/32, 65.6%) have been related to several major outbreaks in the US and Europe in the past (Kathariou, 2003; Liu, 2008). In general, ECs is defined as a small number of isolates of a presumably common ancestor (Chen and Knabel, 2007). Specific ECs continue to be associated with sporadic cases around the world (Neves et al., 2008; Mammina et al., 2009; Lomonaco et al., 2013). Recently, Cantinelli et al. (2013) conclude that the "epidemic clone" denominations represent a redundant but largely incomplete nomenclature system for MLST-defined clones, which must be regarded as successful genetic groups that are widely distributed across time and space. In our study, *L. monocytogenes* isolates of MLST-CC1, including ST1 and ST807 were both ECI which was consistent with this conclusion. However, the high occurrence of multiple virulence-associated genes and ECI markers in *L. monocytogenes* isolates from Chinese RTE food could pose a significant health risk as these isolates can be pathogenic and potentially capable of causing an epidemic.

Certainly, the presence of these virulence-associated genes does not mean a particular strain is virulent, as these genes are normally present in *L. monocytogenes* but if absence of these genes lead to avirulence. Over the past decade, previous studies identified naturally occurring PMSC mutations in *inlA* and have demonstrated that these mutations are responsible for virulence attenuation (Van Stelten and Nightingale, 2008). *L. monocytogenes* strains with PMSCs were frequently isolated from RTE foods in the US, although *inlA* PMSCs were markedly underrepresented among ECs (Van Stelten et al., 2010). In our study, the sequencing of *inlA* genes from isolates revealed that three mutations resulted in PMSCs: type 6, 11 and a novel type at position 326 (GAA→TAA), which means most of strains (32/35, 91.4%) are capable of producing full-length InlA required for host cell invasion. Generally, PMSCs have been reported 30%-45% of prevalence in food but 5% of prevalence in clinical isolates (Jacquet et al., 2004; Van Stelten et al., 2010; Chen et al., 2011). Three mutations were both harbored by the lineage II strains (2 strains with ST9, one strain with ST121) (**Figure 2**), which further confirmed that 1/2a and 1/2c (lineage II) more frequently possess PMSCs than 1/2b or 3b serotypes, with 4b strains rarely having PMSCs (Orsi et al., 2011). PMSC seems to be more frequently observed in ST9 and ST121 isolates as previous reports (Ragon et al., 2008; Ciolacu et al., 2014). ST9

and ST121 include isolates from several different countries and from several different environmental, clinical and food source (Ragon et al., 2008; Parisi et al., 2010; Holch et al., 2013). Some *L. monocytogenes* strains can persist for a longtime in food processing. The repeatedly lost by convergent evolution in the genetically homogeneous of these genotypes, may be attribute to the selective advantage by the loss of a functional InlA protein or a relaxed selective constraint on maintaining InlA function (Ragon et al., 2008). It is worth mentioning that the novel PMSCs type at position 326 (GAA→TAA), indicated that variation occurred in the LLR domain were located in repeats 10 (**Figure 3**). This domain promotes interaction with human surface receptor, E-cadherin, and has been reported as highly conserved, especially from repeats 7 to 15 (Ragon et al., 2008). To date, at least 18 distinct mutations in *inlA* leading to PMSCs have been observed in *L. monocytogenes* isolates worldwide, only four of them (type 5, 16, 17, and 18) were found in LLR (Van Stelten et al., 2010). Compared with the inlA variants, *L. monocytogenes* retained the ability for localized recombination is clearly provided by the *inlA* gene coding InlA, in agreement with previous reports (Nightingale et al., 2005b; Orsi et al., 2007). The substitution of some amino acid were consistent between lineage I strains and II, such as lysine replaced into arginine at position 3 in all lineage I strains (**Figure 3**). Additionally, the close relationship between *inlA* sequencing and MLST typing (**Figure 2**) suggested that housekeeping genes and virulence-associated genes had some similar genetic characterization, representing monophyletic origin. In other words, our data suggest that *L. monocytogenes* evolved and generated different STs related to some degree of evolutionary consistent.

In summary, the research findings suggest that *L. monocytogenes* isolated from Chinese RTE food showed genetic relatedness and virulence attribute. Serotype, lineage, MLST types, even antibiotic resistance showed some degree of genetically relationship. Most MLST sequence types (10/14, 71.4%) found in this study have been linked to human listeriosis around the world. All isolates carried several important virulence genes (*inlA*, *inlB*, *inlC*, *inlJ*, and *hly*), whereas 38.8% (31/80) of strains harbored listeriolysin S genes (*llsX*). Besides, all 4b-4d-4e isolates belonged to ECI showed a high potential to cause human diseases. Further study of *inlA* sequencing showed most of selected strains (32/35, 91.4%) contained PMSC-lacking *inlA* gene sequences required for the encoding of InlA factor and bacterial invasion of the host cell. Based on the genetic properties observed in *L. monocytogenes* isolates, it is reasonable to assume *L. monocytogenes* found in Chinese retail RTE food have potential to cause listeriosis, and is concerning. These data have significant implications for the epidemiological and public health studies of this pathogen.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: QW, JZ, SW. Performed the experiments: SW. Analyzed the data: SW, MC. Contributed reagents/materials/analysis tools: WG. Contributed to the writing of the manuscript: SW, QW.

FUNDING

We would like to acknowledge the financing support of National Natural Science Foundation of China (No. 31471664), the Science and Technology Projects of Guangdong (2013B050800026), and the Science and Technology Projects of Guangzhou (201504010036).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00168

REFERENCES

Bierne, H., Sabet, C., Personnic, N., and Cossart, P. (2007). Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*. *Microbes Infect.* 9, 1156–1166. doi: 10.1016/j.micinf.2007.05.003

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

Charpentier, E., and Courvalin, P. (1999). Antibiotic resistance in *Listeria* spp. *Antimicrob. Agents Chemother.* 43, 2103–2108.

Chen, J., Zhang, X., Mei, L., Jiang, L., and Fang, W. (2009). Prevalence of *Listeria* in Chinese food products from 13 provinces between 2000 and 2007 and virulence characterization of *Listeria monocytogenes* isolates. *Foodborne Pathog. Dis.* 6, 7–14. doi: 10.1089/fpd.2008.0139

Chen, M., Wu, Q., Zhang, J., Yan, Z., and Wang, J. (2014). Prevalence and characterization of *Listeria monocytogenes* isolated from retail-level ready-to-eat foods in South China. *Food Control* 38, 1–7. doi: 10.1016/j.foodcont.2013.09.061

Chen, Y., and Knabel, S. J. (2007). Multiplex PCR for simultaneous detection of bacteria of the genus *Listeria*, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L.* monocytogenes. *Appl. Environ. Microbiol.* 73, 6299–6304. doi: 10.1128/AEM.00961-07

Chen, Y., Ross, W. H., Whiting, R. C., Van Stelten, A., Nightingale, K. K., Wiedmann, M., et al. (2011). Variation in *Listeria monocytogenes* dose responses in relation to subtypes encoding a full-length or truncated internalin A. *Appl. Environ. Microbiol.* 77, 1171–1180. doi: 10.1128/AEM.01564-10

Ciolacu, L., Nicolau, A. I., Wagner, M., and Rychli, K. (2014). Listeria monocytogenes isolated from food samples from a Romanian black market show distinct virulence profiles. *Int. J. Food Microbiol.* 209, 44–51. doi: 10.1016/j.ijfoodmicro.2014.08.035

Clayton, E. M., Hill, C., Cotter, P. D., and Ross, R. P. (2011). Real-time PCR assay to differentiate listeriolysin S-positive and-negative strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 77, 163–171. doi: 10.1128/AEM.01673-10

Cotter, P. D., Draper, L. A., Lawton, E. M., Daly, K. M., Groeger, D. S., Casey, P. G., et al. (2008). Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes*. *PLoS Pathog.* 4:e1000144. doi: 10.1371/journal.ppat.1000144

EFSA (2015). The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J.* 13:3991. doi: 10.2903/j.efsa.2015.3991

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

Gombas, D. E., Chen, Y., Clavero, R. S., and Scott, V. N. (2003). Survey of *Listeria monocytogenes* in ready-to-eat foods. *J. Food Prot.* 66, 559–569.

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

Jacquet, C., Doumith, M., Gordon, J. I., Martin, P. M., Cossart, P., and Lecuit, M. (2004). A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. *J. Infect. Dis.* 189, 2094–2100. doi: 10.1086/420853

Jeffers, G. T., Bruce, J. L., McDonough, P. L., Scarlett, J., Boor, K. J., and Wiedmann, M. (2001). Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology* 147, 1095–1104. doi: 10.1099/00221287-147-5-1095

Kathariou, S. (2003). "Foodborne outbreaks of listeriosis and epidemic-associated lineages of Listeria monocytogenes," in *Microbial Food Safety in Animal Agriculture: Current Topics*, eds M. E. Torrence and R. E. Isaacson (Oxford, UK: Blackwell Publishing), 243–256. doi: 10.1002/9780470752616.ch25

Knabel, S. J., Reimer, A., Verghese, B., Lok, M., Ziegler, J., Farber, J., et al. (2012). Sequence typing confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. *J. Clin. Microbiol.* 50, 1748–1751. doi: 10.1128/JCM.06185-11

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., et al. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science* 292, 1722–1725. doi: 10.1126/science.1059852

Liu, D. (2008). *Handbook of Listeria monocytogenes.* Boca Raton, FL: CRC Press.

Liu, D., Lawrence, M. L., Austin, F. W., and Ainsworth, A. J. (2007). A multiplex PCR for species-and virulence-specific determination of *Listeria monocytogenes*. *J. Microbiol. Methods* 71, 133–140. doi: 10.1016/j.mimet.2007.08.007

Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., et al. (2013). Novel epidemic clones of *Listeria monocytogenes*, United States, 2011. *Emerg. Infect. Dis.* 19, 147. doi: 10.3201/eid1901.121167

Maiden, M. C. (2006). Multilocus sequence typing of bacteria. *Annu. Rev. Microbiol.* 60, 561–588. doi: 10.1146/annurev.micro.59.030804.121325

Mammina, C., Aleo, A., Romani, C., Pellissier, N., Nicoletti, P., Pecile, P., et al. (2009). Characterization of *Listeria monocytogenes* isolates from human listeriosis cases in Italy. *J. Clin. Microbiol.* 47, 2925–2930. doi: 10.1146/annurev.micro.59.030804.121325

Neves, E., Lourenço, A., Silva, A. C., Coutinho, R., and Brito, L. (2008). Pulsed-field gel electrophoresis (PFGE) analysis of *Listeria monocytogenes* isolates from different sources and geographical origins and representative of the twelve serovars. *Syst. Appl. Microbiol.* 31, 387–392. doi: 10.1016/j.syapm.2008.08.005

Nightingale, K. K., Windham, K., and Wiedmann, M. (2005b). 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

Nightingale, K. K., Windham, K., Martin, K. E., Yeung, M., and Wiedmann, M. (2005a). Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in inlA, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Appl. Environ. Microbiol.* 71, 8764–8772. doi: 10.1128/AEM.71.12.8764-8772.2005

Olier, M. W., 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

Orsi, R. H., Ripoll, D. R., Yeung, M., Nightingale, K. K., and Wiedmann, M. (2007). Recombination and positive selection contribute to evolution of *Listeria monocytogenes* inlA. *Microbiology* 153, 2666–2678. doi: 10.1099/mic.0.2007/007310-0

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

Parisi, A., Latorre, L., Normanno, G., Miccolupo, A., Fraccalvieri, R., Lorusso, V., et al. (2010). Amplified fragment length polymorphism and multi-locus sequence typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. *Food Microbiol.* 27, 101–108. doi: 10.1016/j.fm.2009.09.001

Portnoy, D. A., Chakraborty, T., Goebel, W., and Cossart, P. (1992). Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun.* 60, 1263.

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

Ryser, E. T., and Marth, E. H. (2007). *Listeria, Listeriosis, and Food Safety*. New York, NY: CRC Press.

Salcedo, C., Arreazá, L., Alcala, B., de la Fuente, L., and Vázquez, J. (2003). Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. *J. Clin. Microbiol.* 41, 757–762. doi: 10.1128/JCM.41.2.757-762.2003

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

Shen, J., Rump, L., Zhang, Y., Chen, Y., Wang, X., and Meng, J. (2013). Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. *Food Microbiol.* 35, 58–64. doi: 10.1016/j.fm.2013.02.014

Sullivan, C. B., Diggle, M. A., and Clarke, S. C. (2005). Multilocus sequence typing. *Mol. Biotechnol.* 29, 245–254. doi: 10.1385/MB:29:3:245

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882. doi: 10.1093/nar/25.24.4876

Van Stelten, A., and Nightingale, K. (2008). Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the *Listeria monocytogenes* virulence-associated gene inlA. *Appl. Environ. Microbiol.* 74, 7365–7375. doi: 10.1128/AEM.01138-08

Van Stelten, A., Simpson, J., Ward, T., and Nightingale, 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

Wieczorek, K., Dmowska, K., and Osek, J. (2012). Prevalence, characterization, and antimicrobial resistance of *Listeria monocytogenes* isolates from bovine hides and carcasses. *Appl. Environ. Microbiol.* 78, 2043–2045. doi: 10.1128/AEM.07156-11

Wu, S., Wu, Q., Zhang, J., Chen, M., and Hu, H. (2015b). *Listeria monocytogenes* prevalence and characteristics in retail raw foods in China. *PLoS ONE* 10:e0136682. doi: 10.1371/journal.pone.0136682

Wu, S., Wu, Q., Zhang, J., Chen, M., and Yan, Z. (2015a). Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from retail ready-to-eat foods in China. *Food Control* 47, 340–347. doi: 10.1016/j.foodcont.2014.07.028

Xu, X.-K., Wu, Q.-P., Zhang, J.-M., Deng, M.-Q., and Zhou, Y.-H. (2009). Studies on specific detection of *Listeria monocytogenes* in foods by duplex PCR. *Chin. J. Health Lab. Technol.* 19, 1199–1221.

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 RT and handling Editor declared a current collaboration and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 January 2016
doi: 10.3389/fmicb.2016.00009

Biosurfactant Produced by *Salmonella* Enteritidis SE86 Can Increase Adherence and Resistance to Sanitizers on Lettuce Leaves (*Lactuca sativa* L., *cichoraceae*)

Eliandra M. Rossi[1,2], Luniele Beilke[1], Marília Kochhann[1], Diana H. Sarzi[1] and Eduardo C. Tondo[2]*

[1] Laboratório de Microbiologia, Departamento de Ciências Biológicas e da Saúde, Universidade do Oeste de Santa Catarina, São Miguel do Oeste, Brazil, [2] Laboratório de Microbiologia e Controle de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Salmonella Enteritidis SE86 is an important foodborne pathogen in Southern Brazil and it is able to produce a biosurfactant. However, the importance of this compound for the microorganism is still unknown. This study aimed to investigate the influence of the biosurfactant produced by *S*. Enteritidis SE86 on adherence to slices of lettuce leaves and on resistance to sanitizers. First, lettuce leaves were inoculated with *S*. Enteritidis SE86 in order to determine the amount of biosurfactant produced. Subsequently, lettuce leaves were inoculated with *S*. Enteritidis SE86 with and without the biosurfactant, and the adherence and bacterial resistance to different sanitization methods were evaluated. *S*. Enteritidis SE86 produced biosurfactant after 16 h (emulsification index of 11 to 52.15 percent, $P < 0.05$) and showed greater adherence capability and resistance to sanitization methods when the compound was present. The scanning electron microscopy demonstrated that *S*. Enteritidis was able to adhere, form lumps, and invade the lettuce leaves' stomata in the presence of the biosurfactant. Results indicated that the biosurfactant produced by *S*. Enteritidis SE86 contributed to adherence and increased resistance to sanitizers when the microorganism was present on lettuce leaves.

Keywords: lettuce, microbial adherence and resistance, *Salmonella* Enteritidis SE86, biosurfactant, disinfection

OPEN ACCESS

Edited by:
Rosanna Tofalo, University of Teramo, Italy

Reviewed by:
Mehrdad Mark Tajkarimi, University of North Carolina at Greensboro, USA
Ebrahim Hadavi, Islamic Azad University, Iran

****Correspondence:***
Eliandra M. Rossi
eliandra_bio@yahoo.com.br

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

Received: *27 October 2015*
Accepted: *07 January 2016*
Published: *22 January 2016*

Citation:
Rossi EM, Beilke L, Kochhann M, Sarzi DH and Tondo EC (2016) Biosurfactant Produced by Salmonella Enteritidis SE86 Can Increase Adherence and Resistance to Sanitizers on Lettuce Leaves (Lactuca sativa L., cichoraceae). Front. Microbiol. 7:9. doi: 10.3389/fmicb.2016.00009

INTRODUCTION

Salmonella Enteritidis SE86 is a recognized food pathogen responsible for several foodborne disease (FBD) outbreaks in Southern Brazil (Geimba et al., 2004; Oliveira et al., 2009; Tondo and Ritter, 2012; Capalonga et al., 2014; Tondo et al., 2015). Several studies have been carried out taking into account the importance of this pathogen with the aim of understanding the reasons that it continues to be an important foodborne pathogen in this region since 1999 (Geimba et al., 2004; Capalonga et al., 2014; Tondo et al., 2015). Among all the characteristics that may contribute to that, we may highlight its great acid adaptation capability when the pathogen is exposed to acidic environments and, as a consequence, an increase in virulence (Perez et al., 2012) and ability to survive in simulated gastric fluid, (pH 1.5; 2), besides its resistance to sodium

hypochlorite at 200 and 400 ppm (Machado et al., 2010). A previous study (Machado, 2007) demonstrated that *S.* Enteritidis SE86 was able to produce expressive amounts of biosurfactant during its growth in BHI broth. Nevertheless, the characteristics and functions of this compound have not been studied yet.

The term biosurfactant is described as a "surface active agent" produced by microorganisms (Marchant and Banat, 2012). These are amphiphilic compounds used as detergents or wetting, emulsifying, dispersing, and foaming agents in many industrial formulations (Nitschke and Pastore, 2002). Even though they have been highly used, the physiological function of biosurfactants for microbial cells is still not completely understood, and the way these compounds rule food microorganisms is practically unknown. One of the few studies concerning biosurfactants in foods was published by Mellor et al. (2011), who reported that a biosurfactant produced by *Pseudomonas fluorescens* was able to alter the characteristics of chilled chicken meat (increased decomposition) and the compound facilitated the survival of the bacterium.

The production of biosurfactants is usually associated with the presence of large amounts of microorganisms (Ron and Rosenberg, 2001) and this factor can contribute to increased pathogenicity. Also, several researchers have reported that biosurfactants can contribute to the adherence of pathogens to surfaces and the formation of biofilms (Ron and Rosenberg, 1999; Ron and Rosenberg, 2001; Nitschke and Pastore, 2002). Furthermore, the ability of microorganisms to produce biosurfactants can also be linked with their resistance to sanitizers, because generally they present organic compounds that can protect bacterial cells.

Recently, it was stated that bacteria such as *S.* Enteritidis have a natural tendency to stick to surfaces, which includes lettuce leaves (Lima et al., 2013).

Lettuce (*Lactuca sativa* L., *cichoraceae*) is the most consumed green leaf in the world; this is a plant of easy acquisition, standing out due to its nutritional quality and because it is considered a low cost leafy vegetable (Abreu et al., 2010; Lima et al., 2013). During their growing cycle, lettuces can be contaminated by *Salmonella* and, as a consequence, several cases of salmonellosis have been related to the consumption of lettuces (Horby et al., 2003; Sagoo et al., 2003; Takkinen et al., 2005; Nygard et al., 2008; Irvine et al., 2009). In order to avoid contamination, lettuce leaves must be washed and sanitized before going to the table. However, if a biosurfactant is produced by *Salmonella*, microbial cells can easily adhere to the leaves and be protected against inactivation.

The aim of this study was to investigate the influence of the biosurfactant on adherence and resistance of *S.* Enteritidis SE86 to sanitizers on lettuce leaves.

MATERIALS AND METHODS

Lettuce Samples

All lettuces used in this study were purchased in a supermarket in Porto Alegre, Capital of Rio Grande do Sul, Southern state of Brazil. Before the experiments started, lettuces were transported to the laboratory, inside thermal boxes, at 4°C for a maximum period of one hour. Before experiments, injured leaves were removed and the remaining ones were washed with potable water. Whole lettuce leaves were used for the experiments on resistance to sanitizers described in "Influence of Biosurfactant on the Efficiency of Sanitation Methods Used for Disinfection of Whole Lettuce Leaves Contaminated with *S.* Enteritidis SE86." Slices of lettuce leaves with sizes of 10 cm × 10 cm were used for the experiments of adherence, according to Sagong et al. (2011). This was done in order to express results as number of CFU/cm^2. All sliced leaves were cut similarly, aiming to avoid interference in the results.

Before experiments, whole lettuce leaves and sliced leaves were washed and sanitized with potable water with 200 ppm sodium hypochlorite added, for 15 min (Antunes, 2009). After that, leaves were rinsed with sterile distilled water with 0.5% sodium thiosulfate added (Synth, Diadema-SP).

Microorganism

In this study, we used the *S.* Enteritidis SE86 strain, which was isolated from a cabbage involved with a salmonellosis outbreak in the State of Rio Grande do Sul, Brazil, in 1999. This strain was characterized by Geimba et al. (2004) and presents the same profile and genotypic characteristics of *S.* Enteritidis responsible for several cases of salmonellosis that occurred from 1999 to 2012 in Rio Grande do Sul (Capalonga et al., 2014; Tondo et al., 2015). For the tests, the strain was cultivated in BHI broth (Oxoid, Basingstoke, England) at 36 ± 1°C, for approximately 18 h.

Biosurfactant Production on Lettuce Leaves

Four whole lettuce leaves were submerged in 100 ml of minimal medium containing 4.4 log CFU/ml of *S.* Enteritidis SE86 and incubated at 36 ± 1°C for 120 h.

Aliquots of 6 ml were removed every two hours for up to 60 and, after each time period, aliquots were withdrawn every 24 h for up to 120 h of culture in order to determine the emulsification index (IE24), pH, and bacterial count. Bacterial counts were performed in triplicate by seeding the samples onto plates containing xylose lysine deoxycholate agar (XLD: Merck, Darmstadt, Germany) and incubated at 36 ± 1°C for 24 h. The pH was evaluated by aliquots (10 ml) of the samples and then analyzed with a pH meter (PHTECK). The emulsification index (IE24) was assessed using the method described by Cooper and Goldenberg (1987).

All experiments were repeated three times and the averages were subsequently expressed as the final result.

Preparation of the Inoculum of *S.* Enteritidis SE86 With and Without Biosurfactant

The inoculum of *S.* Enteritidis SE86 without biosurfactant was prepared using 40 ml of BHI broth (Merck, Darmstadt, Germany) incubated at 36 ± 1°C for 72 h. After incubation, the culture was centrifuged at 3500 rpm for 15 min and washed with phosphate buffered saline (PBS) three times. Then, the washed cells were

inoculated in 100 ml minimal medium until they reached a concentration of approximately 8.0 log CFU/ml.

In order to prepare the inoculum of *S.* Enteritidis SE86 with biosurfactant, the compound was partially purified, according to the following procedures. The biosurfactant recovery was prepared by centrifuging at 3500 rpm for 15 min inoculum *S.* Enteritidis SE86 in BHI broth incubated at 36 ± 1°C for 72 h. The supernatant was homogenized with ethanol (–4°C) at 95% concentration 4:1 and stored at 4°C for 24 h. Subsequently, the precipitate (biosurfactant) was recovered by centrifugation at 3500 rpm for 15 min and the supernatant was discarded. After the alcohol had evaporated completely, the pellet was resuspended in sterile distilled water and dialyzed. The dialysis was done using a membrane tube (SIGMA) submitted to constant agitation in distilled water for 24 h (Kumar et al., 2004; Ciapina, 2008; Pacheco et al., 2010). Hundred milliliter of solution with partially purified biosurfactant and 8.0 log CFU/ml of *S.* Enteritidis SE86 were used.

Resistance to Sanitizers of *S.* Enteritidis SE86 With and Without Biosurfactant *In Vitro*

The susceptibility of *S.* Enteritidis SE86 with and without biosurfactant to sanitizers *in vitro* was evaluated using sodium hypochlorite (50 and 200 ppm) and vinegar (2 and 20%). The test was performed according to the methodology recommended by Ordinance 101/93 published by the Brazilian Ministry of Agriculture and Food Supply (Brasil, 1993).

Initially, the concentrations of sodium hypochlorite (Q. Boa®) and vinegar (fermented acetic acid from red wine and alcohol—koller®) were prepared in sterile distilled water. Nine milliliter of each sanitizer were aseptically placed into sterile vials, to which was added 1 ml of bovine serum albumin solution (1%). After that, 0.1 ml inoculum (*S.* Enteritidis SE86 with and without the biosurfactant) was added separately to each tube containing sanitizers and the exposure time was measured. After 5, 10, 15, 20, and 30 min of exposure, an aliquot of 0.01 ml of suspension was transferred into tubes containing BHI broth. The tubes were incubated for 96 h at 36 ± 1°C, and the bacterial growth was checked every 24 h. In the case of bacterial growth, the test was considered positive (resistant). The negative confirmation of results (tubes without growth) was performed through inoculation on trypticase soy agar (TSA agar, Merck, Darmstadt, Germany) incubated at 36 ± 1°C for 24 h.

Each experiment was performed in triplicate on different days.

Adherence of *S.* Enteritidis SE86 to Slices of Lettuce Leaves (*Lactuca sativa L., cichoraceae*)

Adherence of *S.* Enteritidis SE86 to slices of lettuce leaves was assessed using the methods proposed by Lima et al. (2013) with the following adaptation: the slices of lettuce leaves were cleaned, as described in Section "Lettuce Samples".

Before each treatment, three slices of lettuce leaf were immersed in 100 ml of minimal medium containing *S.* Enteritidis SE86 at a concentration of approximately 8.0 log CFU/ml, with and without the biosurfactant, for 15, 30, and 60 min at room temperature (25°C). The preparation of the inoculum of *S.* Enteritidis SE86 with and without biosurfactant is described in Section "Preparation of the Inoculum of *S.* Enteritidis SE86 With and Without Biosurfactant". After that, slices of lettuce leaf were submerged in 100 ml of PBS and immediately sonicated for five minutes, using ultrasonic equipment (LF Equipamentos, Anhangaba SP) with intensity of 40 kHz. Sonication was used in order to remove adhered cells following the methods described by Sinde and Carballo (2000). This method was used because it does not damage cells and is considered very efficient in removing bacteria from biomaterials, especially from rough or irregular surfaces (An and Skowronski, 2000).

The counting of *S.* Enteritidis SE86 was performed on XLD agar incubated at 36 ± 1°C for 24 h. Counts were done in triplicate and each experiment was repeated five times.

Influence of Biosurfactant on the Efficiency of Sanitation Methods Used for Disinfection of Whole Lettuce Leaves Contaminated with *S.* Enteritidis SE86

First of all, 250 g of whole lettuce leaves were immersed into 500 ml of the *S.* Enteritidis SE86 inoculum with and without biosurfactant (prepared as described in Section "Preparation of the Inoculum of *S.* Enteritidis SE86 With and Without Biosurfactant") for 60 min.

Sanitation treatments were performed by immersing artificially contaminated lettuce leaves (25 g) in 500 ml of each treatment solution (i.e., potable water for 30 min; 50 and 200 ppm sodium hypochlorite for 15 and 30 min; 2 and 20% vinegar aqueous solution for 15 min). At the end of the contact time, each treatment solution was drained off, and leaves were rinsed with 200 ml of neutralizing buffer solution (0.5% thiosulfate sodium, Synth, Diadema SP) for 30 s, as recommended by Abadias et al. (2008), and then rinsed with potable water.

The negative and positive controls were non-contaminated lettuce leaves and lettuce leaves artificially contaminated with *S.* Enteritidis SE86, respectively. Washing was carried out only with potable water in order to evaluate of bacterial removal.

After treatments, lettuce leaves (25 g) were blended in a Stomacher bag containing 225 ml 0.1% peptone water (Merck, Darmstadt, Germany) for 60 s. *S.* Enteritidis SE86 counting was carried out on XLD agar after incubation at 36 ± 1°C for 24 h. Typical colonies (black) were counted in triplicate and the identity of the microorganism confirmed by biochemical tests.

All treatments were performed ten times on different days and the measurement of free chlorine in solutions was done using a Spectroquant® Kit (Merck).

Scanning Electron Microscopy of *S.* Enteritidis SE86 on Surface of Lettuce Leaves With and Without Biosurfactant

Lettuce slices (1 cm × 1 cm) were prepared using the central region of lettuce leaves (washed and disinfected as described in Section "Lettuce Samples").

Three artificially contaminated lettuce slices with and without biosurfactant were let for 1 h at room temperature (25°C). After that, leaves were gently washed twice using 0.1% peptone water and fixed with 3.0% glutaraldehyde and 0.05 M phosphate buffer, pH 7.0, for 1 h. The slices were washed four times (15 min each) with phosphate-buffered saline. After that, samples were dehydrated by increasing concentrations of ethanol solution (30, 50, 70, 80, 95, and 100%), with 15 min of contact each, and finally acetone PA for 30 min. The slices were dried with CO_2 in a critical-point drier (CPD 030; Bal-Tec), coated with gold (BAL-TEC SCD 050), and taken for observation on a JSM 5800 scanning electron microscope (SEM). Three lettuce slices submerged only in sterile distilled water were used as negative controls.

Statistical Analysis

The ANOVA test was applied (Assistat 7.7 Beta) with $P < 0.05$, in order to assess significant differences in the adherence of *S.* Enteritidis SE86 and its resistance to washing and disinfecting methods on lettuce leaves.

RESULTS

Production of Biosurfactant on Lettuce Leaves

The results showed that *S.* Enteritidis SE86 produced biosurfactant when in contact with the lettuce leaves for more than 16 h, presenting an emulsification index (IE_{24}) of 11% when bacterial population reached 7.11 log CFU/ml (**Figure 1**). The greater emulsification index was 52.15%, after 120 h of contact with lettuce leaves and when the population was 9.8 log CFU/ml and the pH remained at 7.0 during all experiments. During the preparation of the inoculum in minimal medium, SE86 also produced emulsifier (EI24 46%); however, after 120 h, the IE24 decreased to 3% (results not shown), probably because the energy sources were depleted.

In Vitro Resistance to Sanitizers

The *in vitro* testing of the susceptibility to sanitizers revealed that the biosurfactant was responsible for increasing the survival of *S.* Enteritidis SE86 in 50ppm sodium hypochlorite and 2 and 20% vinegar solution. As an example, SE86 without biosurfactant was completely inactivated by 50 ppm sodium hypochlorite in 15 min, while with biosurfactant, the microorganism survived for 30 min. Similarly, SE86 without surfactant was eliminated by 2 and 20% vinegar after 15 and 0 min of exposure, respectively. Nevertheless, the presence of surfactant made SE86 survive for 20 and 5 min, respectively (**Table 1**). The biosurfactant did not influence the survival of SE86 exposed to 200 ppm sodium hypochlorite.

Influence of Surfactant on the Adherence of *S.* Enteritidis SE86 to Slices of Lettuce Leaf

The results of this study showed that there were significant differences ($P < 0.05$) in the adherence of *S.* Enteritidis SE86 to slices of lettuce leaf when biosurfactant was present (**Table 2**). The highest counts of adhered SE86 were observed after 60 min of contact with slices of lettuce leaf. At that time, average counts of 7.3 log CFU/cm^2 and 4.1 log CFU/cm^2 were obtained on lettuce leaves with and without biosurfactant, respectively.

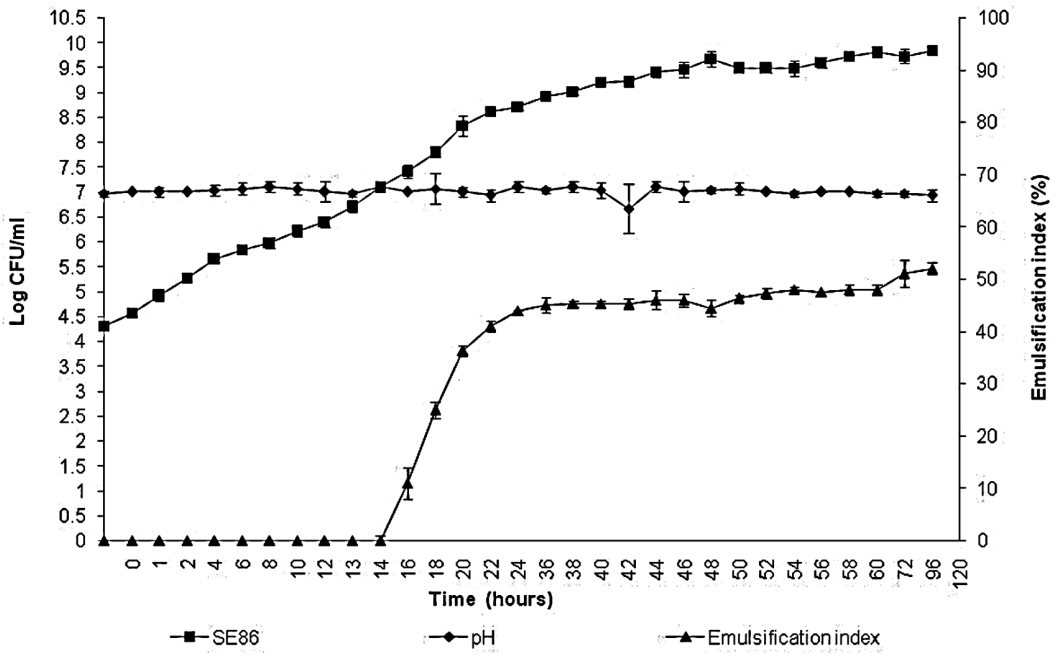


FIGURE 1 | Growth, pH, and emulsification index of *Salmonella* Enteritidis SE86 in minimal medium with whole lettuce leaves for 120 h at 36 ± 1°C.

TABLE 1 | *In vitro* susceptibility testing to disinfectants (200 and 50 ppm sodium hypochlorite and 2 and 20% vinegar solution) of *S.* Enteritidis SE86 with and without biosurfactant.

Sanitizers	Exposure time (minutes) of *S.* Enteritidis SE86 without biosurfactant					Exposure time (minutes) of *S.* Enteritidis SE86 with biosurfactant				
	5	10	15	20	30	5	10	15	20	30
200 ppm sodium hypochlorite	S	S	S	S	S	S	S	S	S	S
50 ppm sodium hypochlorite	R	R	R	S	S	R	R	R	R	R
2% vinegar solution	R	R	R	S	S	R	R	R	R	S
20% vinegar solution	S	S	S	S	S	R	S	S	S	S
Water	R	R	R	R	R	R	R	R	R	R

R: resistant; S: sensitive.

TABLE 2 | Median and standard deviation about the adherence of *S.* Enteritidis SE86 with and without biosurfactant on slices of lettuce leaves at different times.

Time (minutes)	*S.* Enteritidis SE86 without biosurfactant (log CFU/cm^2)	*S.* Enteritidis SE86 with biosurfactant (log CFU/cm^2)
15	3.5 ± 0.3^{c}	6.3 ± 0.2^{b}
30	3.5 ± 0.3^{c}	6.0 ± 0.7^{b}
60	4.1 ± 0.5^{c}	7.3 ± 0.3^{a}

Values represent the means of five replicates. Different letters represent significant differences ($P < 0.05$).

Scanning electron microscopy demonstrated that *S.* Enteritidis SE86 was able to adhere to the slices of lettuce leaf, forms lumps, and enter the stomata when the biosurfactant was present (**Figure 2**).

Resistance to Sanitation Methods of Lettuce Leaves

It was observed that all treatments reduced the amount of *S.* Enteritidis SE86 on lettuces, but lettuces contaminated with *S.* Enteritidis SE86 and with biosurfactant demonstrated higher numbers of survival cells (significant difference $P < 0.05$) than lettuces contaminated with *S.* Enteritidis SE86 without the surfactant (**Figure 3**). Reductions in counts of *S.* Enteritidis SE86 with biosurfactant ranged from 1.0 to 2.8 log CFU/g, whereas the reductions of *S.* Enteritidis SE86 without biosurfactant ranged from 1.3 to 3.3 log CFU/g ($P < 0.05$) (**Table 3**). It was observed that the most effective treatment of lettuce contaminated with *S.* Enteritidis SE86 and biosurfactant was washing it with potable water and submerging it in 200 ppm of sodium hypochlorite for 15 min. This showed a reduction of 2.8 log CFU/g. However, when lettuce leaves were contaminated only with *S.* Enteritidis SE86, the most effective reduction (3.3 log CFU/g) was obtained by the treatment that washed leaves with potable water and sanitized them with 50 ppm sodium hypochlorite for 30 min. This result showed that *S.* Enteritidis SE86 without biosurfactant was inactivated by lower concentrations of sodium hypochlorite.

Salmonella Enteritidis SE86 with biosurfactant was more resistant on lettuce leaves than in *in vitro* tests (**Table 1**; **Figure 3**).

DISCUSSION

Microorganisms develop survival abilities in different environments and biosurfactant production can be an advantage to survive in foods (Mellor et al., 2011). However, the exact physiological function of biosurfactants is not yet completely elucidated (Nitschke and Pastore, 2002; Hamme et al., 2006; Abdel-Mawgoud et al., 2009; Jirku et al., 2015).

Results of the present study demonstrated that *S.* Enteritidis SE86 was able to produce biosurfactant on lettuce leaves and this ability may have facilitated the access to cutin on lettuce leaves, one functional component of the cuticle deposited on the surfaces and within the epidermal walls of aerial parts of plants. Cutin is composed of three dimensional polyesters of long fatty acid chains (Bacic et al., 1988) and the amphipathic property of biosurfactant may facilitate access to nutrients present on lettuce leaves, supplying energy for bacterial growth.

Several research groups have reported that environmental microorganisms are able to produce biosurfactants (Chen et al., 2012; Jain et al., 2013; Ayed et al., 2014; Maoa et al., 2015; Rosa et al., 2015); however, there are no scientific reports showing the production of surfactants by foodborne pathogens. To our knowledge, the present study is the first that demonstrates biosurfactant production by *Salmonella*. Other reports have demonstrated the production of biosurfactants by degradative microorganisms on foods. For example, according to Mellor et al. (2011), the biosurfactant produced by *Pseudomonas fluorescens* contributed to increasing the total bacterial count on chicken stored aerobically for three days, suggesting that the biosurfactant contributed to the bioavailability of nutrients for the bacteria. These researchers suggested that the biosurfactant becomes a competitive advantage for the microorganism to maintain their survival, thereby enhancing the decomposition of chicken meat. Shaheen et al. (2010) have reported that a type of biosurfactant called surfactin may have contributed to the formation of biofilms by *Bacillus cereus* inside milk tanks.

The ability of *Salmonella* to adhere to lettuce leaves was reported by several studies (Wei et al., 2006; Patel and Sharma, 2010; Kroupitski et al., 2011; Lima et al., 2013). Results similar to the ones obtained in this study were found by Kroupitski et al. (2011), who found 7.0 log CFU of *S.* Typhimurium on the central region of lettuce leaves. Also, Lima et al. (2013) demonstrated that the count of *S.* Enteritidis cells that adhered to lettuce leaves

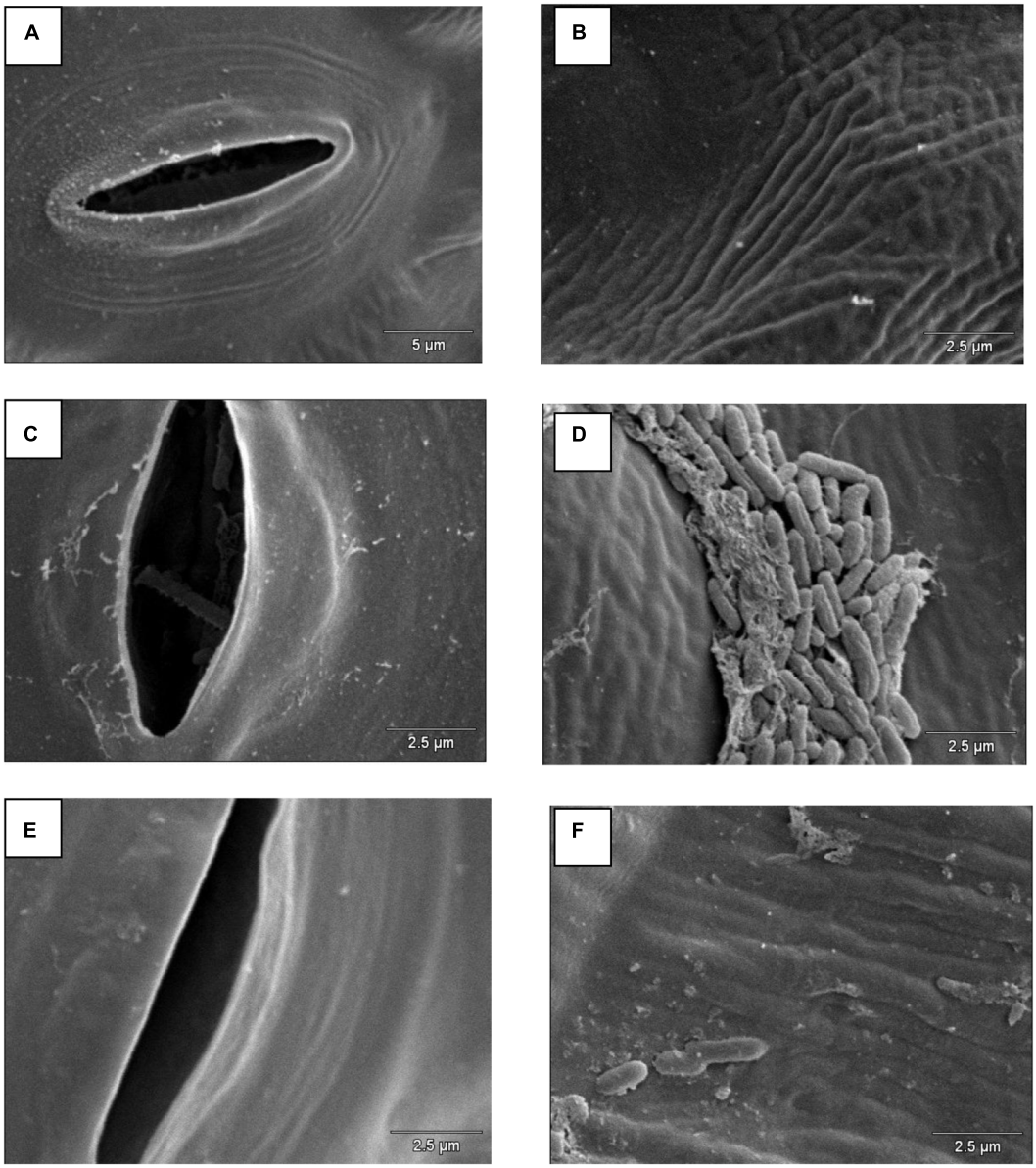


FIGURE 2 | Scanning electron microscopy on lettuce leaf surface infected with *S.* Enteritidis SE86 with and without biosurfactant. Negative control: **(A)** stomata and **(B)** lettuce leaf surface. *S.* Enteritidis SE86 with biosurfactant: **(C)** *S.* Enteritidis SE86 inside a stomata, and **(D)** lumps of *S.* Enteritidis SE86 on lettuce leaf surface. *S.* Enteritidis SE86 without biosurfactant: **(E)** stomata without *S.* Enteritidis SE86 and **(F)** lettuce leaf surface without formation lumps of *S.* Enteritidis SE86.

differed ($P < 0.05$) between the hydroponic and conventional systems, reaching 5.2 ± 0.56 and 4.6 ± 0.26, respectively.

The influence of biosurfactants on bacterial adherence to surfaces has been quite well studied, and the results are variable. Hassan and Frank (2003) stated that Tween 85 surfactant reduced the adherence of *Escherichia coli* O157:H7 to lettuce leaves. Other researchers (Sotirova and Vasileva-Tonkova, 2009) reported that *Pseudomonas aeruginosa* NBIMCC 1390 with rhamnolipid biosurfactant increased cell hydrophobicity to 31% adherence and that these compounds caused changes in the bacterial cell surface.

The results of our study suggest that the biosurfactant contributed to increase the survival of *S.* Enteritidis SE86 on lettuce leaves. According to Wei et al. (2006), high surface adherence of bacterial populations is a competitive tool against other microorganisms.

The results of *in vitro* resistance to sanitizers and resistance to sanitation methods of lettuce leaves showed that *S.* Enteritidis SE86 with biosurfactant is more resistant to antimicrobial activity of the compounds tested.

The bactericidal action of sodium hypochlorite is the result of microbial cell oxidation, after contact of sanitizer and cells

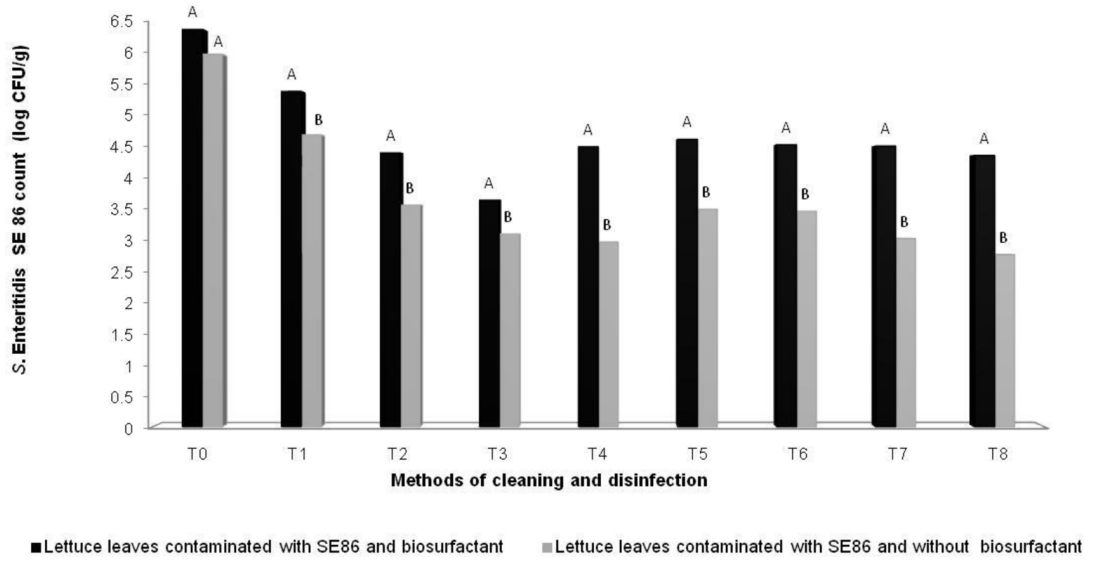


FIGURE 3 | Mean (log CFU/g) of *S.* Enteritidis SE86 on whole lettuce leaves contaminated with *S.* Enteritidis SE86 and biosurfactant (Experiment 1) and *S.* Enteritidis SE86 without biosurfactant (Experiment 2). *Statistical analysis between Experiments 1 and 2. Different letters indicate significant differences ($P < 0.05$). T0: Control positive: lettuce leaves contaminated with *S.* Enteritidis SE86; T1: washing lettuce with potable water; T2: immersion in potable water for 30 min; T3: immersion in 200 ppm of sodium hypochlorite for 15 min; T4: immersion in 200 ppm sodium hypochlorite for 30 min; T5: immersion in 2% vinegar solution for 15 min; T6: immersion in 20% vinegar solution for 15 min; T7: immersion in 50 ppm of sodium hypochlorite for 15 min; T8: immersion in 50ppm of sodium hypochlorite for 30 min.

(Watters et al., 2002; Møretrø et al., 2012; Bermúdez-Aguirre and Barbosa-Cánovas, 2013). According to Møretrø et al. (2012), pH and the presence of organic matter can affect the antimicrobial action of sodium hypochlorite. In our study, it was observed that biosurfactant decreased the antimicrobial action of sodium hypochlorite, probably because this organic compound linked to the sanitizer or avoided the contact of cells with the sanitizer.

TABLE 3 | Reduction (log CFU/g) in counts of *S.* Enteritidis SE86 with and without biosurfactant on whole lettuce leaves after the treatments were performed.

Treatments	Reduction in *S.* Enteritidis SE86 counts on lettuce (log CFU/g) with biosurfactant	Reductions in *S.* Enteritidis SE86 counts on lettuce (log CFU/g) without biosurfactant
Washing with water	1.0^{b}	1.3^{b}
Water (30 min)*	2.0^{c}	2.5^{c}
200 ppm sodium hypochlorite (15 min)*	2.8^{d}	$2.9^{c,d}$
200 ppm sodium hypochlorite (30 min)*	1.9^{c}	$3.1^{c,d}$
2% vinegar solution (15 min)*	1.8^{c}	$2.5^{c,d}$
20% vinegar solution (15 min)*	1.9^{c}	$2.6^{c,d}$
50 ppm sodium hypochlorite (15 min)*	1.9^{c}	$3.0^{c,d}$
50 ppm sodium hypochlorite (30 min)*	2.1^{c}	3.3^{d}

*Log CFU/g: colony forming unit/g converted to $\log_{10}$. *The period of time the solutions spent submerged is shown in parenthesis next to each treatment used. Statistical analysis between treatments. Different letters indicate statistically significant differences between treatments ($P < 0.05$).*

Some studies have shown that acetic acid (vinegar) can reduce the amount of bacteria on foods and surfaces, including whole lettuce leaves (Karapinar and Gonul, 1992; Oliveira et al., 2012). Our study showed that *S.* Enteritidis SE86 in the presence of biosurfactant was more resistant to both vinegar concentrations (solution 2 and 20%). The counts of *S.* Enteritidis SE86 with biosurfactant on whole lettuce leaves sanitized with vinegar solution showed a reduction of 1.8 and 1.9 log CFU/g, whereas the reductions of *S.* Enteritidis SE86 without biosurfactant were 2.5 and 2.6 log CFU/g (**Table 3**).

Vinegars are able to decrease the external and internal pH of cells, inactivating microbial enzymes, and damaging membrane function and metabolic activities such as the transport of nutrients (Chang and Fang, 2007; Ölmez and Kretzschmar, 2009). The less effective antimicrobial action of vinegar solution on lettuce contaminated with *S.* Enteritidis SE86 in the presence of biosurfactant suggested that biosurfactant protected SE86 from contact with the vinegar solution, or this compound was able to neutralize pH action.

The greater resistance of *S.* Enteritidis SE86 in the presence of biosurfactant on lettuce leaves suggests that the biosurfactant production may be a mechanism used by the bacterium to maintain its survival in different environments.

Thus this study demonstrated that *S.* Enteritidis SE86 can use the biosurfactant to increase its adhesion to the surface of lettuce leaves, form lumps, and also to penetrate the stomata of lettuce leaves. These effects may influence the increase of resistance to vinegar and sodium hypochlorite during lettuce sanitization. Furthermore, the surfactant production by adhered cells may protect them, avoiding contact with sanitizers.

CONCLUSION

Based on the results found during this study, it can be concluded that high counts of *S.* Enteritidis SE86 were able to produce biosurfactant on lettuce leaves. The presence of biosurfactant S. Enteritidis SE86 increased the adherence to slices of lettuce leaf and decreased the antimicrobial action of sanitizers (vinegar and sodium hypochlorite) used to sanitize whole lettuce leaves. In addition, when SE86 was added with biosurfactant and was analyzed by scanning electron microscopy, lumps of cells were observed and the bacterium was able to enter the stomata. The same results were not observed in the absence of biosurfactant.

New studies are necessary to investigate other probable functions of biosurfactant produced by SE86. As a perspective of the present study, we suggest investigating the influence of this biosurfactant on the microbial ecology of lettuce leaves, and on the multiplication and survival of SE86 in other foods.

AUTHOR CONTRIBUTIONS

ER: contributes to the development of experimental research, data analysis, and preparation of the article. LB: contributes to the development of experimental research. MK: contributes to the development of experimental research. DS: contributes to the development of experimental research. ET: contributes to the data analysis and preparation of the article.

ACKNOWLEDGMENTS

This research received support from Universidade do Oeste de Santa Catarina, São Miguel do Oeste, Santa Catarina, Brazil, Universidade Federal do Rio Grande do Sul, Brazil and Support Fund for Maintenance and Development of Higher Education (FUMDES) of Santa Catarina, Brazil.

REFERENCES

Abadias, M., Ussal, J., Oliveira, M., Alegre, I., and Vinãs, I. (2008). Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *Int. J. Food Microbiol.* 123, 151–158. doi: 10.1016/j.ijfoodmicro.2007.12.008

Abdel-Mawgoud, A. M., Aboulwafa, M. M., and Hassouna, N. A. H. (2009). Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Appl. Biochem. Biotechnol.* 157, 329–345. doi: 10.1007/s12010-008-8285-1

Abreu, I. M. O., Junqueira, A. M. R., Peixoto, J. R., and Oliveira, S. A. (2010). Qualidade microbiológica e produtividade de alface sob adubação química e orgânica. *Ciênc. Tecnol. Alimentos* 30, 108–118. doi: 10.1590/S0101-20612010000500018

An, Y. H., and Skowronski, P. (2000). "General considerations for studying bacterial adhesion to biomaterials," in *Handbook of Bacterial Adhesion: Principles, Methods and Applications*, eds H. H. An and R. J. Friedman (Berlin: Springer Science and Business Media), 121–132.

Antunes, M. A. (2009). *Contaminação Crescimento e Inativação de Microrganismos na Cadeia de Produção da Alface (Lactuca sativa) Propriedade de Santo Antão*. Ph.D. thesis, Universidade Federal de Viçosa, Minas Gerais.

Ayed, H. B., Hmidet, N., Béchet, M., Chollet, M., Chataigné, G., Jacques, P., et al. (2014). Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. *Process Biochem.* 10, 1699–1707. doi: 10.1016/j.procbio.2014.07.001

Bacic, A., Harris, P. J., and Stone, B. A. (1988). "Structure and function of plant cell walls," in *The Biochemistry of Plants*, eds J. Preiss, W. Stumpf, and P. Michael Conn (Cambridge, MA: Academic Press), 297–373.

Bermúdez-Aguirre, D., and Barbosa-Cánovas, G. V. (2013). Disinfection of selected vegetables under non thermal treatments: chlorine, acid citric, ultraviolet light and ozone. *Food Control* 29, 82–90. doi: 10.1016/j.foodcont.2012.05.073

Brasil (1993). *Ministério da Agricultura, Pecuária e Abastecimento. Portaria n. 101, de 17 de agosto de 1993. Métodos de Análise Microbiológica para Alimentos.* Brasília: Diário Oficial da República Federativa do Brasil, 11937–11945.

Capalonga, R., Ramos, R. C., Both, J. M. C., Soeiro, M. L. T., Longaray, S. M., Haas, S., et al. (2014). Characterization of *Salmonella* Enteritidis involved in foodborne outbreaks in Southern Brazil from 2007 to 2012. *J. Infect. Dev. Ctries.* 8, 811–817.

Chang, J., and Fang, T. J. (2007). Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars Typhimurium in iceberg lettuce and the antimicrobial effect of rice vinegar against *E. coli* O157:H7. *Food Microbiol.* 24, 745–751. doi: 10.1016/j.fm.2007.03.005

Chen, J., Huang, P. T., Zhang, K. Y., and Ding, F. R. (2012). Isolation of biosurfactant producers, optimization and properties of biosurfactant produced by *Acinetobacter* sp. from petroleum-contaminated soil. *J. Appl. Microbiol.* 112, 660–671. doi: 10.1111/j.1365-2672.2012.05242.x

Ciapina, E. M. P. (2008). *Produção de Biossurfactante por Rhodococcus erythropolis em Biorreator de Bancada e Avaliação do seu Efeito na Biodegradação de Borra Oleosa da Indústria do Petróleo*. Ph.D. thesis, Universidade Federal do Rio de Janeiro, Rio de Janeiro.

Cooper, D. G., and Goldenberg, A. B. G. (1987). Surface-active agents from two *Bacillus* species. *Appl. Environ. Microbiol.* 53, 224–229.

Geimba, M. P., Tondo, E. C., de Oliveira, F. A., Canal, C. W., and Brandelli, A. (2004). Serological characterization and prevalence of spvR genes in *Salmonella* isolated from foods involved in outbreaks in Brazil. *J. Food Prot.* 67, 1229–1233.

Hamme, J. D. V., Singh, A., and Ward, O. P. (2006). Physiological aspects: part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnol. Adv.* 24, 604–620. doi: 10.1016/j.biotechadv.2006.08.001

Hassan, A. N., and Frank, J. F. (2003). Influence of surfactant hydrophobicity on the detachment of *Escherichia coli* O157:H7 from lettuce. *Int. J. Food Microbiol.* 87, 145–152. doi: 10.1016/S0168-1605(03)00062-X

Horby, P. W., O'Brien, S. J., Adak, G. K., Graham, C., Hawker, J. I., Hunter, P., et al. (2003). A national outbreak of multi-resistant *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. *Epidemiol. Infect.* 130, 169–178. doi: 10.1017/S0950268802008063

Irvine, W. N., Gillespie, I. A., Smyth, F. B., Rooney, P. J., McClenaghan, A., Devine, M. J., et al. (2009). Investigation of an outbreak of *Salmonella enterica* serovar Newport infection. *Epidemiol. Infect.* 137, 1449–1456. doi: 10.1017/S0950268809002416

Jain, R. M., Mody, K., Joshi, N., Mishra, A., and Jha, B. (2013). Production and structural characterization of biosurfactant produced by an alkaphilic bacterium, *Klebsiella* sp.: evaluation of different carbon sources. *Colloids Surf. B Biointerfaces* 108, 199–204. doi: 10.1016/j.colsurfb.2013.03.002

Jirku, V., Čejková, A., Schreiberová, O., Ježdík, R., and Masák, J. (2015). Multicomponent biosurfactants: a "Green Toolbox" extension. *Biotechnol. Adv.* 33, 1272–1276. doi: 10.1016/j.biotechadv.2015.03.005

Karapinar, M., and Gonul, S. A. (1992). Effects of sodium bicarbonate, vinegar, acetic and citric acids on growth and survival of *Yersinia enterocolitica*. *Int. J. Food Microbiol.* 16, 343–347. doi: 10.1016/0168-1605(92)90036-3

Kroupitski, Y., Pinto, R., Belausov, E., and Sela, S. (2011). Distribution of *Salmonella* typhimurium in romaine lettuce leaves. *Food Microbiol.* 28, 990–997. doi: 10.1016/j.fm.2011.01.007

Kumar, C. G., Joo, S. H., Choi, J. W., Koo, Y. M., and Chang, C. S. (2004). Purification and characterization of an extracellular polysaccharide from haloakalophilic *Bacillus* sp. I-450. *Enzyme Microb. Technol.* 34, 673–681. doi: 10.1016/j.enzmictec.2004.03.001

Lima, P. M., São José, J. P. M., Andrade, N. J., Pires, A. C., and Ferreira, S. O. (2013). Interaction between natural microbiota and physicochemical characteristics of lettuce surfaces can influence the attachment of *Salmonella* Enteritidis. *Food Control* 30, 157–161. doi: 10.1016/j.foodcont.2012.06.039

Machado, T. R. M. (2007). *Avaliação da Aderência ao aço Inoxidável e ao Polietileno por três Sorovares de Salmonella e da Capacidade de Desinfecção*

Dessas Superfícies. Ph.D. dissertations, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, 143.

Machado, T. R. M., Malheiros, P. S., Brandelli, A., and Tondo, E. C. (2010). Avaliação da resistência de *Salmonella* à ação de desinfetantes ácido peracético, quaternário de amônio e hipoclorito de sódio. *Rev. Inst. Adolfo Lutz* 69, 475–481.

Maoa, X., Jianga, R., Xiaoa, W., and Yu, J. (2015). Use of surfactants for the remediation of contaminated soils: a review. *J. Hazard. Mater.* 285, 419–435. doi: 10.1016/j.jhazmat.2014.12.009

Marchant, R., and Banat, I. M. (2012). Biosurfactants: a sustainable replacement for chemical surfactants? *Biotechnol. Lett.* 34, 1597–1605. doi: 10.1007/s10529-012-0956-x

Mellor, G. E., Bentley, J. A., and Dykes, G. A. (2011). Evidence for a role of biosurfactants produced by *Pseudomonas fluorescens* in the spoilage of fresh aerobically stored chicken meat. *Food Microbiol.* 28, 1101–1104. doi: 10.1016/j.fm.2011.02.003

Møretrø, H. E., Live, L., Nesse, L. L., Vestby, L. K., and Langsruda, S. (2012). Control of *Salmonella* in food related environments by chemical disinfection. *Food Res. Int.* 45, 532–544. doi: 10.1016/j.foodres.2011.02.002

Nitschke, M., and Pastore, G. M. (2002). Biossurfactantes: propriedades e aplicações. *Quím. Nova* 25, 772–776. doi: 10.1590/S0100-40422002000500013

Nygard, K., Lassen, J., Vold, L., Andersson, Y., Fisher, I., Lãfdahl, S., et al. (2008). Outbreak of *Salmonella* Thompson infections linked to imported rucola lettuce. *Foodborne Pathog. Dis.* 5, 165–173. doi: 10.1089/fpd.2007.0053

Oliveira, A. B. A., Ritter, A. C., Tondo, E. C., and Cardoso, M. I. (2012). Comparison of different washing and disinfection protocols used by food services in Southern Brazil for lettuce (*Lactuca sativa*). *Food Nutr. Sci.* 3, 28–33. doi: 10.4236/fns.2012.31006

Oliveira, F. A., Geimba, M. P., Pasqualotto, A. P., Brandelli, A., Pasquali, G., Da Silva, W. P., et al. (2009). Clonal relationship among *Salmonella enterica* serovar Enteritidis involved in foodborne outbreaks in Southern Brazil. *Food Control* 20, 606–610. doi: 10.1016/j.foodcont.2008.08.014

Ölmez, H., and Kretzschmar, U. (2009). Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *Food Sci. Technol.* 42, 686–693.

Pacheco, G. J., Ciapina, E. M. P., Gomes, E. B., and Pereira, J. N. (2010). Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. *Braz. J. Microbiol.* 41, 685–693. doi: 10.1590/S1517-83822010000300019

Patel, J., and Sharma, M. (2010). Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *Int. J. Food Microbiol.* 139, 41–47. doi: 10.1016/j.ijfoodmicro.2010.02.005

Perez, K. J., Martins, F. S., Cara, D. C. M., Nicoli, J. R., and Tondo, E. C. (2012). Evaluation of intestinal invasion in germ-free mice challenged with acid-adapted and nonacid-adapted *Salmonella* Enteritidis SE 86 an *Salmonella* Typhimurium ST99. *J. Food Saf.* 32, 108–114. doi: 10.1111/j.1745-4565.2011.00355.x

Ron, E. Z., and Rosenberg, E. (1999). High– and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* 52, 154–162. doi: 10.1007/s002530051502

Ron, E. Z., and Rosenberg, E. (2001). Natural roles of biosurfactants. *Environ. Microbiol.* 3, 229–236. doi: 10.1046/j.1462-2920.2001.00190.x

Rosa, C. F. C., Freire, D. M. G., and Ferraz, H. C. (2015). Biosurfactant microfoam: application in the removal of pollutants from soil. *J. Environ. Chem. Eng.* 3, 89–94. doi: 10.1016/j.jece.2014.12.008

Sagong, H. G., Lee, S. Y., Chang, P. S., Heu, S., Ryu, S., Choi, Y. J., et al. (2011). Combined effect of ultrasound and organic acids to reduce *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* on organic fresh lettuce. *Int. J. Food Microbiol.* 145, 287–292. doi: 10.1016/j.ijfoodmicro.2011.01.010

Sagoo, S. K., Little, C. L., Ward, L., Gillespie, I. A., and Mitchell, R. T. (2003). Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *J. Food Prot.* 66, 403–409.

Shaheen, R., Svensson, B., Andersson, M. A., Cristiansson, A., and Salkinoja-Salomen, M. (2010). Persistence strategies of *Bacillus* cereus spores isolated from dairy silo tanks. *Food Microbiol.* 27, 347–355. doi: 10.1016/j.fm.2009.11.004

Sinde, E., and Carballo, J. (2000). Attachment of *Salmonella* sp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiol.* 17, 439–447. doi: 10.1006/FMIC.2000.0339

Sotirova, A., and Vasileva-Tonkova, E. (2009). Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*. *Microbiol. Res.* 164, 297–303. doi: 10.1016/j.micres.2007.01.005

Takkinen, J., Nakari, U., Johansson, T., Niskanen, T., Siitonen, A., and Kuusi, M. A. (2005). Nationwide outbreak of multiresistant *Salmonella* Typhimurium var Copenhagen DT104B infection in Finland due to contaminated lettuce from Spain, May 2005. *Euro Surveill.* 10, 26.

Tondo, E. C., and Ritter, A. C. (2012). *Salmonella and Salmonellosis in Southern Brazil: A Review of Last Decade Salmonella: Classification, Genetics and Disease Outbreaks*. New York, NY: Nova Science Publishers.

Tondo, E. C., Ritter, A. C., and Casarin, L. S. (2015). "Involvement in foodborne outbreaks, risk factors and options to control *Salmonella* Enteritidis SE86: an important food pathogen in Southern Brazil," in *Salmonella Prevalence, Risk Factors and Treatment Options*, ed. C. B. Hackett (Nova York: Nova Publishers), 65–77.

Watters, L. H., Chinnan, M. S., Walker, S. L., Doyle, M. P., and Lin, C. M. (2002). Consumer acceptance of fresh-cut iceberg lettuce treated with 2% hydrogen peroxide and mild heat. *J. Food Prot.* 65, 1221–1226.

Wei, H., Wolf, G., and Hammes, W. P. (2006). Indigenous microorganisms from iceberg lettuce with adherence and antagonistic potential for use as protective culture. *Innov. Food Sci. Emerg. Technol.* 7, 294–301. doi: 10.1016/j.ifset.2006.02.002

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 December 2015
doi: 10.3389/fmicb.2015.01448

Engineering Bacterial Surface Displayed Human Norovirus Capsid Proteins: A Novel System to Explore Interaction Between Norovirus and Ligands

Mengya Niu[1], Qianqian Yu[1], Peng Tian[2], Zhiyong Gao[3], Dapeng Wang[1] and Xianming Shi[1]*

[1] Department of Food Science and Technology, MOST-USDA Joint Research Center for Food Safety, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China, [2] Produce Safety and Microbiology Research Unit, Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Albany, CA, USA, [3] Beijing Center for Diseases Prevention and Control, Beijing, China

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Tineke H. Jones, Agriculture and Agri-Food Canada, Canada
Miriam Berti, Istituto Zooprofilattico Sperimentale Abruzzo e Molise, Italy

***Correspondence:**
Dapeng Wang
norovirus@163.com

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

Received: *10 October 2015*
Accepted: *04 December 2015*
Published: *22 December 2015*

Citation:
Niu M, Yu Q, Tian P, Gao Z, Wang D and Shi X (2015) Engineering Bacterial Surface Displayed Human Norovirus Capsid Proteins: A Novel System to Explore Interaction Between Norovirus and Ligands. Front. Microbiol. 6:1448. doi: 10.3389/fmicb.2015.01448

Human noroviruses (HuNoVs) are major contributors to acute nonbacterial gastroenteritis outbreaks. Many aspects of HuNoVs are poorly understood due to both the current inability to culture HuNoVs, and the lack of efficient small animal models. Surrogates for HuNoVs, such as recombinant viral like particles (VLPs) expressed in eukaryotic system or P particles expressed in prokaryotic system, have been used for studies in immunology and interaction between the virus and its receptors. However, it is difficult to use VLPs or P particles to collect or isolate potential ligands binding to these recombinant capsid proteins. In this study, a new strategy was used to collect HuNoVs binding ligands through the use of ice nucleation protein (INP) to display recombinant capsid proteins of HuNoVs on bacterial surfaces. The viral protein-ligand complex could be easily separated by a low speed centrifugation step. This system was also used to explore interaction between recombinant capsid proteins of HuNoVs and their receptors. In this system, the VP1 capsid encoding gene (ORF2) and the protruding domain (P domain) encoding gene (3′ terminal fragment of ORF2) of HuNoVs GI.1 and GII.4 were fused with 5′ terminal fragment of INP encoding gene (*inaQn*). The results demonstrated that the recombinant VP1 and P domains of HuNoVs were expressed and anchored on the surface of *Escherichia coli* BL21 cells after the bacteria were transformed with the corresponding plasmids. Both cell surface displayed VP1 and P domains could be recognized by HuNoVs specific antibodies and interact with the viral histo-blood group antigens receptors. In both cases, displayed P domains had better binding abilities than VP1. This new strategy of using displayed HuNoVs capsid proteins on the bacterial surface could be utilized to separate HuNoVs binding components from complex samples, to investigate interaction between the virus and its receptors, as well as to develop an oral vaccine for HuNoVs.

Keywords: human noroviruses, cell surface display, receptor, ice nucleation protein, histo-blood group antigens

INTRODUCTION

Human noroviruses (HuNoVs) are one of major nonbacterial pathogens for foodborne gastroenteritis (Atmar and Estes, 2006). HuNoVs belong to the family of *Caliciviridae*. The single-stranded, positive-sensed RNA viral genome can be divided into three open reading frames (ORFs) (Jiang et al., 1993). The ORF2 encodes the major structural protein (VP1) which have two domains: the shell (S) and the protruding (P) domain (Zheng et al., 2006). The viral capsid is composed of 180 capsid protein monomers organized into 90 dimers (Tan et al., 2008).

Due to a lack of cultivable system or animal models, surrogate viruses such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV) are often used to study the fundamental biology of the viruses such as viral replication pattern and mechanism of infection (Farkas, 2015). Recombinant human norovirus capsid proteins expressed either by eukaryotic or prokaryotic systems are often used for studies for immunogenicity, diagnosis assays, and host-receptor interaction (Gray et al., 1993; Green et al., 1993; Hutson et al., 2003; Huang et al., 2005). The virus-like particles (VLPs), spontaneously formed in a recombinant baculovirus system, have morphological and antigenical similarities to viral particles (Jiang et al., 1992). However, large production of recombinant proteins is still difficult owing to low protein yield and lack of VLPs. Tan et al. applied *E. coli* expression system to produce recombinant norovirus capsid proteins (Tan et al., 2004). They demonstrated that the *E. coli*-expressed capsid proteins maintained the same antigenicity and receptor binding specificity as those of the baculovirus-expressed VLPs, although the *E. coli*-expressed capsid proteins did not form VLPs. Tan et al. further demonstrated that a smaller particle (P particle) could form, which was expressed in *E. coli* (Tan et al., 2004). The P particles expressed *in vitro* is an octahedral nanoparticle formed by 24 copies of P monomers, most likely organized into 12 P dimers. These P particles are easily produced in *E. coli*, extremely stable, and highly immunogenic (Tan and Jiang, 2005b). However, one disadvantage of the bacterial system for expression of recombinant HuNoVs capsid proteins is that the expressed capsid proteins cannot be purified easily. After the fusion tag is removed, gel filtration and anion-exchange chromatography are required for purification of recombinant HuNoVs capsid proteins (Tan et al., 2004). Another disadvantage is that VP1 and P particles expressed by bacteria are either soluble proteins (VP1) or small particles. Therefore, it will be hard to either collect or isolate the viral capsid protein-ligand complex.

Bacterial ice nucleation proteins (INPs) are a family of proteins that enable Gram-negative bacteria to promote crystal formation at relatively high temperatures (Kawahara, 2002). Different INP coding genes from *Pseudomonas syringae*, *Erwinia herbicola*, and *Xanthomonas campestris* were well characterized (Wolber et al., 1986; Schmid et al., 1997; Jung et al., 1998a; Li et al., 2012). INP composes three distinct structural domains: an N-terminal domain, a C-terminal domain and a highly repetitive central domain (Shimazu et al., 2001). So far, INPs have been applied in various perfect bacterial cell surface display systems, including host cells of *E. coli* (Jung et al., 1998b; Kwak et al., 1999; Li et al., 2009), *Salmonella typhi* (Lee et al., 2000), *Vibrio anguillarum* (Xu et al., 2008), *P. syringae* (Shimazu et al., 2003). By transformation of bacteria with the gene encoding a fusion target protein with the anchoring motifs of INP, the target protein could be directly displayed on the surface of the bacteria (Kim and Yoo, 1999; Kwak et al., 1999; Cochet and Widehem, 2000). It was reported that the N-terminal domain of InaQ (named as InaQN) is responsible for the transmembrane transport and membrane-binding activity of INP (Li et al., 2012).

In order to solve the problem of collecting ligands binding to viral capsid proteins, recombinant HuNoVs capsid proteins were displayed on the surface of bacteria with the help of InaQN. It was reported that histo-blood group antigens (HBGAs) have been recognized as receptors for HuNoVs (Hutson et al., 2003; Tan and Jiang, 2005a). Therefore, in this study, Type III porcine gastric mucin (PGM) containing HBGAs (Tian et al., 2008) was used to evaluate the binding efficacy between the viral receptors and displayed HuNoVs VP1s and P domains.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli DH5α and BL21 (ThermoFisher, Shanghai, China) were used as competent cells for recombinant plasmid construction and protein expression. Plasmid pMD19-T (TaKaRa, Dalian, China) inserted with different gene fragments was used for subcloning into the prokaryotic expression plasmid pET-28a (ThermoFisher, Shanghai, China). pCR-TORO/GI.1-ORF2+3 plasmid with inserted gene of HuNoV GI.1 ORF2 was kindly provided by Dr. Peng Tian (PSMRU, WRRC, USDA, CA, USA). pTrc-HisC-inaQ plasmid was kindly provided by Prof. Lin Li (State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China).

Cloning of *inaQn*, HuNoVs GI.1/GII.4 ORF2 and P Domain Coding Fragments

The coding sequence of InaQN domain (*inaQn*) was subcloned from plasmid pTrc-HisC-inaQ according to the previous report (Li et al., 2012).

HuNoV GI.1 ORF2 and its 3′ terminal fragments (named P) were amplified from recombinant plasmid pCR-TORO/GI.1-ORF2+3. A 1584 bp fragment for HuNoV GI.1 ORF2 (GenBank No. M87661) and a 909 bp fragment for HuNoV GI.1 ORF2 3′ terminal were amplified by PCR. Each 20.0 μL PCR reaction including 2.0 μL 10 × PCR Buffer, 1.5 μL 25 mmol/L Mg^{2+}, 1 U *Taq* E (ThemoFisher, Shanghai, China), 1.0 μL 10 mmol/L dNTPs, 1.0 μL 10 mmol/L of upstream and downstream primers respectively, 2.0 μL plasmid and double distilled water (dd H_2O). Thermal cycling condition consists of initial denaturation at 95°C for 5 min, 35 cycles of template denaturation at 95°C for 30 s, primer annealing at 52°C for 30 s, primer extension at 72°C for 1 min 40 s, and final extension at 72°C for 10 min.

Genomic RNA of HuNoV GII.4 strain was extracted by Trizol Kit (ThemoFisher, Shanghai, China) from clinical gastroenteritis samples provided by Beijing Center for Disease Control and Prevention, China. A 1623 bp fragment (GenBank No.

TABLE 1 | Sequences of (RT-) PCR primers.

Gene fragment	Primer	Restriction enzyme site	Sequence(5′-3′)	Amplicon size (bp)
GI.1 ORF2	GI.1F	*Bgl* II	AGATCTATGATGATGGCGTCTAAGG	1584
	GI.1R	*Hin*d III	AAGCTTACAGACCAAGCCTACCTC	
GI.1 P domain	GI.1 P-F	*Bgl* II	AGATCTCAGAAAACCAGGCCCTTC	909
	GI.1 P-R	*Hin*d III	AAGCTTCTAAAGCCAAGCCTTACG	
GII.4 ORF2	GII.4F	*Bgl* II	GGAAGATCTATGAAGATGGCGTCG	1623
	GII.4R	*Eco*R I	CCGGAATTCTTATAAAGCACGTCTG	
GII.4 P domain	GII.4 P-F	*Bgl* II	AGATCTTCAAGAACTAAACCATTCTC	948
	GII.4 P-R	*Eco*R I	GAATTCTTATAGTGCACGCCTACGCC	

TABLE 2 | Recombinant plasmids with restriction enzyme sites.

Recombinant plasmids	Restriction enzyme sites	The length of nucleic acid fragments (bp)
pET28a-1	*Nco* I & *Hin*d III	5246
pET28a-2	*Nco* I & *Eco*R I	5265
pMD19-ORF2(GI.1)	*Bgl* II & *Hin*d III	1584 (GI.1 ORF2)
pMD19-ORF2(GII.4)	*Bgl* II & *Eco*R I	1623 (GII.4 ORF2)
pMD19-P(GI.1)	*Bgl* II & *Hin*d III	909 (GI.1 P domain)
pMD19-P(GII.4)	*Bgl* II & *Eco*R I	948 (GII.4 P domain)
prTc-His C-*inaQ*	*Nco* I & *Bgl* II	525 (InaQN domain)

KM114291) for HuNoV GII.4 ORF2 was amplified by two-step RT-PCR kit (TaKaRa, Dalian, China) in accordance with manufacturer's protocol. The 3′ terminal fragment (948 bp) of ORF2 was amplified according to previous reports (Wang et al., 2008).

All primers used in this study were listed in **Table 1**. PCR products were analyzed by electrophoresis on 1.5% agarose gels and target bands were recycled using AxyPrep DNA gel extraction kit (Corning, Shanghai, China). The recovered nucleic acid fragments were inserted into plasmid pMD19-T, named pMD19-ORF2(GI.1), pMD19-ORF2(GII.4), pMD19-P(GI.1), and pMD19-P(GII.4), respectively. The recombinant plasmids were sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd.

After restriction enzymes digestion (**Table 2**), *inaQn* fragment and ORF2 or ORF2 3′ terminal fragments from GI.1 or GII.4 were inserted into pET-28a to create recombinant plasmids pET28a-inaQn-ORF2(GI.1), pET28a-inaQn-ORF2(GII.4), pET28a-inaQn-P(GI.1), and pET28a-inaQn-P(GII.4), respectively. In addition, pET28a-ORF2(GI.1), pET28a-ORF2(GII.4), pET28a-P(GI.1), and pET28a-P(GII.4) without the *inaQn* gene were also constructed as negative controls. All recombinant plasmids were confirmed by sequencing by Shanghai Majorbio Bio-pharm Technology Co., Ltd.

Expression of Recombinant Plasmids

Recombinant *E. coli* BL21 strains were transformed with different recombinant plasmids, including pET28a-inaQn-ORF2(GI.1), pET28a-inaQn-ORF2(GII.4), pET28a-inaQn-P(GI.1), and pET28a-inaQn-P(GII.4), then cultured in Luria-Bertani (LB) (0.5% yeast extract, 1% trypton, and 1% NaCl) liquid medium containing 100 μg/mL kanamycin with shaking (150 rpm) at 37°C overnight. The transformed BL21 cells (50 μL) were transferred to 5 mL fresh LB medium (100 μg/mL kanamycin) with shaking (150 rpm) at 37°C. When the culture reached to optical density in 600 nm (OD_{600}) of around 0.6, 0.4 mmol/L (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cells were incubated at 16°C for 24 h with shaking (120 rpm). The cells were kept at 4°C for further use.

Preparation of Antibodies for VP1 of HuNoVs GI.1 and GII.4

Expression of Recombinant Capsid Proteins in *E. coli*

E. coli BL21 with recombinant plasmids pET28a-ORF2 (GI.1) and pET28a-ORF2 (GII.4) were grown in LB medium with 100 μg/mL kanamycin with shaking (150 rpm) at 37°C overnight. The next day, the cells (2 mL) were subcultured in fresh LB (200 mL) with 100 μg/mL kanamycin with shaking (150 rpm) at 37°C. The recombinant plasmids were expressed in *E. coli* BL21 at 37°C for 4 h after induction by 0.4 mmol/L IPTG. All induced cells were collected for further use.

Purification of Recombinant Protein and Preparation of Specific Antibodies

Recombinant capsid protein was purified according to previous report with minor modifications (Wang et al., 2008). Briefly, the cells expressing viral capsid proteins at the condition described in Section Expression of Recombinant Capsid Proteins in *E. coli* were washed 3 times using PBS (pH 7.2, NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 2 mmol/L). The cells were resuspended in 20 mL Buffer A [pH 8.0, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 50 mmol/L NaCl, 5% glycerin (v/v), 0.5% Triton X-100 (v/v)] with 100 μg/mL lysozyme (Sigma, St. Louis, USA). After incubation at 37°C for 30 min, cells were subjected to ultrasonic treatment (BILON92-II, Shanghai, China) under the condition of 6 s with 4 s interval for 90 cycles on the ice. Then cell lysate was centrifuged at 10,000 × g for 15 min at 4°C. The precipitate (crude inclusion body) was washed by 10 mL Buffer A. Twenty milliliters lysis buffer (pH 9.0, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 50 mmol/L NaCl, 5% glycerin (v/v), 0.5% Triton X-100 (v/v), and 8 mol/L urea) was added to

resuspend the precipitate and then resolved slowly on the ice for 2 h. After centrifugation at 10,000 × g at 4°C for 30 min, the supernatant was dialyzed in TE buffer (pH 8.0, 10 mmol/L Tris-HCl, 1 mmol/L EDTA) at 4°C for 12 h. The TE buffer was then replaced and continued second dialysis. After dialysis, the supernatant was centrifuged at 10,000 × g at 4°C for 30 min. The recombinant protein in supernatant was analyzed by SDS-PAGE according to our previous report (Wang et al., 2008).

Preparation of Primary Antibody Against VP1

Ten female Balb/c mice (Hubei Provincial Center for Disease Control and Prevention, China), 5–6 weeks old, were immunized subcutaneously with 0.1 mg recombinant VP1 of HuNoV GI.1 or GII.4 every 2 weeks respectively. The sera were collected and used as primary antibodies against the recombinant viral capsid proteins (VP1 and P particles) after 3 months of immunization. The sera were stored at −80°C for further use.

Ethics Statement

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Institutional Animal Care and Use Committee. The protocol was approved by Laboratory Animal Feeding Standard Operation Procedure (SOP-Ani-019-2012), the Institutional Animal Care and Use Committee, and the Laboratory Animal Center of Shanghai Jiao Tong University (Permit Number: A2015020).

Whole Cell Enzyme Immunoassay (EIA)

A modified sandwich EIA was developed to evaluate the recombinant VP1 or P domains display efficiency on the surface of host cells with or without InaQN. Briefly, the transformed cells with recombinant plasmids pET28a-ORF2 (GI.1), pET28a-ORF2 (GII.4), pET28a-P (GI.1), pET28a-P (GII.4) and pET28a-inaQn-ORF2(GI.1), pET28a-inaQn-P(GI.1), pET28a-inaQn-ORF2(GII.4), pET28a-inaQn-P(GII.4), and plasmid pET28a as control were collected after induction of viral protein expression as described in previous Section Expression of Recombinant Plasmids by centrifugation at 5000 × g for 5 min. The pellet was washed with PBS (pH 7.2) twice and adjusted OD_{600} to around 1.0 in 500 μL PBS in a 1.5 mL Eppendorf tube. After centrifugation at 5000 × g for 5 min, the pellet was resuspended with 500 μL primary antibodies against VP1 obtained from Section Preparation of Primary Antibody Against VP1 (1:10,000 diluted in Tris-Buffered Saline and Tween-20 with 1% bovine serum albumin) and incubated for 30 min at 37°C. After washing with Tris-Buffered Saline and Tween-20 (TBST) for three times, the bacterial pellet was resuspended with 500 μL Peroxidase-Conjugated Goat Anti-Mouse IgG (H+L) (Yeasen, Shanghai, China) at a dilution of 1:5000 and incubated for 30 min at 37°C. After three washes with TBST, the bacterial pellet was resuspended in 500 μL PBS. Finally, a 50 μL of suspension was taken and mixed with 50 μL 3,3′,5,5′-tetramethylbenzidine [Friendbio Science and Technology (Wuhan) Co., Ltd., Hubei, China]. After keeping in the dark for 10 min, 50 μL of mixture was transferred to 96 well module (Lantian Biological Equipment Factory, Jiangsu, China), and 50 μL 2 mol/L H_2SO_4 was added to stop the reaction. OD_{450} value was measured using a Sunrise Microplate Reader (Tecan Sunrise, Switzerland). In addition, bacteria transformed with recombinant plasmids without *inaQn* gene including pET28a-ORF2 (GI.1), pET28a-ORF2 (GII.4), pET28a-P (GI.1), and pET28a-P (GII.4) were also tested.

Measuring the HBGAs Binding Abilities

Type III PGM purchased from Sigma (St, Louis, MI, USA) was used as viral receptor. Each well of the Nunc Immuno Module (VWR, CA, USA) was coated with 100 μL of PGM (1 mg/mL in 0.05 mol/L carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight. After being washed with PBS (pH 7.2) for 3 times, the wells were blocked with 120 μL of 1% bovine serum albumin (BSA) in PBS at 37°C for 1 h. The wells were washed with PBS (pH 7.2) for 3 times and used immediately.

The transformed bacteria were cultured as described in Section Expression of Recombinant Plasmids. The cells with recombinant plasmids pET28a-inaQn-ORF2(GI.1), pET28a-inaQn-P(GI.1), pET28a-inaQn-ORF2(GII.4), pET28a-inaQn-P(GII.4) were collected by centrifugation at 3000 × g for 5 min and diluted to 10^2 or 10^3 CFU/mL in PBS. The diluted cells (100 μL per well) were added into 48 wells, half of which were coated with PGM, and the remainder was not coated with PGM. After incubation at 37°C for 30 min, 10 μL suspension from each well was taken onto the LB agar plate containing 100 μg/mL kanamycin for overnight at 37°C. After incubation, the colonies were counted. In addition, recombinant plasmids without *inaQn* gene were constructed as a control including pET28a-ORF2 (GI.1), pET28a-ORF2 (GII.4), pET28a-P (GI.1), and pET28a-P (GII.4). The binding ratio was calculated by comparing the difference of colony forming units (CFU) before and after binding with or without PGM.

$$\begin{aligned} \text{Binding ratio} &= n/N \times 100\% \\ n &= (\text{CFU in well without PGM}) \\ &\quad -(\text{CFU in well with PGM}) \\ N &= \text{CFU in well without PGM} \end{aligned}$$

Statistical Analysis

IBM SPSS Statistics Software (version 19) was used for analyzing statistics. Each experiment was repeated at least three times ($N = 3$) as independent replicates in triplicates within each experiment ($n = 3$). One-way ANOVA was utilized for data analysis. Differences in means were considered significant when the $p < 0.05$.

RESULTS

The Fusion Viral Proteins Expressed on the Surface of Transformed Cells

A modified sandwich EIA was developed to evaluate the recombinant VP1 or P domains display efficiency on the surface of host cells with or without InaQN (**Figure 1**). The results indicated the fusion protein could display on the surface of bacterial cells and maintain the antigenicity of HuNoVs (**Figure 1**). The absorbance was significantly higher in bacteria

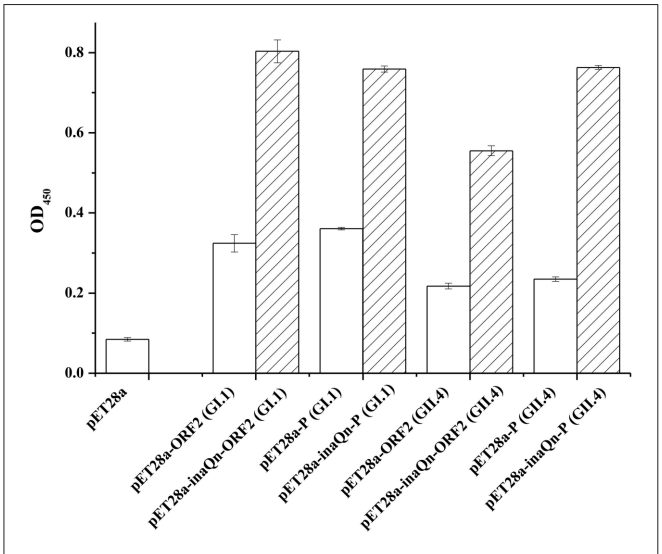


FIGURE 1 | Absorbance of 450 nm (OD_{450}) in whole cell EIA assay. The whole cell EIA results of recombinant HuNoVs VP1/P domains (□) and recombinant InaQN-HuNoVs VP1/P domains (▨). Bars represent standard error.

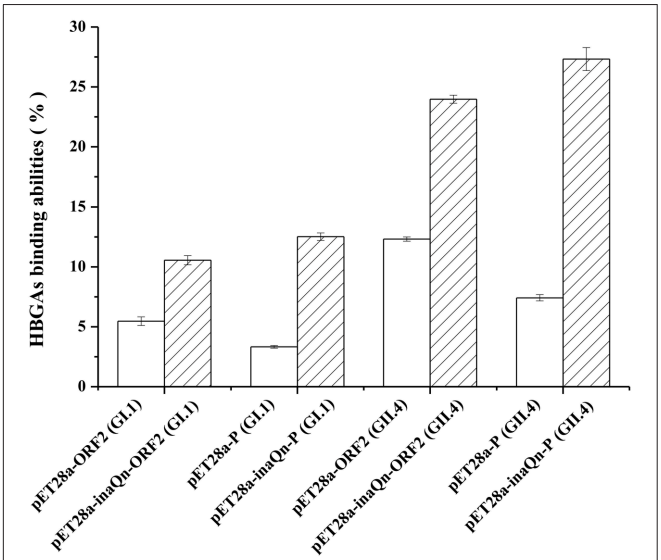


FIGURE 2 | HBGAs binding assay. HBGAs binding abilities of *E. coli* with recombinant HuNoVs VP1/P domains (□) expression and recombinant InaQN-HuNoVs VP1/P domains expression (▨). Bars represent standard error.

transformed with plasmids of *inaQn* gene fused with ORF2 or P domain coding gene than that of plasmids of ORF2 or P domain coding gene without *inaQn* gene ($p < 0.01$). The P/N (Positive result/Negative result) ratio of pET28a-inaQn-ORF2 (GI.1), pET28a-inaQn-P (GI.1), pET28a-inaQn-ORF2 (GII.4), and pET28a-inaQn-P (GII.4) were 9.45, 8.93, 6.53, and 8.98 respectively. Without the *inaQn*, the P/N ratio of pET28a-ORF2 (GI.1), pET28a-P (GI.1), pET28a-ORF2 (GII.4), and pET28a-P (GII.4) were 3.81, 4.25, 2.55, and 2.77 respectively. The presence of *inaQn* gene significantly increased display of HuNoVs capsid proteins on the surface of the transformed bacterial cells. There was no significant difference in signals of displayed VP1 and P domains of GI.1 transformed bacteria ($p > 0.05$). However, the signals of displayed P domains were significantly higher than VP1 in GII.4 transformed bacteria ($p < 0.01$). No significant difference was found among pET28a-inaQn-ORF2 (GI.1) ($OD_{450} = 0.803 \pm 0.029$), pET28a-inaQn-P (GI.1) ($OD_{450} = 0.759 \pm 0.008$), and pET28a-inaQn-P (GII.4) ($OD_{450} = 0.763 \pm 0.005$) ($p > 0.05$). However, absorbance signal of pET28a-inaQn-ORF2 (GII.4) ($OD_{450} = 0.555 \pm 0.012$) was significantly lower than the residual groups ($p < 0.01$).

Binding of HBGAs with Surface Displayed VP1 and P Domains

To identify the HBGAs receptor-binding capacity, *E. coli* BL21 cells were transformed with plasmids pET-28a, pET28a-ORF2 (GI.1), pET28a-inaQn-ORF2 (GI.1), pET28a-P (GI.1), pET28a-inaQn-P (GI.1), pET28a-ORF2 (GII.4), pET28a-inaQn-ORF2 (GII.4), pET28a-P (GII.4), and pET28a-inaQn-P (GII.4). The HBGAs binding capacity was evaluated by colony counting assay. When bacteria transformed with HuNoVs capsid protein encoding genes were added to PGM coated wells, the binding capacity between the HBGAs in PGM and displayed VP1 or P domains was determined by comparing the binding ratio of CFU in wells with or without PGM (**Figure 2**). The HBGAs binding capacity was $10.5 \pm 0.4\%$, $12.5 \pm 0.3\%$, $24.0 \pm 0.3\%$, and $27.3 \pm 0.9\%$ in bacteria transformed with pET28a-inaQn-ORF2 (GI.1), pET28a-inaQn-P (GI.1), pET28a-inaQn-ORF2 (GII.4), and pET28a-inaQn-P (GII.4), respectively. The HBGAs binding capacity was $5.5 \pm 0.4\%$, $3.3 \pm 0.1\%$, $12.3 \pm 0.2\%$, and $7.4 \pm 0.3\%$ in bacteria transformed with pET28a-ORF2 (GI.1), pET28a-P (GI.1), pET28a-ORF2 (GII.4), and pET28a-P (GII.4) without *inaQn*, respectively. Therefore, the HBGAs binding capacity of InaQN fused VP1s and P particles was significantly higher than groups without InaQN ($p < 0.01$). The HBGAs binding capacities of displayed P domains from both genotypes are slightly higher than displayed VP1. However, the HBGAs binding capacities of both displayed VP1 and P domains of GI.1 were significantly lower than that of GII.4.

DISCUSSION

In this study, cell surface display systems for HuNoVs capsid protein VP1 and P domains were constructed using InaQN as a carrier protein. To our knowledge, it is the first report on HuNoVs displayed the surface of transformed bacteria. Our results indicated that displayed VP1 or P domains of HuNoVs were successfully anchored on the surface of bacterial cells. The surface displayed VP1 or P domains maintained the antigenicity (**Figure 1**). The cell surface display using INP has been used in vaccine development. For example, a recombinant oral vaccine for hepatitis C has been tested (Lee et al., 2000). Therefore, this system could be a good candidate for vaccine development. Currently, we are in the process of using these cell surface displayed VP1 and P domains for vaccine candidate for HuNoVs.

The HBGAs have been recognized as receptors or co-receptors for HuNoVs (Huang et al., 2003). In this study, we demonstrated that the displayed viral proteins could interact with the HBGAs viral receptors (**Figure 2**). The HBGAs binding capacity of the displayed VP1 and P domains from GII.4 were significantly higher than displayed VP1 and P domains from GI.1, indicating GII.4 P domains or VP1 may fold more perfectly and be easily recognized by viral receptors than GI.1 P domains or VP1. Recently, sialic acids have been identified as additional cellular receptors/co-receptor for Tulane virus (Tan et al., 2015) and for HuNoVs (Rydell et al., 2009). It is possible that other receptors or ligands for HuNoVs have not been identified. VLP and P particles are limited for valuing in collection or isolation of HuNoVs receptors or ligands. VLP and P particles could not be used to get viral particle-ligand complex unless ultracentrifugation is applied. It is not practical to collect or isolate a viral particle-ligand complex by ultracentrifugation due to the small sample size. In this study, the results demonstrated that the viral capsid protein-receptor complex could be easily collected by low speed centrifugation. The cell surface display system we described in this paper could be an alternative method to replace the ultracentrifugation to collect the viral capsid binding ligands.

There was some background binding in bacteria expressing the viral capsid proteins (VP1 and P domains) without the *inaQn* gene. The background noise could be a result of non-specific binding of the viral capsid proteins on bacteria or nonspecific binding of the antibodies to bacteria as the antibodies were made from viral capsid proteins, which expressed in *E. coli* BL21. The background should be reduced significantly when the targets are viral receptors or ligands. Currently, we are in the process of isolating candidate ligands for HuNoVs using this approach.

This new strategy of using cell surface displayed HuNoVs capsid proteins will provide a new approach to isolate HuNoVs ligands, a new approach to characterize interaction between HuNoVs and receptors or ligands, a faster way to construct VP1 or P domains from a new strain or mutated strains. Since a couple of viral antigens displayed on the bacterial surface using INP have been proved to be an attractive platform for production of vaccine (Kim and Yoo, 1999; Kwak et al., 1999; Lee et al., 2000) and *E. coli*-expressed norovirus P particle could be a platform for antibody production (Tan et al., 2011), we believe that cell surface displayed HuNoVs capsid proteins can also be a good candidate for vaccine development.

AUTHOR CONTRIBUTIONS

DW and MN designed the experiments. MN, QY, and DW carried out experiments. MN, DW, and XS analyzed sequencing data and experimental results. ZG provided HuNoV GII.4 clinical sample and analyzed the viral RNA sequence. PT provided plasmid pCR-TORO/GI.1-ORF2+3. MN, DW, and PT wrote and modified the manuscript. XS provided laboratory equipment and place.

ACKNOWLEDGMENTS

This work was jointly supported by the grant No. 2012AA101601 from the Ministry of Science and Technology of China and the grant No. 31301475 from the National Natural Science Foundation of China. We want to thank Prof. Lin Li kindly provided plasmid pTrc-HisC-inaQ (State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China).

REFERENCES

Atmar, R. L., and Estes, M. K. (2006). The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. North Am.* 35, 275–290. doi: 10.1016/j.gtc.2006.03.001

Cochet, N., and Widehem, P. (2000). Ice crystallization by *Pseudomonas syringae*. *Appl. Microbiol. Biotechnol.* 54, 153–161. doi: 10.1007/s002530000377

Farkas, T. (2015). Rhesus enteric calicivirus surrogate model for human norovirus gastroenteritis. *J. Gen. Virol.* 96, 1504–1514. doi: 10.1099/jgv.0.000020

Gray, J. J., Jiang, X., Morgan-Capner, P., Desselberger, U., and Estes, M. K. (1993). Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. *J. Clin. Microbiol.* 31, 1022–1025.

Green, K., Lew, J., Jiang, X., Kapikian, A., and Estes, M. K. (1993). Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J. Clin. Microbiol.* 31, 2185–2191.

Huang, P., Farkas, T., Marionneau, S., Zhong, W., Ruvoën-Clouet, N., Morrow, A. L., et al. (2003). Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *J. Infect. Dis.* 188, 19–31. doi: 10.1086/375742

Huang, P., Farkas, T., Zhong, W., Tan, M., Thornton, S., Morrow, A. L., et al. (2005). Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. *J. Virol.* 79, 6714–6722. doi: 10.1128/JVI.79.11.6714-6722.2005

Hutson, A. M., Atmar, R. L., Marcus, D. M., and Estes, M. K. (2003). Norwalk virus-like particle hemagglutination by binding to H histo-blood group antigens. *J. Virol.* 77, 405–415. doi: 10.1128/JVI.77.1.405-415.2003

Jiang, X., Wang, M., Graham, D. Y., and Estes, M. K. (1992). Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* 66, 6527–6532.

Jiang, X., Wang, M., Wang, K., and Estes, M. K. (1993). Sequence and genomic organization of Norwalk virus. *Virology* 195, 51–61. doi: 10.1006/viro.1993.1345

Jung, H. C., Lebeault, J. M., and Pan, J. G. (1998a). Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. *Nat. Biotechnol.* 16, 576–580. doi: 10.1038/nbt0698-576

Jung, H. C., Park, J. H., Park, S. H., Lebeault, J. M., and Pan, J. G. (1998b). Expression of carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein. *Enzyme Microb. Technol.* 22, 348–354. doi: 10.1016/S0141-0229(97)00224-X

Kawahara, H. (2002). The structures and functions of ice crystal-controlling proteins from bacteria. *J. Biosci. Bioeng.* 94, 492–496. doi: 10.1016/S1389-1723(02)80185-2

Kim, E. J., and Yoo, S. K. (1999). Cell surface display of hepatitis B virus surface antigen by using *Pseudomonas syringae* ice nucleation protein. *Lett. Appl. Microbiol.* 29, 292–297. doi: 10.1046/j.1365-2672.1999.00612.x

Kwak, Y. D., Yoo, S. K., and Kim, E. J. (1999). Cell surface display of human immunodeficiency virus type 1 gp120 on *Escherichia coli* by using ice nucleation protein. *Clin. Diagn. Lab. Immunol.* 6, 499–503.

Lee, J. S., Shin, K. S., Pan, J. G., and Kim, C. J. (2000). Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat. Biotechnol.* 18, 645–648. doi: 10.1038/76494

Li, Q. Q., Yan, Q., Chen, J., He, Y., Wang, J., Zhang, H., et al. (2012). Molecular characterization of an ice nucleation protein variant (inaQ) from *Pseudomonas syringae* and the analysis of its transmembrane transport activity in *Escherichia coli*. *Int. J. Biol. Sci.* 8, 1097–1108. doi: 10.7150/ijbs.4524

Li, Q. Q., Yu, Z., Shao, X., He, J., and Li, L. (2009). Improved phosphate biosorption by bacterial surface display of phosphate-binding protein utilizing ice nucleation protein. *FEMS Microbiol. Lett.* 299, 44–52. doi: 10.1111/j.1574-6968.2009.01724.x

Rydell, G. E., Nilsson, J., Rodriguez-Diaz, J., Ruvoën-Clouet, N., Svensson, L., Le Pendu, J., et al. (2009). Human noroviruses recognize sialyl Lewis x neoglycoprotein. *Glycobiology* 19, 309–320. doi: 10.1093/glycob/cwn139

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J. R., and Jann, A. (1997). Molecular organisation of the ice nucleation protein InaV from *Pseudomonas syringae*. *FEBS Lett.* 414, 590–594. doi: 10.1016/S0014-5793(97)01079-X

Shimazu, M., Mulchandani, A., and Chen, W. (2001). Cell surface display of organophosphorus hydrolase using ice nucleation protein. *Biotechnol. Prog.* 17, 76–80. doi: 10.1021/bp0001563

Shimazu, M., Nguyen, A., Mulchandani, A., and Chen, W. (2003). Cell surface display of organophosphorus hydrolase in *Pseudomonas putida* using an ice-nucleation protein anchor. *Biotechnol. Prog.* 19, 1612–1614. doi: 10.1021/bp0340640

Tan, M., Fang, P., Chachiyo, T., Xia, M., Huang, P., Fang, Z., et al. (2008). Noroviral P particle: structure, function and applications in virus-host interaction. *Virology* 382, 115–123. doi: 10.1016/j.virol.2008.08.047

Tan, M., Huang, P., Xia, M., Fang, P., Zhong, W., McNeal, M., et al. (2011). Norovirus P particle, a novel platform for vaccine development and antibody production[J]. *Virology* 85, 753–764. doi: 10.1128/JVI.01835-10

Tan, M., and Jiang, X. (2005a). Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. *Trends Microbiol.* 13, 285–293. doi: 10.1016/j.tim.2005.04.004

Tan, M., and Jiang, X. (2005b). The P domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors. *J. Virol.* 79, 14017–14030. doi: 10.1128/JVI.79.22.14017-14030.2005

Tan, M., Wei, C., Huang, P., Fan, Q., Quigley, C., Xia, M., et al. (2015). Tulane virus recognizes sialic acids as cellular receptors. *Sci. Rep.* 5:11784. doi: 10.1038/srep11784

Tan, M., Zhong, W., Song, D., Thornton, S., and Jiang, X. (2004). *E. coli*-expressed recombinant norovirus capsid proteins maintain authentic antigenicity and receptor binding capability. *J. Med. Virol.* 74, 641–649. doi: 10.1002/jmv.20228

Tian, P., Engelbrektson, A., and Mandrell, R. (2008). Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with porcine gastric mucin conjugated to magnetic beads. *J. Appl. Environ. Microbiol.* 74, 4271–4276. doi: 10.1128/AEM.00539-08

Wang, D. P., Wu, Q. P., Kou, X. X., Yao, L., and Zhang, J. (2008). Distribution of norovirus in oyster tissues. *J. Appl. Microbiol.* 105, 1966–1972. doi: 10.1111/j.1365-2672.2008.03970.x

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhove, J., van Montagu, M., and Warren, G. J. (1986). Identification and purification of a bacterial ice-nucleation protein. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7256–7260. doi: 10.1073/pnas.83.19.7256

Xu, Y., Liu, Q., Zhou, L., Yang, Z., and Zhang, Y. (2008). Surface display of GFP by *Pseudomonas syringae* truncated ice nucleation protein in attenuated *Vibrio anguillarum* strain. *Mar. Biotechnol.* 10, 701–708. doi: 10.1007/s10126-008-9108-7

Zheng, D. P., Ando, T., Fankhauser, R. L., Beard, R. S., Glass, R. I., and Monroe, S. S. (2006). Norovirus classification and proposed strain nomenclature. *Virology* 346, 312–323. doi: 10.1016/j.virol.2005.11.015

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.

frontiers in Microbiology

REVIEW
published: 27 November 2015
doi: 10.3389/fmicb.2015.01361

Classification and Taxonomy of Vegetable Macergens

Bukola R. Aremu [1] *and Olubukola O. Babalola* [1,2]*

[1] *Department of Biological Sciences, Faculty of Agriculture, Science and Technology, North-West University, Mmabatho, South Africa,* [2] *Food Security and Safety Niche Area, Faculty of Agriculture, Science and Technology, North-West University, Mmabatho, South Africa*

Macergens are bacteria capable of releasing pectic enzymes (pectolytic bacteria). These enzymatic actions result in the separation of plant tissues leading to total plant destruction. This can be attributed to soft rot diseases in vegetables. These macergens primarily belong to the genus *Erwinia* and to a range of opportunistic pathogens namely: the *Xanthomonas* spp., *Pseudomonas* spp., *Clostridium* spp., *Cytophaga* spp., and *Bacillus* spp. They consist of taxa that displayed considerable heterogeneity and intermingled with members of other genera belonging to the *Enterobacteriaceae*. They have been classified based on phenotypic, chemotaxonomic and genotypic which obviously not necessary in the taxonomy of all bacterial genera for defining bacterial species and describing new ones These taxonomic markers have been used traditionally as a simple technique for identification of bacterial isolates. The most important fields of taxonomy are supposed to be based on clear, reliable and worldwide applicable criteria. Hence, this review clarifies the taxonomy of the macergens to the species level and revealed that their taxonomy is beyond complete. For discovery of additional species, further research with the use modern molecular methods like phylogenomics need to be done. This can precisely define classification of macergens resulting in occasional, but significant changes in previous taxonomic schemes of these macergens.

Keywords: classification, macergens, pectolytic, proteolytic, taxonomy, species

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Ivonne Delgadillo,
University of Aveiro, Portugal
Rosanna Tofalo,
Università degli Studi di Teramo, Italy

***Correspondence:**
Olubukola O. Babalola
olubukola.babalola@nwu.ac.za

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

Received: *21 August 2015*
Accepted: *16 November 2015*
Published: *27 November 2015*

Citation:
Aremu BR and Babalola OO (2015) Classification and Taxonomy of Vegetable Macergens. Front. Microbiol. 6:1361. doi: 10.3389/fmicb.2015.01361

INTRODUCTION

Marcergens are soft rot causing bacteria, responsible for plant tissue maceration resulting in total tissue collapse (Beattie, 2006; Bhai et al., 2012). Soft rot diseases of vegetables are the most characteristic symptom of tissue maceration in a plant. These begin as small water soaked lesion, expands and intensifies until the tissue turns soft and watery (Reddy, 2015). Apparently, the outer surface of the diseased plant might stay unbroken, while tanning and depressed, or enclosed in an exuding bacterial mucus layer (Heyman et al., 2013). Foul smells are common owing to the discharge of explosive complexes through tissue degradation. Best bacterial growth follows plant cell lysis in these diseases (Rich, 2013). Soft-rotting bacteria are distinguished for the speed at which they stimulate soft rot. Warehoused crop may turn to liquid in only a few hours (Reddy, 2015). These pathogens usually enter through wound spots or natural openings such as lenticels and persist

Abbreviation: DNA: Deoxy Ribonucleic Acid; ITS: Internal transcribed spacer; MLSA: Multilocus Sequence Analysis; NADH: Nicotinamide Adenine Dinucleotide (Reduced); RNA: Ribonucleic Acid; rDNA: Ribosomal Deoxy Ribonucleic Acid; rRNA: Ribosomal Ribonucleic Acid

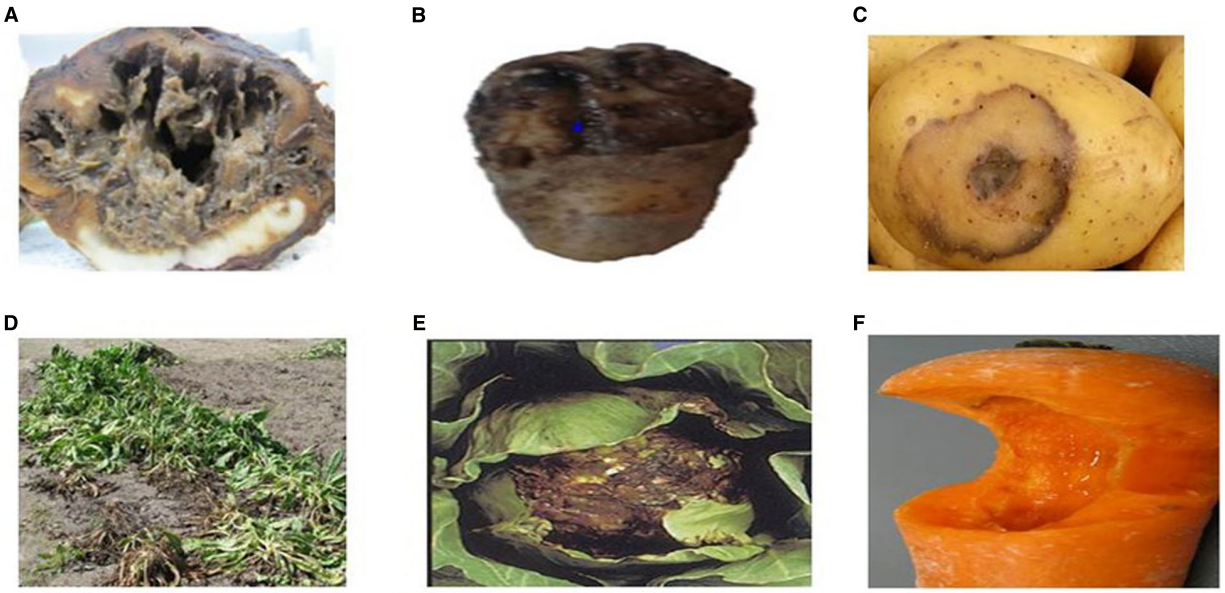


FIGURE 1 | Unmarketable Vegetable as a Result of Macergen Infestation (A). Chicory root affected by soft rot diseases, **(B,C)**. Potato with soft rot diseases, **(D)**. Chicory leaves with soft rot disease, **(E)**. Cabbage with soft rot disease, **(F)**. Carrot with soft rot disease. Adapted from Lindsey du Toit, Washington State University, North Carolina Cooperative Extension Sevice (Lan et al., 2013).

in the intercellular spaces and vascular tissues till the environmental conditions become fit for disease development. Parenchymatous tissues are macerated by massive quantities of pectic exoenzymes exudates produced during this period. These enzymes comprise of cellulolytic enzymes, pectate lyases, and pectin methylesterases, which are responsible for the total tissue destruction (Parthiban et al., 2012).

Soft rot can be found worldwide, anywhere ample storage tissues of vegetables and ornamentals are found (Golkhandan et al., 2013; Elbanna et al., 2014). Potatoes, carrots, and onions are among the most affected vegetables, along with tomato and cucumber (Mir et al., 2010) (**Figure 1**). Soft rot of fleshy vegetables and ornamental plants can be caused by more than six genera of pectolytic bacteria comprising; *Erwinia*, *Pseudomonas*, *Clostridium*, *Bacillus*, *Cytophaga*, and *Xanthomonas* (Elbanna et al., 2014). The estimated rate of infection of macergens on harvested crop ranges from 15 to 30%. *Erwinia* are the major macergens causing tissue degradation in vegetables (Choi and Kim, 2013; Waleron et al., 2014). Although, *Erwinia* are the primary macergens, it is not a single taxon. It is reclassified into genera such as *Pectobacterium* and *Dickeya* (Brady et al., 2012; Nabhan et al., 2012; Czajkowski et al., 2013). Macergens comprise of multiple groups ranging from the very complex *Pseudomonas*, a gamma-*proteobacteria* to as diverse as *Bacillus* and *Clostridium* which are firmicutes. *Bacillus* spp., *Clostridium* spp., *Pseudomonas marginalis*, and *Pantoea agglomerans* only cause soft rot when conditions are favorable to do so, thus are secondary invader called opportunistic pathogens (da Silva, 2013). Among all these pectolytic bacteria, soft rot *Erwinias* are the most important primary macergens that can macerate both the growing and harvested crop (Baz et al., 2012). All other bacteria are referred to as secondary because they can only destroy the parenchymatous tissues of plant under extreme environmental conditions or secondary invaders after *Erwinias* or other pathogens have infected the plant.

These macergens infect and destroy plant tissues both pre- and post-harvest and this species causes the greatest damage to harvested vegetables (Lee et al., 2012). There is need to ensure a continuous cold chain from immediately after post-harvest, to retail for successful management of these ubiquitous spoilage bacteria that only thrive well at temperatures of 20°C and above (Tournas, 2005). The fluorescent *Pseudomonads* (*P. fluorescens* and *P. viridiflava*) can macerate plant parenchymatous tissues at a temperature below 4°C. This cause higher occurrence of these bacteria on decayed vegetables both at wholesale and retail markets. These soft-rotting fluorescent *Pseudomonads* and *Erwinia* therefore become the major threat to commercial fresh product operations and fresh vegetables precisely, from the farm to retail and wholesale outlets (Saranraj et al., 2012). There are currently no commercial agents available specifically for controlling soft rot (Yaganza et al., 2014).

Despite advances in vegetable production and disease management, many challenges face growers of vegetables, out of which the major one is the damage caused by macergens (Wu et al., 2012). Macergens damage the tissues of vegetable thereby reducing the quality, yield, shelf-life and consumer satisfaction of these plants (Akhtar, 2015). They usually cause great economic losses due to their ability to infect and macerate vegetable tissues at any point in time, be it, the field, transit, storage or marketing period (Lee et al., 2012). In the nature of

TABLE 1 | List of interesting *Erwinia* species.

Erwinia species	Sources	Reference
Erwinia amylovora	Apple, pear	Ashmawy et al. (2015)
Erwinia ananas	Honeydew melon	Wells et al. (1987)
Erwinia cacticida	Sunflower	Valenzuela-Soto et al. (2015)
Erwinia carotovora	Carrots, potatoes, cucumbers, onions, tomatoes, lettuce	Nazerian et al. (2013), Akbar et al. (2015)
Erwinia chrysanthemi	Potatoes	van der Wolf et al. (2014)
Erwinia papaya	Papaya	Gardan et al. (2004)
Erwinia cypripedii	Papaya	Leu et al. (1980)
Erwinia herbicola	Tomatoes	Ibrahim and AL- Saleh (2010)
Erwinia mallotivora	Papaya	Amin et al. (2011)
Erwinia nigrifluens	Walnut, hazelnut	Frutos (2010)
Erwinia persicinus	Bananas, cucumbers, and tomatoes	O'Hara et al. (1998)
Erwinia psidii	Guava, Eucalyptus	Pomini et al. (2005), Coutinho et al. (2011)
Erwinia quercina	Oaks	Shang et al. (2015)
Erwinia rhapontici	Rhubarb, garlic, tomato, onions, cucumber	Dowson (1941), Huang et al. (2003)
Erwinia rubrifaciens	Walnut, hazelnut	Frutos (2010)
Erwinia stewartii	Sweet corn	Roper (2011)
Erwinia tracheiphila	Pumpkin, watermelon	Sanogo et al. (2011)
Erwinia uredovora	Rice	Yan et al. (2010)
Erwinia tasmiensis	Pear	Thapa et al. (2012)
Erwinia billingiae	Pear	Kube et al. (2005)
Erwinia wasabiae	Potatoes	Moleleki et al. (2013)
Erwinia brasiliense	Potatoes	van der Merwe et al. (2010)
Erwinia betavasculorum	Sugarbeet	Nedaienia and Fassihiani (2011)
Erwinia oleae	Olive	Moretti et al. (2011)
Erwinia pyrifoliae	Pear	Shrestha et al. (2003)
Erwinia atrosepticum	Potatoes	Kwasiborski et al. (2013)
Erwinia uzenensis	Pear	Matsuura et al. (2012)
Erwinia odoriferum	Chicory, potato	Waleron et al. (2014)
Erwinia piriflorinigrans	Pear	López et al. (2011)
Erwinia toletana	Olive	Rojas et al. (2004)

today's worldwide market, there are extremely high expectations for growers to provide ample supplies of high-quality, disease-free produce that have extended shelf-life (Kewa, 2012; Cheverton, 2015). The traditional methods to identify these macergens are extremely slow, more complex and obsolete (Hawks, 2005). Also, resistance genes active against macergens have been found in multiple host species, but their sequences and mechanisms remain unknown (Nykyri et al., 2012). Hence, means of quick identification of these bacteria are very essential. But the understanding of the taxonomy of these macergens will go a long way in shedding light to understand their biology and ultimately to the best method of controlling them. At present, there is very few knowledge available on the biology, ecology and epidemiology of macergens affecting vegetables in lowland and highland tropics. In order to increase crop production an assessment of biology, ecology and epidemiology of these bacteria need to be successfully implemented. Thus, this review focuses on the classification and taxonomy of the macergens to the species level. This is very important for more exploration in biotechnology.

TYPES OF MICROORGANISMS ON VEGETABLES

The majority of Gram negative rods identified from raw vegetables were fluorescent *Pseudomonads* spp., *Klebsiella* spp., *Serratia* spp., *Flavobacterium* spp., *Xanthomonads* spp., *Chronobacterium* spp., and *Alcaligenes* (Elbanna et al., 2014). In vegetables like broccoli, cabbage, mungbean sprouts and carrot, Gram positive rods were predominantly isolated. *Coryneform* bacteria and catalase negative *cocci* were also predominantly isolated from broccoli, raw peas and raw sweet corn. In India, the mesophilic microflora of potatoes mainly comprised Gram positive bacteria, *Bacillus* spp., and *Micrococcus* spp. as fluorescent *Pseudomonads*, *Cytophaga* spp., *Flavobacterium* spp., *Xanthomonas*. spp., and *Erwinia* spp. *Leuconostoc mesenteroides* was the most common and abundant species found in vegetables amongst lactic acid bacteria (Andrews and Harris, 2000).

TAXONOMY OF MACERGENS

Genus *Erwinia*

Erwinia belongs to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacteriales* and family *Enterobacteriaceae*. For the past several decades, *Enterobacteria* that macerate and decay plants tissues, often referred to as the pectolytic Erwinias, were placed in genus *Erwinia*. Named after the eminent plant pathologist, Erwinin F. Smith. They are non-spore forming, facultative Gram negative rod-shaped anaerobes of approximately 0.7 × 1.5 μm in size with peritrichous flagella. This genus contains diverse set of group of organisms represented in **Table 1**. Since its establishment many new genera have been generated from *Erwinia*.

Nomenclature of *Erwinia*

Traditionally two species (*Erwinia carotovora* and *Erwinia chrysanthemi*) are circumscribed as the important plant pathogenic strains, but has been reclassification into a new genus, *Pectobacterium*, with multiple species being proposed (Gardan et al., 2003). *Pectobacterium* spp. (Waldee, 1945; formerly *Erwinia carotovora*) and *Dickeya* spp. (formerly *Erwinia chrysanthemi*) species are related to soft rot *Enterobacteria* pathogens with broad host ranges. These species formerly were known as the soft rot *Erwinia* spp., but several studies have shown that the soft rot *Enterobacteria* and *E. amylovora*, the type strain of the *Erwinia* genus, are too divergent to be included in one clade. Therefore, the soft rot *Erwinia* spp. were moved to two new genera as *Pectobacterium* and *Dickeya* (Nabhan et al., 2013). *Pectobacterium* and *Dickeya* spp. are considered broad-host range pathogens in part because, they have been isolated from so many plant species and in part because, single strains are pathogens of numerous plant species under experimental conditions. Within the genus *Pectobacterium*, there are five major clades designated I, II, III, IV, and V, which differs from previous studies. These comprise five subspecies or species-level clades of *Pectobacterium* namely; *Pectobacterium carotovorum* subsp. *carotovorum* (syn. *Erwinia carotovorum* subsp. *carotovorum*), *Pectobacterium atrosepticum* (syn. *Erwinia carotovorum* subsp.

atrosepticum) *Pectobacterium wasabiae* (syn. *Erwinia carotovorum* subsp. *wasabiae*), *Pectobacterium betavasculorum* (syn. *Erwinia carotovorum* subsp. *betavasculorum*), and *Pectobacterium carotovorum* subsp. *brasiliense* (Hauben et al., 2005; Nabhan et al., 2012). The reconstructed phylogenies agree that *P. atrosepticum*, *P. betavasculorum*, and *P. wasabiae* do form individual clades and place the *brasiliensis* strains in an individual clade.

Previous suggestions to separate the pectolytic *Enterobacteria* into the genus *Pectobacterium* has not found favor among phytobacteriologists. Initially the suggestion was made by Waldee (1945), who recommended the segregation on the basis of the unique pectolytic activity of the bacteria. Consequently, Hauben et al. (1998) revived the suggestion to support the proposal by adding evidence from the 16S ribosomal DNA sequence analysis of various plant-associated members of the *Enterobacteriaceae*. Although the phenotypic characterization and analysis of a single DNA fragment might have been considered insufficient for the subdivision at the generic level, the DNA-DNA hybridization study conducted by Gardan et al. (2003) provides further stimulation to change in favor of the new nomenclature. Samson et al. (2005), have proposed several new species from new genus, *Dickeya* for *E. chrysanthemi*, comprising of six genomic species namely: *Dickeya dianthicola*, *D. dadantii*, *D. zeae*, *D. chrysanthemi*, *D. dieffenbachiae*, *D. paradisiaca*.

A recently initiated multi-locus sequencing project, as well as DNA hybridization data from the 1970s, supports the transfer of *E. carotovora* and *E. chrysanthemi* to two separate genera as well as the elevation of some soft rot *Erwinia* subgroups to the species level (Brady et al., 2012).

All the phylogenetic analyses completed to date have suffered from the small number of strains available for some *Enterobacteria* species, which makes it difficult to determine the relatedness of these taxa. Unfortunately, the naming and re-naming of species has caused considerable confusion in the literature, resulting in manuscripts being published with names that were used for only a few years. Since *Erwinia* has remained the preferred name used in the literature, the comprehensive phylogenetic study of the entire group of soft rot *Enterobacteria* remains uncompleted (Charkowski, 2006; Elbanna et al., 2014). The pectolytic *Erwinia* are ubiquitous in environments that support plant growth, and because they may be found in association with asymptomatic plants, they have been viewed as opportunistic pathogens analogous to medical bacteria that infect only immunologically compromised individuals. *Pectobacterium carotovorum*, in the family *Enterobacteriaceae*, is a highly diverse species consisting of at least two valid names, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* and a suggested third taxon, *P. carotovorum* subsp. *brasiliense* (De Boer et al., 2012). Despite the lack of valid *carotovorum* publication, the *P. carotovorum* subsp. *brasiliense* name has been used in more than 10 publications since first published in 2004 as *Erwinia carotovora* subsp. *brasiliense* (Ma et al., 2007). Assigning strains to this taxon was based mainly on the genetic information of the 16S-23S intergenic spacer region of the rRNA operon, partial sequence of 16S rRNA gene and multilocus sequence analysis (MLSA) of housekeeping genes and MALDI-TOF characterization (Wensing et al., 2012). **Table 2** depicts the molecular method employed in the characterization of *Pectobacterium* and *Dickeya* species. *Pectobacterium carotovorum* subsp. *brasiliense* was first described as causing blackleg disease on potatoes (*Solanum tuberosum* L.) in Brazil and has since been described as also causing soft rot in *Capsicum annum* L., *Ornithogalum* spp., and *Daucus carota* subsp. *Sativus*. Strains of this taxon were isolated in the USA, Canada, South Africa, Peru, Germany, Japan, Israel, and Syria (Ngadze et al., 2012; Moleleki et al., 2013).

Genus *Pseudomonas* was first described in 1894 as one of the most diverse and ubiquitous bacterial genera whose species have been isolated worldwide from soil, decayed plant materials and rhizopheric region, quite a numerous plant species (Migula, 1894). They comprise a heterogeneous group of species which were grouped into five groups based on RNA homology (Saranraj et al., 2012). The RNA-homology group I belong to the fluorescent group because of their ability of producing pyoverdines. Pectolytic *Pseudomonas* belongs to this rRNA group I organism of gamma *Proteobacteria*. They are non-sporulating, Gram-negative, strict aerobic, rod-shape with polar flagella (Özen and Ussery, 2012). The strains of these bacteria called *P. marginalis* or *P. fluorescens* can be attributed to soft rot diseases in vegetables. The very complex groups of fluorescent, oxidase positive soft rot *Pseudomonas* are opportunistic macergens. **Table 3** represents the molecular methods for the description of *Pseudomonas* species belonging to macergens.

Nomenclature of *Pseudomonas*

The nomenclature of bacteria in the genus *Pseudomonas* has changed considerably during the last decennia. *P. marginalis* or *P. fluorescens* are pectinolytic that cause strains soft rot on a wide range of hosts. The taxonomic and phytopathogenic status of *P. marginalis* is not well known. However, these are biochemically and phenotypically indistinguishable from saprophytic strains of *P. fluorescens* biovars II, *P. putida*, and *P. chlororaphis* (now includes *P. aureofaciens*). Based on their ability to degrade pectin and macerate the plant parenchymatous tissues they are referred to as *P. marginalis*. Recently, based on 16S rRNA analysis Anzai et al. (2000) came up with 57 strains of *Pseudomonas* sensu stricto with seven subclusters: *P. syringae* group, *P. chlororaphis* group, *P. fluorescens* group, *P. putida* group, *P. stutzeri* group, *P. aeruginosa* group, and *P. pertucinogena* group (Novik et al., 2015). Also, in the same genus *Pseudomonas*, some species have been found to be misclassified for instance *P. aureofaciens* and *P. aurantiaca*, which were reclassified into *P. chlororaphis* (Peix et al., 2007).

Ever since the discovery of genus *Pseudomonas*, it has undergone several taxonomic changes not only as far as the number of species included, but also as far as the criteria used for their definition and delineation. In Bergey's Manual of Systematic Bacteriology's current edition, an extensive list of methods used in *Pseudomonas* taxonomy was integrated (Palleroni, 2005). These methods, which consist of cell morphology and structure, cell wall composition, pigment types, nutritional and metabolic characteristics, susceptibility to different compounds, antibiotic production, pathogenicity of other organisms, antigenic structure and genetic and ecological studies

TABLE 2 | Molecular methods of identifying macergens.

Macergens	Molecular methods	Isolation sources	Reference
Pectobacterium carotovora	AFLP, MLSA, MLST, PFGE, MALDI-TOF MS, qPCR	Potatoes	Nabhan et al. (2012), Ngadze et al. (2012), Šalplachta et al. (2015), Humphris et al. (2015)
Pectobacterium atrosepticum	AFLP, RFLP, RAPD, qPCR, MALDI-TOF MS	Potatoes	Ngadze et al. (2012), Duarte et al. (2004), Pritchard et al. (2013), Šalplachta et al. (2015)
Pectobacterium wasabiae	AFLP, MLST, RAPD, qPCR	Horse radish, potatoes, crucifer	Avrova et al. (2002), De Boer et al. (2012), Kim et al. (2012)
Pectobacterium odoriferum	AFLP, MLSA, MLST	Potatoes, celery	Avrova et al. (2002), Waleron et al. (2014)
Pectobacterium betavasculorum	AFLP, MLST, 16S rRNA, qPCR	Potatoes	Avrova et al. (2002), De Boer et al. (2012), van der Merwe et al. (2010), Humphris et al. (2015)
Pectobacterium brasiliense	MLST, 16S-23S rDNA, qPCR, MALDI-TOF MS	Potatoes	De Boer et al. (2012), Czajkowski et al. (2015), Werra et al. (2015)
Dickeya chrysanthemi	16S—23S rDNA, RFLP of recA, AFLP, rep-PCR, 16S rDNA, MLST, DNA–DNA hybridization, qPCR, MALDI-TOF MS	Potatoes	Laurila et al. (2008), Waleron et al. (2002), Avrova et al. (2002), Sławiak et al. (2009), Ma et al. (2007), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya dianthicola	rep-PCR, 16S rDNA, PFGE, MALDI-TOF MS, DNA–DNA hybridization, qPCR,	Potatoes	Sławiak et al. (2009), Degefu et al. (2013), Šalplachta et al. (2015), Samson et al. (2005), Pritchard et al. (2013)
Dickeya dadantii	rep-PCR, 16S rDNA, PFGE, DNA–DNA hybridization, qPCR, MALDI-TOF MS	Potatoes,	Sławiak et al. (2009), Degefu et al. (2013), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya zeae	rep-PCR, 16S rDNA, RPLP, PFGE, DNA–DNA hybridization, qPCR, MALDI-TOF MS	Potatoes, maize	Sławiak et al. (2009), Samson et al. (2005), Degefu et al. (2013), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya dieffenbachiae	rep-PCR, 16S rDNA, AFLP, PFGE, DNA–DNA hybridization, MALDI-TOF MS	Potatoes	Sławiak et al. (2009), Samson et al. (2005), Degefu et al. (2013), Šalplachta et al. (2015)
Dickeya paradisiaca	rep-PCR, 16S rDNA, AFLP, PFGE, qPCR, MALDI-TOF MS	Potatoes, banana, maize	Sławiak et al. (2009), Degefu et al. (2013), Samson et al. (2005), Pritchard et al. (2013), Šalplachta et al. (2015)
Dickeya solani	rep-PCR, PFGE, RFLP, qPCR, MALDI-TOF	Potatoes, tomato, maize,	van der Wolf et al. (2014), Degefu et al. (2013), Waleron et al. (2013a), Pritchard et al. (2013), Šalplachta et al. (2015)

PFGE: Pulse-field gel electrophoresis; 16S-23S intergenic transcribed region of the rRNA operon; MLSA: multilocus sequence analysis of housekeeping genes; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AFLP: amplified fragment length polymorphism; MLST: multilocus sequence tagging; RFLP: restriction fragment length polymorphism; RAPD: random amplification of polymorphic DNA; rep-PCR: repetitive sequence-based PCR 3.2 Genus Pseudomonas*.*

revealed the efforts for characterizing *Pseudomonas* species. The phenotypic taxonomic markers comprise a set of tests, namely: cell shape, flagella type, consumption of carbon sources such as organic acids, polyalcohols and amino acids, ability to grow in different culture conditions, antibiotic resistance, production of antibiotic substances and exocellular enzymes (Palleroni, 2005).

In *Pseudomonas* taxonomy, the effectiveness of chemotaxonomic studies has been confirmed, such as quinone systems, fatty acid, protein, polar lipid or polyamine profiles, which are usually useful in the taxonomy of most bacterial groups. Generally, *Pseudomonas* species were reclassified by chemotaxonomic markers into other genera such as *P. mephitica* into *Janthinobacterium lividum* (Kämpfer et al., 2008). Janse et al. (1992), used whole fatty acid analysis in the study of a broad collection opportunistic phytopathogenic to clarify the taxonomic position of some *P. marginalis* strains included in the *P. fluorescens* group. Also, Janse et al. (1992) reported that other bacteria (*P. putida*, *P. aureofaciens*, and *P. tolaasii*) within the fluorescent oxidase positive pseudomonads group also exhibit pectinolytic ability. Hence, they are referred to as *P. fluorescens* supercluster. The study of polyamine composition in Proteobacteria revealed putrescine as the main polyamine present in the *P. fluorescens* complex, thus help in the delineation of species from this group. Recently, the polar lipid patterns of representative species of genus *Pseudomonas* were analyzed which showed the presence of phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine as major polar lipids (Cámara et al., 2007).

Siderotyping an interesting taxonomic tool was used in characterizing fluorescent and then to non-fluorescent based on the isoelectrophoretic. Characterization of the major siderophores and pyoverdines and determination of strains pyoverdine mediated iron uptake specificity led to characterization of several *Pseudomonas* strains at species level, through species-specific pyoverdines (Novik et al., 2015). Mass spectrometry for the determination of molecular mass of pyoverdines has helped recently to improve siderotyping resolution power and accuracy (Meyer et al., 2008).

Currently fluorescent spectroscopy fingerprinting, the most modern techniques for biomolecules analysis are being applied to *Pseudomonas* taxonomy, by emission spectra of three intrinsic fluorophores (NADH, tryptophan, and the complex of aromatic amino acids and nucleic acid), which have been able to differentiate *Pseudomonas* at genus level from

TABLE 3 | Molecular methods for the description of *Pseudomonas* species belonging to macergens.

Macergens	Molecular methods	Isolation sources	Reference
Pseudomonas. fluorescens	RFLP ITS1, 16S rRNA gene, WC-MALDI-TOF MS	Wheat	Franzetti and Scarpellini (2007), Mulet et al. (2012)
Pseudomonas marginalis	16S rRNA	Onion	Achbani et al. (2014)
Pseudomonas putida	16S rRNA, MLSA	Potato	Delfan et al. (2012), Mulet et al. (2010)
Pseudomonas chlororaphis	16S rRNA, MLSA WC-MALDI-TOF MS	Sugarbeet and spring wheat	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas aureofaciens	16S rRNA, MLSA, WC-MALDI-TOF MS	Corn	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas syringae	16S–23S rDNA, 16S rRNA, MLSA	Kiwifruit, cucumber, tomato	Rees-George et al. (2010), Mulet et al. (2010)
Pseudomonas stutzeri	16S rRNA, MLSA	Ginseng	Mulet et al. (2010)
Pseudomonas aeruginosa	RFLP ITS1, 16S rRNA gene, MLST	Tomato, lettuce, celery	Franzetti and Scarpellini (2007)
Pseudomonas pertucinogena	16S rRNA, MLSA	Wheat	Mulet et al. (2010)
Pseudomonas aurantiaca	16S rRNA, MLSA, WC-MALDI-TOF MS	Cotton	Mulet et al. (2010), Mulet et al. (2012)
Pseudomonas corrugata	rep-PCR fingerprinting, MLSA	Tomato	Trantas et al. (2015)
Pseudomonas cichorii	16S rRNA, MLSA	Tomato	Mulet et al. (2010)

16S-23S intergenic transcribed region of the rRNA operon; MLSA: multilocus sequence analysis of housekeeping genes; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AFLP: amplified fragment length polymorphism; MLST: multilocus sequence tagging; RFLP: restriction fragment length polymorphism; rep-PCR: repetitive sequence-based PCR.

TABLE 4 | Macergens host pathogenicity.

Macergens	Disease symptoms	Host range	Reference
Erwinia carotovora	Soft rot	Wide	Nabhan et al. (2012), Nabhan et al. (2013)
Erwinia carotovora ssp. atrosepticum	Soft rot	Potato	Baz et al. (2012), Ngadze et al. (2012)
Erwinia carotovora ssp. brasiliensis	Soft rot	Potato	Moleleki et al. (2013), Zhao et al. (2013)
Erwinia carotovora ssp. carotovora	Soft rot	Sugar beet	Waleron et al. (2013b)
Erwinia carotovora ssp. odorifera	Soft rot	Chicory	Lan et al. (2013)
Erwinia carotovora E. chrysanthemi	Soft rot	Wide	Brady et al. (2012)
Erwinia cypripedii	Brown rot	Cypripedium	Horst (2013)
Erwinia rhapontici	Crown rot	Rhubarb	Brady et al. (2012)
Erwinia carcinogenesis	Soft rot	Giant cactus	Ma et al. (2007)
Pseudomonas marginalis	Soft rot	Lettuce, cabbage	Gašic et al. (2014)
Pseudomonas fluorescens	Soft rot	Pepper, potato	Bhai et al. (2012), Czajkowski et al. (2012)
Pseudomonas viridiflava	Soft rot	Carrot, pepper,	Almeida et al. (2013), Mitrev et al. (2014)
Pseudomonas putida	Soft rot	Lettuce, ginger	Krejzar et al. (2008), Moreira et al. (2013)
Xanthomonas campestris	Black rot	Crucifers	Kifuji et al. (2013), Vicente and Holub (2013)
Xanthomonas campestris	Soft rot	Tomato, pepper	Singh et al. (2012)
Xanthomonas. campestris aberrans	Soft rot	Brassica	Gupta et al. (2013)
Xanthomonas axonopodis vesicatoria	Soft rot	Tomato	Sharma and Agrawal (2014)
Xanthomonas axonopodis phaseoli	Black rot	Bean	Porch et al. (2012), Dutta et al. (2013)
Xanthomonas axonopodis dieffenbachia	Soft rot	Tomato, pea	Ismail et al. (2012), Czajkowski et al. (2014)
Xanthomonas. axonopodis citri	Soft rot	Potato	Terta et al. (2012)

Burkholderia, *Xanthomonas*, or *Stenotrophomonas* with very high sensitivity, and moreover at species level *P. chlororaphis*, *P. lundensis*, *P. fragi*, *P. taetrolens* and *P. stutzeri* grouped separately from *P. putida*, *P. pseudoalcaligenes*, and *P. fluorescens*, which correlate with the phylogenetic clusters earlier obtained by Anzai et al. (2000); Peix et al. (2007), and Tourkya et al. (2009).

Hence, other gene sequences like housekeeping genes have been used in the last decade as phylogenetic molecular markers in taxonomic studies such as the *rec*A, *atp*D, *car*A, *gyr*B, and *rpo*B, whose effectiveness has been demonstrated in genus *Pseudomonas* for species differentiation (Hilario et al., 2004). For instance, the effectiveness of *rpo*B has been reported in discriminating closely related *Pseudomonas*, with a phylogenetic resolution of the *rpo*B tree roughly three times higher than that of the 16S rRNA gene tree (Tayeb et al., 2005). These genes also enhanced differentiation of subspecies within *P. chlororaphis* (Hilario et al., 2004; Peix et al., 2007). Nevertheless, the analysis of housekeeping genes is not frequently used so far in *Pseudomonas* species description, but only *gyr*B, *rpo*B and *rpoD* have been integrated in the current description of *P. xiamenensis* (Lai and Shao, 2008).

16S-23S rRNA intergenic spacer is another phylogenetic marker used increasingly in taxonomic studies for discrimination of very closely related bacteria, at species and intraspecific levels, even at the strain level because of its high variability both in size and sequence (Sakamoto et al., 2001). This region can be amplified by using universal primers, and specific protocols (Locatelli et al., 2002). The efficacy of this phylogenetic marker has been reported in the differentiation of *Pseudomonas* species (Guasp et al., 2000). The selection of the minimal principles necessary for species delineation and description is selected for each bacterial genus by a committee created by experts in the given genus. The methods used in the taxonomy of the genus *Pseudomonas* and its related genera have been standardized by the subcommittee on the taxonomy. However, the minimal

standards for genus *Pseudomonas* species description are yet to be cleared after the 2002 meeting of this subcommittee (De Vos and Yabuuchi, 2002). Hence, the new species description of this genus must be based on the general minimal standards for bacterial species characterization (Stackebrandt et al., 2002). These general minimal standards needed for the classification of new species and/or subspecies must comprise 16S rRNA sequencing, DNA-DNA hybridization, fatty acid analysis and phenotypic classification.

Genus *Xanthomonas*

The genus *Xanthomonas* belong to the family *Xanthomonadaceae*. This family composed of 10 genera that dwell in an extreme environment. The genus *Xanthomonas* belongs to the gamma proteolytic subdivision (Mbega et al., 2014). They are Gram-negative, aerobic, rod-shape, motile, non-spore forming with a single polar flagellum, comprises of 27 species infecting more than 400 dicots and monocots plant species (Rodriguez et al., 2012).

Nomenclature of *Xanthomonas*

Traditionally, genus *Xanthomonas* is referred to as a taxon of pathogenic plant bacteria (Dye et al., 1974; Bradbury, 1984). *Xanthomonas* usually produce some extracellular polysaccharide namely: xanthan and xanthomonadin, a membrane-bound, brominated, aryl-polyene, yellow pigment (Adriko et al., 2014). This yellow pigment is responsible for their pathogenicity and virulence (Subramoni et al., 2006). However, the yellow-pigmented *X.* spp. (*X. campestris*) are the only one associated with tissue maceration of the post-harvest vegetables and fruits (Liao and Wells, 1987). They are opportunistic macergens because they are entering through natural openings or after infection of the plant by *Erwinia* spp.

Genetically, it can be differentiated into over 141 pathovars (pv.) based on specificity range (Swings and Civerolo, 1993). But *Xanthomonas* classification of *X. campestris* pathovar was based on the host pathogenicity system (**Table 4**)

Initially, this genus undergone diverse taxonomic and phylogenetic studies based on their phenotype and host specificity. Until Vauterin et al. (1995) revised the reclassification of *Xanthomonas* by DNA-DNA hybridization into 20 species based on their genomic relatedness. Phenotypic fingerprinting techniques such as 50S-polyacrylamide gel electrophoresis (50S-PAGE) of cellular proteins and gas chromatographic analysis of fatty acid methyl esters (FAME) reasonable supported these genomic groups to an extent. Hence, both techniques are useful tools in specific and interspecific differentiation of *Xanthomonas* levels (Rademaker et al., 2000).

Other analyses like Multi-Locus Sequence Analysis (MLSA), Amplified Fragment Length Polymorphism (AFLP) were also used in characterisation of this genus, revealing the complexity and diversity of the genus previously described by DNA-DNA hybridization (Ferreira-Tonin et al., 2012; Hamza et al., 2012). Not quite long, the phylogeny of species representing the principal lineages of the genus *Xanthomonas* were reported based on their genome (Rodriguez et al., 2012). The 16S ribosomal DNA sequences and MLSA classified *Xanthomonas* species into two major groups (Vicente and Holub, 2013). Group I comprising: *X. albilineans*, *X. hyacinth*, *X. theicola*, *X. sacchari* and *X. translucens*, and Group II made up of *X. arboricola*, *X. axonopodis*, *X. bromi*, *X. campestris*, *X. cassavae*, *X. codiaei*, *X. cucurbitae*, *X. fragariae*, *X. hortorum*, *X. melonis*, *X. oryzae*, *X. pisi*, *X. populi*, *X. vasicola*, and *X. vesicatoria* (Rodriguez et al., 2012). Thus, taxonomy of this genus are still subjected to debate since the last decade (Rodriguez et al., 2012; Vandroemme et al., 2013; Lamichhane, 2014).

CONCLUSION

The taxonomy of all these macergens is far from being complete because of the controversial issues arising from their classification which were based on host pathogenicity (**Table 1**). This may be affected by the sudden change in the ecosystem. This classification is not based on scientific research perspective for defined taxa and the consequences brought about by these marcergens may become difficult to understand. It is majorly based on symptoms that is similar in all the macergens, and this is unreliable according to (Sławiak et al., 2013). Although, some scientific method like MLSA were used for the classification they have limitation of single locus analysis. Thus, a proper classification is imperative, in order to reflect an understanding of their existing natural diversity and relationships among them. This will help plant breeders, farmers, and legislators to ensure quick and effective disease diagnosis and management, in order to avoid unnecessary destruction of economically valuable crops. The knowledge of genomic diversity within the macergens pathovars is necessary for host resistance disease based management strategies for the plant breeders.

As a concluding comment, we would like to stress that we applaud further developments in molecular methods of analyzing macergens for a better classification of these macergens. It is our belief, however, that any future progress in taxonomy as a scientific discipline will depend only on the availability of new experimental data that will broaden and refined the view on bacterial diversity.

AUTHOR CONTRIBUTIONS

BR involved in data collection from internet, drafting of the manuscript or revising it critically for important intellectual content; have given final approval of the version to be published; and agree 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. OO involved in collection of data, drafting of the manuscript, revising it critically, responsible for any aspect of the article and also help in the general supervision of the article.

ACKNOWLEDGMENTS

The authors would like to thank the North West University and National Research Foundation, South Africa for funds (Grant no. UID81192 OO Babalola) that have supported research in their laboratory.

REFERENCES

Achbani, E., Sadik, S., El Kahkahi, R., Benbouazza, A., and Mazouz, H. (2014). First report on *Pseudomonas marginalis* bacterium causing soft rot of onion in morocco. *Atlas J. Biol.* 3, 218–223. doi: 10.5147/ajb.2014.0136

Adriko, J., Mbega, E. R., Mortensen, C. N., Wulff, E. G., Tushemereirwe, W. K., Kubiriba, J., et al. (2014). Improved PCR for identification of members of the genus *Xanthomonas*. *Eur. J. Plant Pathol.* 138, 293–306. doi: 10.1007/s10658-013-0329-x

Akbar, A., Ahmad, M., Khan, S. Z., and Ahmad, Z. (2015). Characterization of the causal organism of soft rot of tomatoes and other vegetables and evaluation of its most aggressive isolates. *Am. J. Plant Sci.* 6, 511. doi: 10.4236/ajps.2015.64055

Akhtar, S. (2015). "Advances in conventional breeding approaches for postharvest quality improvement in vegetables," in *Postharvest Biology and Technology of Horticultural Crops: Principles and Practices for Quality Maintenance*, ed. M. W. Siddiqui (New Jersey, USA: Apple Academic Press Inc.), 141–176.

Almeida, I., Maciel, K., Neto, J. R., and Beriam, L. (2013). *Pseudomonas* viridiflava in imported carrot seeds. *Australas. Plant Dis. Notes* 8, 17–19. doi: 10.1007/s13314-012-0086-2

Amin, N. M., Bunawan, H., Redzuan, R. A., and Jaganath, I. B. S. (2011). *Erwinia mallotivora* sp., a new pathogen of papaya (*Carica papaya*) in Peninsular Malaysia. *Int. J. Mol. Sci.* 12, 39–45. doi: 10.3390/ijms12010039

Andrews, J. H., and Harris, R. F. (2000). The ecology and biogeography of microorganisms on plant surface. *Annu. Rev. Phytopathol.* 38, 145–180. doi: 10.1146/annurev.phyto.38.1.145

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H., and Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.* 50, 1563–1589. doi: 10.1099/00207713-50-4-1563

Ashmawy, N. A., Zaghloul, T. I., and El-Sabagh, M. A. (2015). Isolation and molecular characterization of the fire blight pathogen, *Erwinia amylovora*, isolated from apple and pear orchards in Egypt. *Plant Pathol. J.* 14, 142. doi: 10.3923/ppj.2015.142.147

Avrova, A. O., Hyman, L. J., Toth, R. L., and Toth, I. K. (2002). Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. *Appl. Environ. Microbiol.* 68, 1499–1508. doi: 10.1128/AEM.68.4.1499-1508.2002

Baz, M., Lahbabi, D., Samri, S., Val, F., Hamelin, G., Madore, I., et al. (2012). Control of potato soft rot caused by *Pectobacterium carotovorum* and *Pectobacterium atrosepticum* by Moroccan actinobacteria isolates. *World J. Microb. Biotechnol.* 28, 303–311. doi: 10.1007/s11274-011-0820-5

Beattie, G. A. (2006). "Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances," in *Plant-associated Bacteria*, ed. S. S. Gnanamanickam (Netherlands: Springer), 1–56. doi: 10.1007/978-1-4020-4538-7_1

Bhai, R., Kishore, V., Kumar, A., Anandaraj, M., and Eapen, S. (2012). Screening of rhizobacterial isolates against soft rot disease of ginger (*Zingiber officinale* Rosc.). *J. Spices Aromat. Crops* 14, 130–136.

Bradbury, J. F. (1984). "Genus II. *Xanthomonas* dowson 1939, 187," in *Bergey's Manual of Systematic Bacteriology*, eds N. R. Krieg and J. G. Holt (Baltimore: Williams & Wilkins), 199–210.

Brady, C. L., Cleenwerck, I., Denman, S., Venter, S. N., Rodríguez-Palenzuela, P., Coutinho, T. A., et al. (2012). Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth, 1967) Hauben et al. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. quercina comb. nov., *Lonsdalea quercina* subsp. *iberica* subsp. nov., and *Lonsdalea quercina* subsp. *britannica* subsp. nov., emendation of the description of the genus *Brenneria*, reclassification of *Dickeya dieffenbachiae* as *Dickeya dadantii* subsp. *dieffenbachiae* comb. nov., and emendation of the description of *Dickeya dadantii*. *Int. J. Syst. Evol. Microbiol.* 62, 1592–1602. doi: 10.1099/ijs.0.035055-0

Cámara, B., Strömpl, C., Verbarg, S., Spröer, C., Pieper, D. H., and Tindall, B. J. (2007). *Pseudomonas reinekei* sp. nov., *Pseudomonas moorei* sp. nov. and *Pseudomonas mohnii* sp. nov., novel species capable of degrading chlorosalicylates or isopimaric acid. *Int. J. Syst. Evol. Microb.* 57, 923–931. doi: 10.1099/ijs.0.64703-0

Charkowski, A. O. (2006). "The soft rot *Erwinia*," in *Plant-Associated Bacteria*, ed. S. S. Gnanamanickam (Heidelberg, NL: Springer), 423–505. doi: 10.1007/978-1-4020-4538-7_13

Cheverton, P. (2015). *Key Account Management: Tools and Techniques for Achieving Profitable Key Supplier Status*. Great Britain: Kogan Page Publishers.

Choi, O., and Kim, J. (2013). *Pectobacterium carotovorum* subsp. *brasiliense* causing soft rot on paprika in Korea. *J. Phytopathol.* 161, 125–127. doi: 10.1111/jph.12022

Coutinho, T. A., Brady, C. L., Van Der Vaart, M., Venter, S. N., Telechea, N., Rolfo, M., et al. (2011). A new shoot and stem disease of *Eucalyptus* species caused by *Erwinia psidii*. *Australas. Plant Pathol.* 40, 55–60. doi: 10.1007/s13313-010-0013-y

Czajkowski, R., De Boer, W., Van Der Zouwen, P., Kastelein, P., Jafra, S., De Haan, E., et al. (2013). Virulence of '*Dickeya solani*' and *Dickeya dianthicola* biovar-1 and-7 strains on potato (*Solanum tuberosum*). *Plant Pathol.* 62, 597–610. doi: 10.1111/j.1365-3059.2012.02664.x

Czajkowski, R., De Boer, W., Van Veen, J., and Van Der Wolf, J. (2012). Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. *biovar* 3 *in vitro* and in planta. *Plant Pathol.* 61, 169–182. doi: 10.1111/j.1365-3059.2011.02486.x

Czajkowski, R., Ozymko, Z., and Lojkowska, E. (2014). Isolation and characterization of novel soilborne lytic bacteriophages infecting *Dickeya* spp. *biovar* 3 ('*D. solani*'). *Plant Pathol.* 63, 758–772. doi: 10.1111/ppa.12157

Czajkowski, R., Pérombelon, M., Jafra, S., Lojkowska, E., Potrykus, M., Van Der Wolf, J., et al. (2015). Detection, identification and differentiation of *Pectobacterium* and *Dickeya* species causing potato blackleg and tuber soft rot: a review. *Ann. Appl. Biol.* 166, 18–38. doi: 10.1111/aab.12166

da Silva, W. L. (2013). *Sweetpotato Storage Root Rots: Flooding-Associated Bacterial Soft Rot Caused by Clostridium spp. and Infection by Fungal End Rot Pathogens Prior to Harvest*. Ph.D. thesis, Louisiana State University, Louisiana, USA.

De Boer, S. H., Li, X., and Ward, L. J. (2012). *Pectobacterium* spp. associated with bacterial stem rot syndrome of potato in Canada. *Phytopathology* 102, 937–947. doi: 10.1094/PHYTO-04-12-0083-R

De Vos, P., and Yabuuchi, E. (2002). International committee on systematic bacteriology: subcommittee on the taxonomy of *Pseudomonas* and related organisms international committee on systematic bacteriology: subcommittee on the taxonomy of *Pseudomonas* and related organisms. *Int. J. Syst. Evol. Microbiol.* 52, 2329. doi: 10.1099/ijs.0.02575-0

Degefu, Y., Potrykus, M., Golanowska, M., Virtanen, E., and Lojkowska, E. (2013). A new clade of *Dickeya* spp. plays a major role in potato blackleg outbreaks in North Finland. *Ann. Appl. Biol.* 162, 231–241.

Delfan, A., Etemadifar, Z., Bouzari, M., and Emtiazi, G. (2012). Screening of novel bacteriophage infection in *Pseudomonas putida* isolated from potato disease. *Jundishapur J. Microbiol.* 5, 550–554. doi: 10.5812/jjm.3786

Dowson, W. (1941). The identification of the bacteria commonly causing soft rot in plants. *Ann. Appl. Biol.* 28, 102–106. doi: 10.1111/j.1744-7348.1941.tb07543.x

Duarte, V., De Boer, S., Ward, L., and Oliveira, A. (2004). Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *J. Appl. Microbiol.* 96, 535–545. doi: 10.1111/j.1365-2672.2004.02173.x

Dutta, B., Block, C., Stevenson, K., Sanders, F. H., Walcott, R., and Gitaitis, R. (2013). Distribution of phytopathogenic bacteria in infested seeds. *Seed Sci. Technol.* 41, 383–397. doi: 10.15258/sst.2013.41.3.06

Dye, D., Lelliott, R., Buchanan, R., and Gibbons, N. (1974). "Genus II. *Xanthomonas Dowson* 1939, 187," in *Bergey's Manual of Determinative Bacteriology*, eds R. E. Buchanan, and N. E. Gibbons (Baltimore: Williams & Wilkins Company), 243–249.

Elbanna, K., Elnaggar, S., and Bakeer, A. (2014). Characterization of *Bacillus altitudinis* as a new causative agent of bacterial soft rot. *J. Phytopathol.* 162, 712–722. doi: 10.1111/jph.12250

Ferreira-Tonin, M., Rodrigues-Neto, J., Harakava, R., and Destéfano, S. A. L. (2012). Phylogenetic analysis of *Xanthomonas* based on partial rpoB gene sequences and species differentiation by PCR-RFLP. *Int. J. Syst. Evol. Microbiol.* 62, 1419–1424. doi: 10.1099/ijs.0.028977-0

Franzetti, L., and Scarpellini, M. (2007). Characterisation of *Pseudomonas* spp. isolated from foods. *Ann. Microbiol.* 57, 39–47. doi: 10.1007/BF03175048

Frutos, D. (2010). Bacterial diseases of walnut and hazelnut and genetic resources. *J. Plant Pathol.* 92, S79–S85.

Gardan, L., Christen, R., Achouak, W., and Prior, P. (2004). *Erwinia papayae* sp. nov., a pathogen of papaya (*Carica papaya*). *Int. J. Syst. Evol. Microbiol.* 54, 107–113. doi: 10.1099/ijs.0.02718-0

Gardan, L., Gouy, C., Christen, R., and Samson, R. (2003). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and

Pectobacterium wasabiae sp. nov. *Int. J. Syst. Evol. Microbiol.* 53, 381–391. doi: 10.1099/ijs.0.02423-0

Gašić, K., Gavrilović, V., Dolovac, N., Trkulja, N., Živković, S., Ristić, D., et al. (2014). *Pectobacterium carotovorum* subsp. carotovorum-the causal agent of broccoli soft rot in Serbia. *Pesticidi Fitomedicina* 29, 249–255. doi: 10.2298/PIF1404249G

Golkhandan, E., Kamaruzaman, S., Sariah, M., Zainal Abidin, M., and Nasehi, A. (2013). Characterisation of *Pectobacterium carotovorum* causing soft rot on *Kalanchoe gastonis-bonnierii* in Malaysia. *Archives Phytopathol. Plant Prot.* 46, 1809–1815. doi: 10.1080/03235408.2013.778452

Guasp, C., Moore, E. R., Lalucat, J., and Bennasar, A. (2000). Utility of internally transcribed 16S-23S rDNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. *Int. J. Syst. Evol. Microbiol.* 50, 1629–1639. doi: 10.1099/00207713-50-4-1629

Gupta, M., Vikram, A., and Bharat, N. (2013). Black rot-A devastating disease of crucifers: a review. *Agric. Rev.* 34, 269–278. doi: 10.5958/j.0976-0741.34.4.012

Hamza, A., Robene-Soustrade, I., Jouen, E., Lefeuvre, P., Chiroleu, F., Fisher-Le Saux, M., et al. (2012). MultiLocus sequence analysis-and amplified fragment length polymorphism-based characterization of xanthomonads associated with bacterial spot of tomato and pepper and their relatedness to *Xanthomonas* species. *Syst. Appl. Microbiol.* 35, 183–190. doi: 10.1016/j.syapm.2011.12.005

Hauben, L. M., Edward, R. B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., et al. (1998). Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *Syst. Appl. Microbiol.* 21, 384–397. doi: 10.1016/S0723-2020(98)80048-9

Hauben, L., Van Gijsegem, F., and Swings, J. (2005). "Genus XXIV. *Pectobacterium* Waldee 1945, 469AL emend," in *Bergey's Manual of Systematic Bacteriology*, 2nd Edn, eds L. Hauben, E. R. B. Moore, I. Vauterin, M. Steenakcers, J. Mergaert, J. Verdonck et al. (New York: Springer), 721–730.

Hawks, B. (2005). Agricultural bioterrorism protection act of 2002: possession, use, and transfer of biological agents and toxins; final rule. *Federal Regist.* 70, 13241–13292.

Heyman, F., Blair, J., Persson, L., and Wikström, M. (2013). Root rot of pea and faba bean in southern Sweden caused by *Phytophthora pisi* sp. nov. *Plant Dis.* 97, 461–471. doi: 10.1094/PDIS-09-12-0823-RE

Hilario, E., Buckley, T. R., and Young, J. M. (2004). Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of atpD, carA, recA, and 16S rDNA. *Antonie Van Leeuwenhoek* 86, 51–64. doi: 10.1023/B:ANTO.0000024910.57117.16

Horst, R. K. (ed) (2013). "Bacterial diseases," in *Westcott's Plant Disease Handbook*, (New York, USA: Springer), 69–90.

Huang, H., Hsieh, T., Erickson, R., and Erickson, R. (2003). Biology and epidemiology of *Erwinia rhapontici*, causal agent of pink seed and crown rot of plants. *Plant Pathol. Bull.* 12, 69–76.

Humphris, S. N., Cahill, G., Elphinstone, J. G., Kelly, R., Parkinson, N. M., Pritchard, L., et al. (2015). Detection of the bacterial potato pathogens *Pectobacterium* and *Dickeya* spp. using conventional and real-time PCR. *Plant Pathol. Techniques Protocols* 1302, 1–16. doi: 10.1007/978-1-4939-2620-6_1

Ibrahim, Y. E., and AL- Saleh, M. A. (2010). Isolation and characterization of *Erwinia herbicola* associated with internal discoloration of tomato fruits (*Lycopersicon esculentum* Mill) in Saudi Arabia. *Emirates J. Food Agric.* 22, 475–482. doi: 10.9755/ejfa.v22i6.4665

Ismail, M. E., Abdel-Monaim, M. F., and Mostafa, Y. M. (2012). Identification and pathogenicity of phytopathogenic bacteria associated with soft rot disease of girasole tuber. *Not. Sci. Biol.* 4, 75–81. doi: 10.5897/jbr11.015

Janse, J. D., Derks, J. H. J., Spit, B. E., and Van Der Tuin, W. R. (1992). Classification of fluorescent soft rot *Pseudomonas* Bacteria, Including *P. marginalis* Strains, using whole cell fatty acid analysis. *Syst. Appl. Microbiol.* 15, 538–553. doi: 10.1016/S0723-2020(11)80114-1

Kämpfer, P., Falsen, E., and Busse, H. (2008). Reclassification of *Pseudomonas mephitica* Claydon and Hammer 1939 as a later heterotypic synonym of *Janthinobacterium lividum* (Eisenberg 1891) De Ley et al. 1978. *Int. J. Syst. Evol. Microbiol.* 58, 136–138. doi: 10.1099/ijs.0.65450-0

Kewa, J. L. (2012). *Supplying Customer Requirements in the Fresh Produce Chain in the Highlands of Papua New Guinea*. Ph.D. thesis, Lincoln University, Lincoln.

Kifuji, Y., Hanzawa, H., Terasawa, Y., and Nishio, T. (2013). QTL analysis of black rot resistance in cabbage using newly developed EST-SNP markers. *Euphytica* 190, 289–295. doi: 10.1007/s10681-012-0847-1

Kim, M. H., Cho, M. S., Kim, B. K., Choi, H. J., Hahn, J. H., Kim, C., et al. (2012). Quantitative real-time polymerase chain reaction assay for detection of *Pectobacterium wasabiae* using YD repeat protein gene-based primers. *Plant Dis.* 96, 253–257. doi: 10.1094/PDIS-06-11-0511

Krejzar, V., Mertelík, J., Pánková, I., Kloudová, K., and Kudela, V. (2008). *Pseudomonas marginalis* associated with soft rot of *Zantedeschia* spp. *Plant Prot. Sci.* 44, 85–90.

Kube, M., Beck, A., Zinder, S., Kuhl, H., Reinhardt, R., and Adrian, L. (2005). Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat. Biotechnol.* 23, 1269–1273. doi: 10.1038/nbt1131

Kwasiborski, A., Mondy, S., Beury-Cirou, A., and Faure, D. (2013). Genome sequence of the *Pectobacterium atrosepticum* strain CFBP6276, causing blackleg and soft rot diseases on potato plants and tubers. *Genome Announc.* 1, e00374-00313. doi: 10.1128/genomeA.00374-13

Lai, Q., and Shao, Z. (2008). *Pseudomonas xiamenensis* sp. nov., a denitrifying bacterium isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* 58, 1911–1915. doi: 10.1099/ijs.0.65459-0

Lamichhane, J. R. (2014). *Xanthomonas arboricola* diseases of stone fruit, almond, and walnut trees: progress toward understanding and management. *Plant Dis.* 98, 1600–1610. doi: 10.1094/PDIS-08-14-0831-FE

Lan, W. W., Nishiwaki, Y., Akino, S., and Kondo, N. (2013). Soft rot of root chicory in Hokkaido and its causal bacteria. *J. General Plant Pathol.* 79, 182–193. doi: 10.1007/s10327-013-0440-z

Laurila, J., Ahola, V., Lehtinen, A., Joutsjoki, T., Hannukkala, A., Rahkonen, A., et al. (2008). Characterization of *Dickeya* strains isolated from potato and river water samples in Finland. *Eur. J. Plant Pathol.* 122, 213–225. doi: 10.1007/s10658-008-9274-5

Lee, J.-H., Shin, H., Ji, S., Malhotra, S., Kumar, M., Ryu, S., et al. (2012). Complete genome sequence of phytopathogenic *Pectobacterium carotovorum* subsp. *carotovorum* bacteriophage PP1. *J. Virol.* 86, 8899–8900. doi: 10.1128/JVI.01283-12

Leu, L., Lee, C., and Huang, T. (1980). Papaya black rot caused by *Erwinia cypripedii*. *Plant Prot. Bull. Taiwan* 22, 377–384.

Liao, C. H., and Wells, J. M. (1987). Diversity of pectolytic, fluorescent pseudomonads causing soft rots of fresh vegetables at produce markets. *Phytopathology* 77, 673–677. doi: 10.1094/Phyto-77-673

Locatelli, L., Tarnawski, S., Hamelin, J., Rossi, P., Aragno, M., and Fromin, N. (2002). Specific PCR Amplification for the Genus *Pseudomonas* Targeting the 3′ Half of 16S rDNA and the Whole 16S–23S rDNA Spacer. *Syst. Appl. Microbiol.* 25, 220–227. doi: 10.1078/0723-2020-00110

López, M. M., Roselló, M., Llop, P., Ferrer, S., Christen, R., and Gardan, L. (2011). *Erwinia piriflorinigrans* sp. nov., a novel pathogen that causes necrosis of pear blossoms. *Int. J. Syst. Evol. Microbiol.* 61, 561–567. doi: 10.1099/ijs.0.020479-0

Ma, B., Hibbing, M. E., Kim, H. S., Reedy, R. M., Yedidia, I., Breuer, J., et al. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytopathology* 97, 1150–1163. doi: 10.1094/PHYTO-97-9-1150

Matsuura, T., Mizuno, A., Tsukamoto, T., Shimizu, Y., Saito, N., Sato, S., et al. (2012). *Erwinia uzenensis* sp. nov., a novel pathogen that affects European pear trees (*Pyrus communis* L.). *Int. J. Syst. Evol. Microbiol.* 62, 1799–1803. doi: 10.1099/ijs.0.032011-0

Mbega, E. R., Mabagala, R., Adriko, J., Lund, O. S., Wulff, E. G., and Mortensen, C. N. (2014). Five species of xanthomonads associated with bacterial leaf spot symptoms in tomato from Tanzania. *Eur. J. Plant Pathol.* 138, 293–306. doi: 10.1094/PDIS-01-12-0105-PDN

Meyer, J. M., Gruffaz, C., Raharinosy, V., Bezverbnaya, I., Schäfer, M., and Budzikiewicz, H. (2008). Siderotyping of fluorescent *Pseudomonas*: molecular mass determination by mass spectrometry as a powerful pyoverdine siderotyping method. *Biometals* 21, 259–271. doi: 10.1007/s10534-007-9115-6

Migula, N. (1894). Arbeiten aus dem Bakteriologischen. *Inst. Technischen Hochschule Karlsruhe* 1, 235–238.

Mir, S. A., Zargar, M. Y., Sheikh, P. A., Bhat, K. A., Bhat, N. A., and Masoodi, S. D. (2010). Studies on status and host range of soft rot disease of cabbage (*Brassica oleracea* var Capitata) Kashmir Valley. *J. Phytol.* 2, 55–59.

Mitrev, S., Karov, I., Kovacevik, B., and Kostadinovska, E. (2014). *Pseudomonas* population causing tomato pith necrosis in the Republic of Macedonia. *J. Plant Pathol.* 96, 589–592. doi: 10.4454/JPP.V96I3.002

Moleleki, L. N., Onkendi, E. M., Mongae, A., and Kubheka, G. C. (2013). Characterisation of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. *Eur. J. Plant Pathol.* 135, 279–288. doi: 10.1007/s10658-012-0084-4

Moreira, S. I., Dutra, D. D. C., Rodrigues, A. C., Oliveira, J. R. D., Dhingra, O. D., and Pereira, O. L. (2013). Fungi and bacteria associated with post-harvest rot of ginger rhizomes in Espírito Santo, Brazil. *Trop. Plant Pathol.* 38, 218–226.

Moretti, C., Hosni, T., Vandemeulebroecke, K., Brady, C., De Vos, P., Buonaurio, R., et al. (2011). *Erwinia oleae* sp. nov., isolated from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*. *Int. J. Syst. Evol. Microbiol.* 61, 2745–2752. doi: 10.1099/ijs.0.026336-0

Mulet, M., Gomila, M., Scotta, C., Sánchez, D., Lalucat, J., and García-Valdés, E. (2012). Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus *Pseudomonas*. *Syst. Appl. Microbiol.* 35, 455–464. doi: 10.1016/j.syapm.2012.08.007

Mulet, M., Lalucat, J., and García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ. Microbiol.* 12, 1513–1530. doi: 10.1111/j.1462-2920.2010.02181.x

Nabhan, S., De Boer, S. H., Maiss, E., and Wydra, K. (2013). *Pectobacterium aroidearum* sp. nov., a soft rot pathogen with preference for monocotyledonous plants. *Int. J. Syst. Evol. Microbiol.* 63, 2520–2525. doi: 10.1099/ijs.0.046011-0

Nabhan, S., Wydra, K., Linde, M., and Debener, T. (2012). The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. *Plant Pathol.* 61, 498–508. doi: 10.1111/j.1365-3059.2011.02546.x

Nazerian, E., Sijam, K., Ahmad, Z. A. M., and Vadamalai, G. (2013). Characterization of *Pectobacterium carotovorum* subsp. *carotovorum* as a new disease on Lettuce in Malaysia. *Australas. Plant Dis. Notes* 8, 105–107. doi: 10.1007/s13314-013-0107-9

Nedaienia, R., and Fassihiani, A. (2011). Host range and distribution of *Pectobacterium betavasculorum* the causal agent of bacterial vascular necrosis and root rot of sugarbeet in fars province. *Iran. J. Plant Pathol.* 47, 47–48.

Ngadze, E., Brady, C. L., Coutinho, T. A., and Van Der Waals, J. E. (2012). Pectinolytic bacteria associated with potato soft rot and blackleg in South Africa and Zimbabwe. *Eur. J. Plant Pathol.* 134, 533–549. doi: 10.1007/s10658-012-0036-z

Novik, G., Savich, V., and Kiseleva, E. (2015). "An insight into beneficial *Pseudomonas* bacteria," in *Microbiology in Agriculture and Human Health*, (Europe: InTech), 73–105. doi: 10.5772/60502

Nykyri, J., Niemi, O., Koskinen, P., Nokso-Koivisto, J., Pasanen, M., Broberg, M., et al. (2012). Revised phylogeny and novel horizontally acquired virulence determinants of the model soft rot phytopathogen *Pectobacterium wasabiae* SCC3193. *PLOS Pathog.* 8:e1003013. doi: 10.1371/journal.ppat.1003013

O'Hara, C. M., Steigerwalt, A. G., Hill, B. C., Miller, J. M., and Brenner, D. J. (1998). First report of a human isolate of *Erwinia persicinus*. *J. Clin. Microbiol.* 36, 248–250.

Özen, A. I., and Ussery, D. W. (2012). Defining the *Pseudomonas* genus: where do we draw the line with *Azotobacter*? *Microb. Ecol.* 63, 239–248. doi: 10.1007/s00248-011-9914-8

Palleroni, N. J. (2005). Genus IX. *Stenotrophomonas* Palleroni and Bradbury 1993, 608VP. *Bergey's Manual Syst. Bacteriol.* 2, 107–115.

Parthiban, V., Prakasam, V., and Prabakar, K. (2012). Enzymatic changes in carrot roots induced by *Erwinia carotovora* var. *carotovora*. *Int. J. Pharma Biosci.* 3, 246–252.

Peix, A., Valverde, A., Rivas, R., Igual, J. M., Ramírez-Bahena, M. H., Mateos, P. F., et al. (2007). Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* 57, 1286–1290. doi: 10.1099/ijs.0.64621-0

Pomini, A. M., Manfio, G. P., Araújo, W. L., and Marsaioli, A. J. (2005). Acyl-homoserine lactones from *Erwinia psidii* R. IBSBF 435T, a guava phytopathogen (*Psidium guajava* L.). *J. Agric. Food Chem.* 53, 6262–6265. doi: 10.1021/jf050586e

Porch, T. G., Urrea, C. A., Beaver, J. S., Valentin, S., Peña, P. A., and Smith, J. R. (2012). Registration of TARS-MST1 and SB-DT1 multiple-stress-tolerant black bean germplasm. *J. Plant Regist.* 6, 75–80. doi: 10.3198/jpr2010.08.0501crg

Pritchard, L., Humphris, S., Saddler, G., Parkinson, N., Bertrand, V., Elphinstone, J., et al. (2013). Detection of phytopathogens of the genus *Dickeya* using a PCR primer prediction pipeline for draft bacterial genome sequences. *Plant Pathol.* 62, 587–596. doi: 10.1111/j.1365-3059.2012.02678.x

Rademaker, J. L., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., et al. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *Int. J. Syst. Evol. Microbiol.* 50, 665–677. doi: 10.1099/00207713-50-2-665

Reddy, P. P. (2015). *Plant Protection in Tropical Root and Tuber Crops*. (New Delhi, IND: Springer). doi: 10.1007/978-81-322-2389-4

Rees-George, J., Vanneste, J. L., Cornish, D. A., Pushparajah, I. P. S., Yu, J., Templeton, M. D., et al. (2010). Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S–23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. *Plant Pathol.* 59, 453–464. doi: 10.1111/j.1365-3059.2010.02259.x

Rich, A. E. (2013). *Potato Diseases*. New York, USA: Academic Press.

Rodriguez, R., Luis, M., Grajales, A., Arrieta-Ortiz, M., L., Salazar, C., et al. (2012). Genomes-based phylogeny of the genus *Xanthomonas*. *BMC Microbiol.* 12:43. doi: 10.1186/1471-2180-12-43

Rojas, A. M., Rios, J. E., Fischer-Le Saux, M., Jimenez, P., Reche, P., Bonneau, S., et al. (2004). *Erwinia toletana* sp. nov., associated with *Pseudomonas savastanoi*-induced tree knots. *Int. J. Syst. Evol. Microbiol.* 54, 2217–2222. doi: 10.1099/ijs.0.02924-0

Roper, M. C. (2011). *Pantoea stewartii* subsp. *stewartii*: lessons learned from a xylem-dwelling pathogen of sweet corn. *Mol. Plant Pathol.* 12, 628–637. doi: 10.1111/j.1364-3703.2010.00698.x

Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I., and Benno, Y. (2001). Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. *Microbiol. Immunol.* 45, 39. doi: 10.1111/j.1348-0421.2001.tb01272.x

Šalplachta, J., Kubesová, A., Horký, J., Matoušková, H., Tesařová, M., and Horká, M. (2015). Characterization of *Dickeya* and *Pectobacterium* species by capillary electrophoretic techniques and MALDI-TOF MS. *Anal. Bioanal. Chem.* 407, 7625–7635. doi: 10.1007/s00216-015-8920-y

Samson, R., Legendre, J., Christen, R., Fischer-Le Saux, Achouak, W., and Gardan, L. (2005). Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov. and *Dickeya zeae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 55, 1415–1427. doi: 10.1099/ijs.0.02791-0

Sanogo, S., Etarock, B., and Clary, M. (2011). First report of bacterial wilt caused by *Erwinia tracheiphila* on pumpkin and watermelon in New Mexico. *Plant Dis.* 95, 1583–1583. doi: 10.1094/PDIS-06-11-0507

Saranraj, P., Stella, D., and Reetha, D. (2012). Microbial spoilage of vegetables and its control measures: a review. *Int. J. Nat. Prod. Sci.* 2, 1–12.

Shang, J., Liu, B., and He, W. (2015). A new method to detect Lonsdalea quercina in infected plant tissues by real-time PCR. *Forest Pathol.* 45, 28–35. doi: 10.1111/efp.12125

Sharma, D., and Agrawal, K. (2014). Incidence and histopathological study of *Xanthomonas axonopodis*. *J. Agric. Technol.* 10, 233–242.

Shrestha, R., Koo, J., Park, D., Hwang, I., Hur, J., and Lim, C. (2003). *Erwinia pyrifoliae*, a causal endemic pathogen of shoot blight of Asian pear tree in Korea. *Plant Pathol. J.* 19, 294–300. doi: 10.5423/PPJ.2003.19.6.294

Singh, U., Singh, R. P., and Kohmoto, K. (2012). "Pathogenesis and host specificity in plant pathogenic bacteria," in *Prokaryotes*, ed. G. Meurant (Great Britain: Elsevier Science Ltd), 19–23.

Sławiak, M., Van Beckhoven, J. R., Speksnijder, A. G., Czajkowski, R., Grabe, G., and Van Der Wolf, J. M. (2009). Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp. strains isolated from potato in Europe. *Eur. J. Plant Pathol.* 125, 245–261. doi: 10.1007/s10658-009-9479-2

Sławiak, M., Van Doorn, R., Szemes, M., Speksnijder, A., Waleron, M., Van Der Wolf, J., et al. (2013). Multiplex detection and identification of bacterial pathogens causing potato blackleg and soft rot in Europe, using padlock probes. *Ann. Appl. Biol.* 163, 378–393. doi: 10.1111/aab.12075

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., et al. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 52, 1043–1047. doi: 10.1099/00207713-52-3-1043

Subramoni, S., Jha, G., and Sonti, R. (2006). "Virulence functions of xanthomonads," in *Plant-Associated Bacteria*, ed. S. Gnanamanickam (Heidelberg, NL: Springer), 535–571. doi: 10.1007/978-1-4020-4538-7_15

Swings, J. G., and Civerolo, E. L. (1993). *Xanthomonas*. London: Chapman & Hall.

Tayeb, A. L., Ageron, E., Grimont, F., and Grimont, P. (2005). Molecular phylogeny of the genus *Pseudomonas* based on rpoB sequences and application for the identification of isolates. *Res. Microbiol.* 156, 763–773. doi: 10.1016/j.resmic.2005.02.009

Terta, M., Azelmat, S., M'hand, R. A., Achbani, E. H., Barakate, M., Bouteau, F., et al. (2012). Molecular typing of *Pectobacterium carotovorum* isolated from potato tuber soft rot in Morocco. *Ann. Microbiol.* 62, 1411–1417. doi: 10.1007/s13213-011-0391-6

Thapa, S. P., Park, D. H., Kim, W. S., Choi, B. S., Lim, J. S., Choi, I. Y., et al. (2012). Comparative genomics of Japanese *Erwinia pyrifoliae* strain Ejp617 with closely related *Erwinias*. *Genome* 56, 83–90. doi: 10.1139/gen-2012-0094

Tourkya, B., Boubellouta, T., Dufour, E., and Leriche, F. (2009). Fluorescence spectroscopy as a promising tool for a polyphasic approach to pseudomonad taxonomy. *Curr. Microbiol.* 58, 39–46. doi: 10.1007/s00284-008-9263-0

Tournas, V. H. (2005). Spoilage of vegetable crops by bacteria and fungi and related health hazards. *Crit. Rev. Microbiol* 31, 33–44. doi: 10.1080/10408410590886024

Trantas, E., Sarris, P., Pentari, M., Mpalantinaki, E., Ververidis, F., and Goumas, D. (2015). Diversity among *Pseudomonas corrugata* and *Pseudomonas mediterranea* isolated from tomato and pepper showing symptoms of pith necrosis in Greece. *Plant Pathol.* 64, 307–318. doi: 10.1111/ppa.12261

Valenzuela-Soto, J., Maldonado-Bonilla, L., Hernández-Guzmán, G., Rincón-Enríquez, G., Martínez-Gallardo, N., Ramírez-Chávez, E., et al. (2015). Infection by a coronatine-producing strain of *Pectobacterium cacticidum* isolated from sunflower plants in Mexico is characterized by soft rot and chlorosis. *J. Gen. Plant Pathol.* 81, 368–381. doi: 10.1007/s10327-015-0606-y

van der Merwe, J. J., Coutinho, T. A., Korsten, L., and Van Der Waals, J. E. (2010). *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. *Eur. J. Plant Pathol.* 126, 175–185. doi: 10.1007/s10658-009-9531-2

van der Wolf, J. M., Nijhuis, E. H., Kowalewska, M. J., Saddler, G. S., Parkinson, N., Elphinstone, J. G., et al. (2014). *Dickeya solani* sp. nov., a pectinolytic plant-pathogenic bacterium isolated from potato (*Solanum tuberosum*). *Int. J. Syst. Evol. Microbiol.* 64, 768–774. doi: 10.1099/ijs.0.052944-0

Vandroemme, J., Cottyn, B., Baeyen, S., De Vos, P., and Maes, M. (2013). Draft genome sequence of *Xanthomonas fragariae* reveals reductive evolution and distinct virulence-related gene content. *BMC Genom.* 14:829. doi: 10.1186/1471-2164-14-829

Vauterin, L., Hoste, B., Kersters, K., and Swings, J. (1995). Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* 45, 472–489. doi: 10.1099/00207713-45-3-472

Vicente, J. G., and Holub, E. B. (2013). *Xanthomonas campestris* pv. campestris (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. *Mol. Plant Pathol.* 14, 2–18. doi: 10.1111/j.1364-3703.2012.00833.x

Waldee, E. L. (1945). Comparative studies of some peritrichous phytopathogenic bacteria. *Iowa State College J. Sci.* 19, 435–484.

Waleron, M., Czajkowski, R., Waleron, K., and Lojkowska, E. (2013a). Restriction fragment length polymorphism-based identification of '*Dickeya* Solani', A new genetic clade of *Dickeya* spp. *J. Plant Pathol.* 95, 609–613.

Waleron, M., Waleron, K., and Lojkowska, E. (2013b). Occurrence of *Pectobacterium wasabiae* in potato field samples. *Eur. J. Plant Pathol.* 137, 149–158. doi: 10.1007/s10658-013-0227-2

Waleron, M., Waleron, K., and Lojkowska, E. (2014). Characterization of *Pectobacterium carotovorum* subsp. odoriferum causing soft rot of stored vegetables. *Eur. J. Plant Pathol.* 139, 457–469. doi: 10.1007/s10658-014-0403-z

Waleron, M., Waleron, K., Podhajska, A. J., and Łojkowska, E. (2002). Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analysis of a recA gene fragment. *Microbiology* 148, 583–595. doi: 10.1099/00221287-148-2-583

Wells, J., Sheng, W., Ceponis, M., and Chen, T. (1987). Isolation and characterization of strains of *Erwinia ananas* from honeydew melons. *Phytopathology* 77, 511–514. doi: 10.1094/Phyto-77-511

Wensing, A., Gernold, M., and Geider, K. (2012). Detection of *Erwinia* species from the apple and pear flora by mass spectroscopy of whole cells and with novel PCR primers. *J. Appl. Microbiol.* 112, 147–158. doi: 10.1111/j.1365-2672.2011.05165.x

Werra, P. D., Bussereau, F., Keiser, A., and Ziegler, D. (2015). First report of potato blackleg caused by *Pectobacterium carotovorum* subsp. *brasiliense* in Switzerland. *Plant Dis.* 99, 551–552. doi: 10.1094/PDIS-07-14-0742-PDN

Wu, J., Liu, X., Diao, Y., Ding, Z., and Hu, Z. (2012). Authentication and characterization of a candidate antagonistic bacterium against soft rot of *Amorphophallus konjac*. *Crop. Prot.* 34, 83–87. doi: 10.1016/j.cropro.2011.12.008

Yaganza, E.-S., Tweddell, R., and Arul, J. (2014). Postharvest application of organic and inorganic salts to control potato (*Solanum tuberosum* L.) Storage softrot: plant tissue–salt physicochemical interactions. *J. Agric. Food Chem.* 62, 9223–9231. doi: 10.1021/jf5017863

Yan, H., Yu, S., Xie, G., Fang, W., Su, T., and Li, B. (2010). Grain discoloration of rice caused by *Pantoea ananatis* (synonym *Erwinia uredovora*) in China. *Plant Dis.* 94, 482–482. doi: 10.1094/PDIS-94-4-0482B

Zhao, Y., Li, P., Huang, K., Wang, Y., Hu, H., and Sun, Y. (2013). Control of postharvest soft rot caused by *Erwinia carotovora* of vegetables by a strain of *Bacillus amyloliquefaciens* and its potential modes of action. *World J. Microbiol. Biotechnol.* 29, 411–420. doi: 10.1007/s11274-012-1193-0

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 November 2015
doi: 10.3389/fmicb.2015.01238

The Membrane Proteins Involved in Virulence of *Cronobacter sakazakii* Virulent G362 and Attenuated L3101 Isolates

YingWang Ye[1,2,3,4] †, Jina Gao[1†], Rui Jiao[1†], Hui Li[1,2,3,4 †], Qingping Wu[2,3,4]*, Jumei Zhang[2,3,4] and Xian Zhong[2,3,4]*

[1] School of Biotechnology and Food Engineering, Hefei University of Technology, Hefei, China, [2] State Key Laboratory of Applied Microbiology Southern China, Guangzhou, China, [3] Guangdong Provincial Key Laboratory of Microbiology Culture Collection and Application, Guangzhou, China, [4] Guangdong Institute of Microbiology, Guangzhou, China

Cronobacter sakazakii is an opportunistic foodborne pathogen and the virulence differences were previously documented. However, information about membranous proteins involved in virulence differences was not available. In this study, virulent characterization such as biofilm formation and flagella motility between virulent *C. sakazakii* isolate G362 and attenuated L3101 were determined. Then, two-dimensional gel electrophoresis (2-DE) technology was used to preliminarily reveal differential expression of membranous proteins between G362 and L3101. On the mass spectrometry (MS) analysis and MASCOT research results, fourteen proteins with differential expression were successfully identified. At the threshold of twofold changes, five out of eight membranous proteins were up-regulated in G362. Using RT-PCR, the expression abundance of the protein (*enzV, ompX, lptE, pstB,* and *OsmY*) genes at mRNA levels was consistent with the results by 2-DE method. The findings presented here provided novel information and valuable knowledge for revealing pathogenic mechanism of *C. sakazakii*.

Keywords: membranous proteins, *Cronobacter sakazakii*, virulence factors, two-dimensional gel electrophoresis (2-DE)

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università degli Studi di Teramo, Italy

Reviewed by:
Learn-Han Lee, Monash University Malaysia, Malaysia
Maria Schirone, Università degli Studi di Teramo, Italy

****Correspondence:***
Yingwang Ye
yeyw04@mails.gucas.ac.cn;
Qingping Wu
wuqp203@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: *23 August 2015*
Accepted: *26 October 2015*
Published: *09 November 2015*

Citation:
Ye Y, Gao J, Jiao R, Li H, Wu Q, Zhang J and Zhong X (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

INTRODUCTION

The genus of *Cronobacter* is a group of opportunistic pathogens linked with life-threatening infections in neonates (Holý and Forsythe, 2014). *Cronobacter* sp. was isolated from various kinds of samples including food, soil, environments, clinical samples (Gurtler et al., 2005). In addition, these infections often occurred among poor immunity or low-birth weight newborn through consumption of contaminated powdered infant formula (Muytjens et al., 1983; Tall et al., 2014). The *Cronobacter* genus consists of *Cronobacter sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. condiment*, *C. universalis*, and *C. dublinensis* (Joseph et al., 2012a,b). Furthermore, *C. sakazakii* is the predominant species within *Cronobacter* isolated from powdered milk (Xu et al., 2014).

Membranous proteins were required for adhesion, invasion, and the release of toxin of pathogens, and further played important roles on host-pathogen interaction or host infections in foodborne pathogens (Allaoui et al., 1993; Monteville et al., 2003; Ellis and Kuehn, 2010).

Transmembrane signal proteins of two-component regulatory system in gram-negative pathogens contributed to immune escape or biofilm formation (Krachler et al., 2011). In addition, outer membrane proteins A (OmpA) and X (OmpX) in *C. sakazakii* played critical roles in adhesion to and invading human cells (Mohan Nair and Venkitanarayanan, 2007; Mohan Nair et al., 2009; Kim et al., 2010). Kothary et al. (2007) reported that *zpx* gene encoding the cell-bound zinc-containing metalloprotease might play important roles in dissemination of *Cronobacter* into the systemic circulation. However, little information about membranous proteins involved in virulence differences among *C. sakazakii* isolates is available.

To determine the potential virulence factors of pathogenic bacteria, the use of global techniques such as proteomics was widely applied in exploring different factors between virulent and attenuated bacteria (Srinivasa Rao et al., 2004; Hecker et al., 2010; Niemann et al., 2011).

In this study, the virulent characterization including biofilm formation, flagella motility, and O serotypes between G362 and L3101 was determined. Then, the 2-DE technology coupled with mass spectrometry was applied to identify global molecular information about differentially expressed membranous proteins. Finally, Real-time PCR was used to further validate the expression abundance of these novel membranous protein genes between G362 and L3101.

MATERIALS AND METHODS

Cronobacter sakazakii Isolates

The virulence differences between *C. sakazakii* G362 and L3101 from food samples were previously determined through intraperitoneal injection and histopathologic analysis (small intestine, kidney, and liver; Ye et al., 2015). The significant

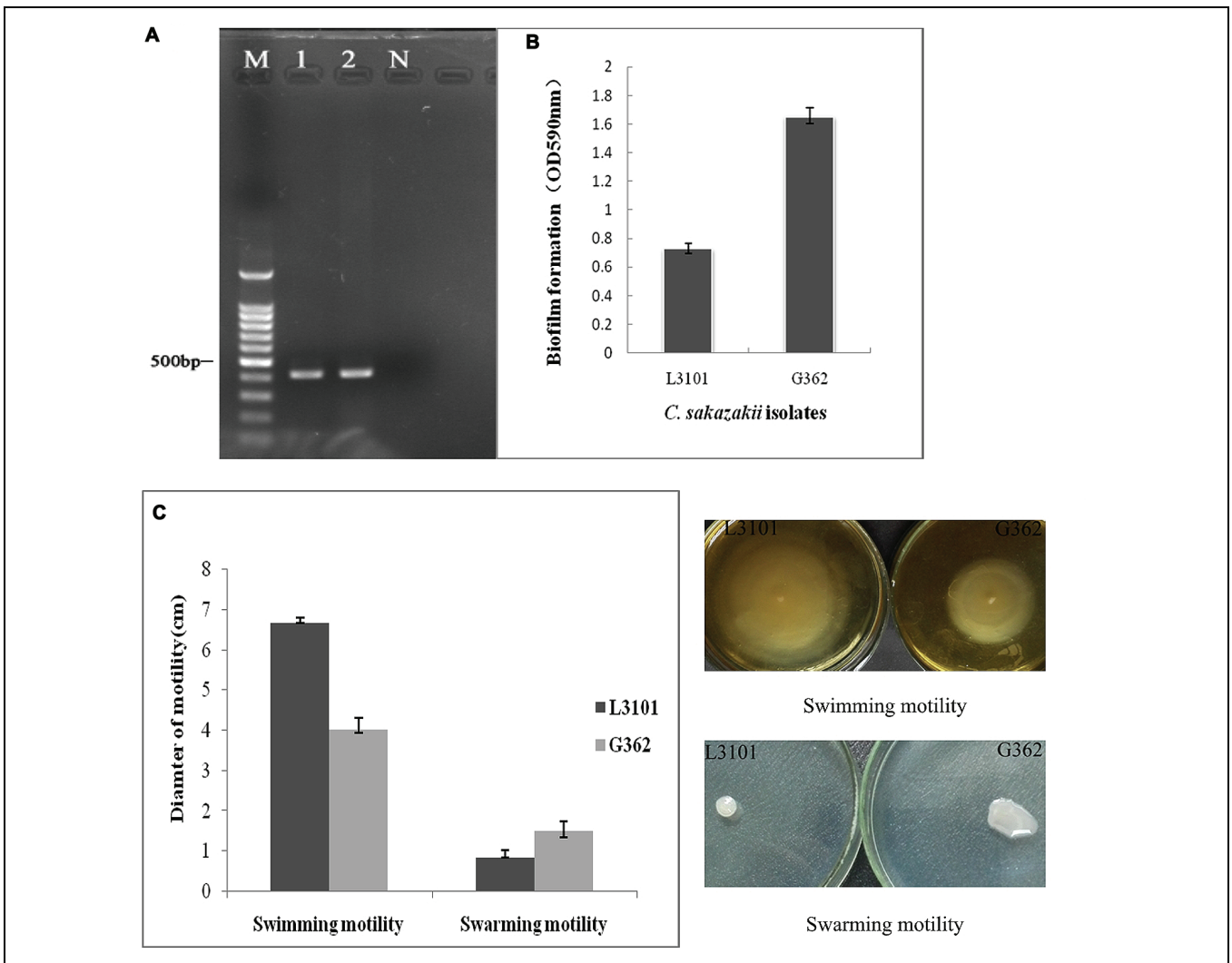


FIGURE 1 | The phenotypic characterization (O serotypes, biofilm formation, and motility) of *Cronobacter sakazakii* virulent G362 and attenuated L3101 isolate. (A) Detection of O serotypes by PCR; **(B)** Biofilm formation using crystal violet staining (CVS); **(C)** Detection of swimming and swarming motility.

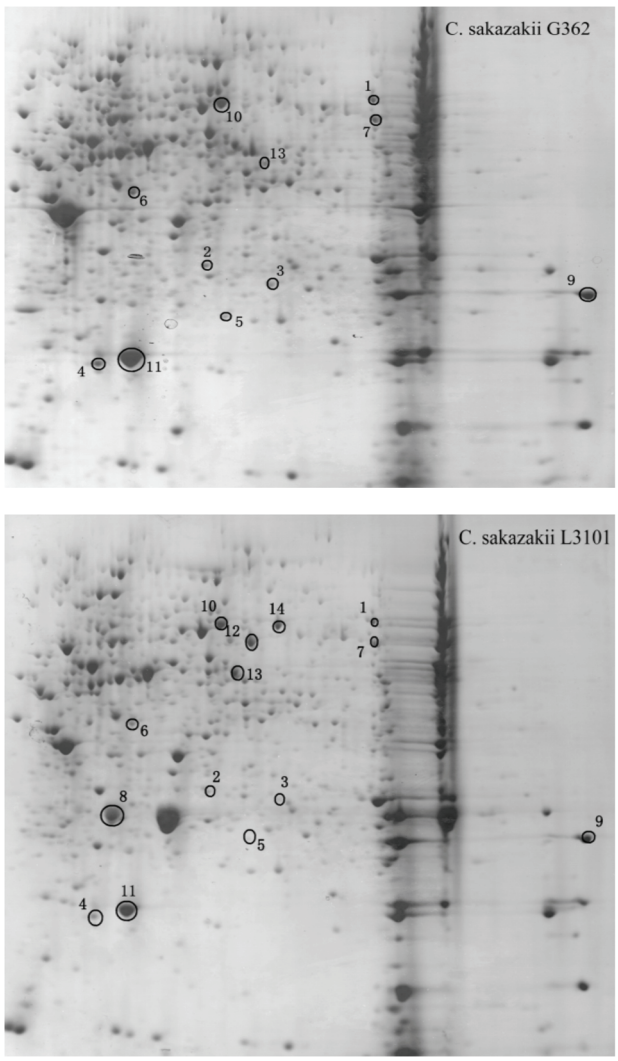


FIGURE 2 | Membranous proteomics profiles of virulent (G362) and attenuated (L3101) isolates. pH 3-10 (from left to right).

morphological change in small intestine, liver, and kidney further reveals the virulence differences between G362 and L3101.

Different Phenotypic Characterization between G362 and L3101

The biofilm formation between G362 and L3101 was determined using crystal violet staining (CVS) method as described by Hochbaum et al. (2011). The O serotypes were detected as described by Sun et al. (2012). In addition, the primers, PCR conditions, and PCR mixtures also referred to the description by Sun et al. (2012).

In addition, the motility of two isolates (G362 and L3101) was also determined. Media consisting of 10 g/L tryptose, 5 g/L NaCl, and 1.5 g/L agarose was used to test the swimming motility. Swimming plate was inoculated with *C. sakazakii* isolates from an overnight culture of TSA agar using a sterile toothpick and incubated at 37°C for 8 h. Media (8 g/L nutrient broth, 5 g/L D-glucose, and 5 g/L Difco bacto-agar) was made to determine the swarming motility. Swarming plate was inoculated with *C. sakazakii* isolates from swimming plate using a sterile toothpick and incubated at 37°C for 12 h. The diameter of motility at the agar dish interface was measured in triplicate.

Membranous Proteins Extraction and Quantification

Two *C. sakazakii* isolates (G362 and L3101) were inoculated into tryptic soy broth (TSB, Huankai, Guangzhou) for incubation at 37°C for 16 h. Two grams (wet weight) of G362 and L3101 were suspended in 5 ml of phosphate buffer saline (PBS, pH7.4) at 4°C. Then the cell suspension was sonicated in an ice bath (SONICS VC-505). Following the sonication process, Triton X-114 was applied for membranous proteins extraction using the method described by González de la Vara and Alfaro (2009). Then, two different phase collections and the insoluble pellet were performed to analyze SOD activity (Huang et al., 2006). Acetone precipitation was employed to remove the excess salts in the aqueous phase collection and detergent phase collection respectively. After lysis buffer (5 M urea, 2 M thiourea, 2% SB3-10, 2% CHAPS, 65 mM DTT, 40 mM Tris; Bio-Rad, USA) dissolution and centrifuged at 18, 000 $\times$ *g* for 1 h at 4°C, membrane proteins supernatant was stored at −80°C for use. Proteins qualification was measured by the bicinchoninic acid (BCA) assay as described by Krieg et al. (2005).

2-DE Analysis

For each sample, 100 μl (8 μg/μl) of membrane proteins extract, diluted with 200 μl of rehydration buffer (7 M urea, 2 M thiourea, 1% ASB-14, 65 mM DTT, 0.2% Bio-Lyte (w/v), 40 mM Tris, 0.001% Bromophenol blue; Bio-Rad, USA), was applied to a reswelling cassette with 17 cm immobiline dry strip (pH 3-10 NL, Bio-Rad, USA). Rehydration was allowed to proceed at 20°C to 16 h under silicone oil. The isoelectric focusing (IEF) was performed using the PROTEAN IEF system (Bio-Rad, USA). Each IPG strip current limit during IEF was set 50 μA at 20°C. The focusing was conducted in next five steps: (1) constant voltage at 250 V for 1 h; (2) constant voltage at 500 V for 30 min; (3) constant voltage at 1000 V for 2 h; (4) under linear ramping mode voltage increased from 1000 to 10, 000 V in 5 h; (5) at last constant voltage at 10, 000 V until the total volt-hours reached 68,000 V-h. The following steps including equilibration, SDS-PAGE electrophoresis, coomassie brilliant blue stain (CBB-stained), gel analysis, and subsequent MS analysis were performed as described by Cordwell et al. (2008) with modification. In brief, spots in gels between isolates corresponding to the same protein identifications were detected using PD-Quest (Bio-Rad) and the relative spot intensities calculated. Significantly changed spots were selected by rate increased/decreased twofold. Then Protein spots of interest were subjected to in-gel digestion, and then cut from the gels for mass spectrometric analysis. Protein identities were based on a combination of peptide fingerprint and MS/MS spectra. MS

TABLE 1 | Identification and relative quantification of differentially expressed membranous proteins by MALDI-TOF/TOF MS for virulent *Cronobacter sakazakii* G362 compared to L3101.

Spot No.[a]	Identified protein	Mascot score[b]	UniProt No.[c]	Calculated pI[d]	Mass (Da)[e]	Sequence coverage	Matched peptide[f]	Protein localizaion	Increase or decrease (fold change)
(1)	Putative multidrug resistance protein MdtD	71	A7MHI9	10.18	50787.54	14%	8	Inner membrane	↑(2.04)
(2)	Osmolarity sensory histidine kinase EnvZ	48	K8D7W7	6.35	49995.71	8%	4	Inner membrane	↑(2.63)
(3)	LPS-assembly lipoprotein LptE	263	A7MQS1	6.91	20160.72	20%	8	Outer membrane	↑(2.01)
(4)	Outer membrane protein X	313	I2EKJ1	4.84	18244.18	43%	12	Outer membrane	↑(2.20)
(5)	Osmotically inducible protein OsmY	182	K8CMY3	6.76	21320.04	32%	7	Outer membrane	↑(2.06)
(6)	Outer membrane protein A	129	M1JW19	5.20	37080.54	22%	8	Outer membrane	↑(3.43)
(7)	Putative ABC transporter permease	89	I2EF46	9.21	34737.27	48%	5	Membrane	↑(2.49)
(8)	Phosphate import ATP-binding protein PstB	92	F5VNR7	6.24	29110.44	11%	3	Membrane	↓(Only in attenuated strains)
(9)	50S ribosomal protein L3	386	A7MPI7	9.87	22241.48	54%	8	Cytoplasmic	↑(2.67)
(10)	Putative maltose-6′-phosphate glucosidase	107	A7MQ03	5.75	49315.43	17%	5	Cytoplasmic	↑(2.18)
(11)	DNA protection during starvation protein	183	A7MEY6	5.49	18589.09	21%	4	Cytoplasmic	↑(2.33)
(12)	GDP-mannose 4,6-dehydratase	264	A7MHG3	5.84	42046.81	35%	12	Cytoplasmic	↓(Only in attenuated strains)
(13)	GDP-L-fucose synthase	114	A7MHG4	5.90	35896.77	25%	7	Cytoplasmic	↓(2.75)
(14)	Methylthioribose kinase	93	A7MKY0	5.83	44372.46	15%	9	Cytoplasmic	↓(Only in attenuated strains)

[a]*The number refer to the spot numbers as given in* ***Figure 1****.*
[b]*MOWSE scores of the highest confident matches ($P < 0.05$).*
[c]*Accession numbers in uniprot protein database (available at http://www.uniprot.org).*
[d]*pI, the predicted isoelectric point calculated from the protein sequence.*
[e]*The predicted molecular weight calculated from the protein sequence.*
[f]*Number of experimental peptides matched to the predicted protein peptides.*

TABLE 2 | Primers used in the study by real-time PCR.

Genes	Sequences of primers (5′–3′)	size (bp)
OmpX	F:AAAAAGACCGCACTGAAGATGG R:TATCAGTGCCGTTGGTAGCCT	150
enzV	F:TCCATCAGGGCGATTTCTC R:GAATAATGCCTTTACCGACCTG	150
LptE	F:CCTCGGTCTTCCAGAACGGT R:GCGGCTTTGTCATACATCTCCT	196
OsmY	F:GCTGAGCGGCTTTGTTGA R:GATTTCGCTGGTGGTGGC	163
pstB	F:CGAAAACATTCTGAACCACTCC R:CGTTCCATAATGCGGCTTTG	197
16S rRNA	F-ACGAGTGGCGGACGGTGA R-TCAGTTCCAGTGTGGCTGG	238

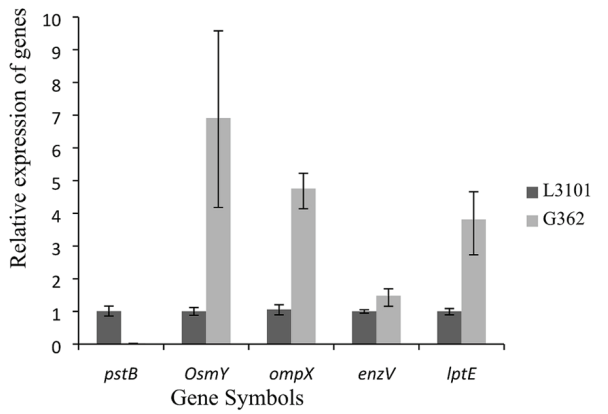


FIGURE 3 | Relative fluorescence quantitative PCR (RT-PCR) of membrane protein genes involved in virulence differences between G362 and L3101.

and MS/MS data were searched using MASCOT version 1.9.05 (Matrix Science) as search engine against the NCBI database. *C. sakazakii* species database was defined as a matching species. For each isolate sample, the 2-D gels in triplicate were analyzed.

Expression Abundance of Membranous Protein Genes by Real Time PCR

The overnight culture (1.0 ml) of *C. sakazakii* isolates was subject to extraction of RNA using bacterial RNA extraction kit (BIOMIGA, USA). Then, cDNA was obtained using first-strand cDNA synthesis kit (BIOMIGA, USA) according to the manufacturer' instructions. The RT-PCR mixture consisted of 2 μl cDNA, 10 μl 2 × SYBR Green qPCR mix (Giagen, Beijing), 0.2 μM for each primer, and RNase-free water in a final volume of 20 μl. The PCR program was initiated at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 59°C for 20 s, 72°C for 25 s using ABI Stepone plus (Applied Biosystems). The expression abundance of targeted genes between G362 and L3101 was determined in triplicate using $\Delta\Delta C_t$ method.

RESULTS AND DISCUSSION

Cronobacter sakazakii is an important foodborne pathogen associated with severe infections in poor immunity or low-birth weight newborn. The virulence differences of *C. sakazakii* G362 and L3101were previously documented by Ye et al. (2015). In this study, the virulent characterization including biofilm formation, O serotypes, and motility was further determined. In **Figure 1A**, both G362 and L3101 were O5 serotype by PCR assay (Sun et al., 2012). Xu et al. (2014) found that O2 serotype in *C. sakazakii* is the predominant serotype among isolates from powdered milk. However, the correlation between O serotypes and virulence was not revealed. Additionally, the significant differences of biofilm formation between G362 and L3101 were observed in **Figure 1B**, which might be involved in their adhesion to surfaces. The biofilm formation was associated with virulence in *Candida* (Hasan et al., 2009), *Escherichia coli* (Naves et al., 2008), and *Pseudomonas* (Ghadaksaz et al., 2015). Interestingly, the differences of motility (swimming and swarming) in **Figure 1C** were also founded between G362 and L3101 at 37°C for 8 h (swimming motility) and 12 h (swarming motility). Swimming ability of L3101 is stronger than that of G362, while the weakest swarming motility was also observed in L3101. This is the first report to determine the relationship between flagella motility and biofilm formation in *C. sakazakii*. Our result preliminarily indicated that swimming motility had negative effects on the biofilm formation, while swarming motility contributed to the biofilm formation. The biofilm formation required flagella motility in *Pseudomonas aeruginosa* (Barken et al., 2008), and motility and adhesion to surface was also connected in *E. coli* (Wood et al., 2006). However, flagella motility was not necessarily required for biofilm formation in *P. aeruginosa* (Caiazza et al., 2007) and *Burkholderia pseudomallei* (Lee et al., 2010). In *C. sakazakii*, the regulation of biofilm formation and flagella motility remains to be revealed.

Thereafter, to reveal the membranous proteins involved in virulence differences among *C. sakazakii* isolates, the membranous proteomic profiles of G362 and L3101 were firstly constructed using the 2-DE technology (**Figure 2**). Changes of membranous proteins in intensity (>twofold) were considered as significant differences. The expression abundance of 14 proteins was successfully identified between G362 and L3101. In **Table 1**, 8 out of 14 differentially expressed proteins was confirmed as membrane-associated proteins and expression abundance of seven membranous proteins was increased in G362. The different expression abundance of genes of membranous proteins including enzV, ompX, lptE, pstB, and OsmY were detected using real time PCR. The primers were listed in **Table 2** and 16S rRNA gene was used as internal control. From **Figure 3**, the expression of five membranous protein genes at mRNA levels using real-time PCR was consistent to results by 2-D method. The relative expression abundance of *osmF, ompX, enzV,* and *lptE* genes in G362 were 6.88, 4.49, 1.48, and 3.82 fold changes than those in L3101, respectively, while the expression of *pstB* in L3101 was 61.38 fold changes

than that in G362. The relative expression of five factors by real-time PCR was consistent with results using 2-DE method.

Based on proteomic analysis, the outer membrane proteins (ompA and ompX) were increased in G362 compared to those in L3101. The ompA and ompX proteins were reported to enhance adherence and invasion of human cells (Mohan Nair and Venkitanarayanan, 2007; Mohan Nair et al., 2009; Liu et al., 2012). Outer membrane A of *E. coli* has been reported enhanced the invasion of brain microvascular endothelial (BMEC) by assisting through the blood-brain barrier (Prasadarao et al., 1996). Our findings also indicated that ompA and ompX might be involved in virulence differences of *C. sakazakii* at membranous protein levels.

Besides, the expression abundance of novel proteins of LPS-assembly lipoprotein LptE, Osmolarity sensory histidine kinase EnvZ, and Osmotically inducible protein OsmY in G362 were increased compared to those in L3101. The LPS-assembly lipoprotein LptE encoded by the gene *lptE* in *E. coli* is essential for the biosynthesis of lipopolysaccharide, resulting in strong immunogenicity with host cells (Chng et al., 2010). Inner membrane osmolarity sensory histidine kinase EnvZ-OmpR pair in *C. sakazakii* would be activated in response to environmental osmoic stress (Amalaradjou and Venkitanarayanan, 2011). The receptor ompR could influence bacterial biofilm formation and flagella motility by regulating genes *ompF* and *ompC*. Further findings indicated that virulence of *ompR* mutants decreased in *Salmonella typhimurium* (Bernardini et al., 1990; Raczkowska et al., 2011). Additionally, Osmotically inducible protein OsmY also played important roles in pathogenic bacteria. In *S. typhimurium* and *E. coli*, OsmY was indirectly associated with virulence behavior (Bader et al., 2003; Dong and Schellhorn, 2009). In addition, putative multidrug resistance protein MdtD and putative ABC transporter permease were also up-regulated in G362. Some authors reported mdtABCD together with BaeSR two-component system contributed to resisting to host defenses through membrane drug-efflux pump (Baranova and Nikaido, 2002; Zoetendal et al., 2008; Leblanc et al., 2011). ABC transporter permease, a multi-pass membrane protein, was responsible for oligogalacturonide transport, but its detailed mechanism involved in virulence of bacteria is not documented.

Interestingly, the expression abundance of some proteins like phosphate import ATP-binding protein PstB, GDP-L-fucose synthase and methylthioribose kinase were increased in L3101. To date, the correlation of these proteins and bacterial virulence was not documented.

In summary, the relationship between flagella motility and biofilm formation was preliminarily determined, which indicated that motility was not necessarily required for biofilm formation. Then, membranous proteomic profiles between virulent and attenuated *C. sakazakii* isolate were firstly constructed. In spite of ompA and ompX, some novel factors (envZ, LptE, MdtD, and OsmY) were successfully identified by proteomics which were potentially involved in virulence differences. The findings here provided novel insights and better knowledge for revealing pathogenic mechanisms of *C. sakazakii*. However, the detailed roles and regulation of these potential virulence factors of *C. sakazakii*, and study on interaction between host and bacteria are also urgent for exploration of pathogenic mechanism.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the National Natural Science Foundation of China (31201292) and Guangdong Province, Chinese Academy of comprehensive strategic cooperation project (2012B090400017).

REFERENCES

Allaoui, A., Sansonetti, P. J., and Parsot, C. (1993). MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasions. *Mol. Microbiol.* 7, 59–68. doi: 10.1111/j.1365-2958.1993.tb01097.x

Amalaradjou, M. A. R., and Venkitanarayanan, K. (2011). Proteomic analysis of the mode of antibacterial action of trans-cinnamaldehyde against *Cronobacter sakazakii* 415. *Foodborne Pathog. Dis.* 8, 1095–1102. doi: 10.1089/fpd.2011.0841

Bader, M. W., Navarre, W. W., Shiau, W., Nikaido, H., Frye, J. G., McClelland, M., et al. (2003). Regulation of *Salmonella* typhimurium virulence gene expression by cationic antimicrobial peptides. *Mol. Microbiol.* 50, 219–230. doi: 10.1046/j.1365-2958.2003.03675.x

Baranova, N., and Nikaido, H. (2002). The BaeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. *J. Bacteriol.* 184, 4168–4176. doi: 10.1128/JB.184.15.4168-4176.2002

Barken, K. B., Pamp, S. J., Yang, L., Gjermansen, M., Bertrand, J. J., Klausen, M., et al. (2008). Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.* 10, 2331–2343.1. doi: 10.1111/j.1462-2920.2008.01658.x

Bernardini, M., Fontaine, A., and Sansonetti, P. (1990). The two-component regulatory system ompR-envZ controls the virulence of *Shigella flexneri*. *J. Bacteriol.* 172, 6274–6281.

Caiazza, N. C., Merritt, J. H., and Brothers, K. M. (2007). Inverse regulation of biofilms formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 189, 3603–3612. doi: 10.1128/JB.01685-06

Chng, S. S., Gronenberg, L. S., and Kahne, D. (2010). Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry* 49, 4565–4567. doi: 10.1021/bi100493e

Cordwell, S. J., Alice, C. L. L., Touma, R. G., Scott, N. E., Falconer, L., Jones, D., et al. (2008). Identification of membrane-associated proteins from *Campylobacter jejuni* strains using complementary proteomics technologies. *Proteomics* 8, 122–139. doi: 10.1002/pmic.200700561

Dong, T., and Schellhorn, H. E. (2009). Global effect of RpoS on gene expression in pathogenic *Escherichia coli* O157: H7 strain EDL933. *BMC Genomics* 10:349. doi: 10.1186/1471-2164-10-349

Ellis, T. N., and Kuehn, M. J. (2010). Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* 74, 81–94. doi: 10.1128/MMBR.00031-09

Ghadaksaz, A., Fooladi, A. A. I., Hosseini, H. M., and Amin, M. (2015). The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates. *J. Appl. Biomed.* 13, 61–68. doi: 10.1016/j.jab.2014.05.002

González de la Vara, L. E., and Alfaro, B. L. (2009). Separation of membrane proteins according to their hydropathy by serial phase partitioning with Triton X-114. *Anal. Biochem.* 387, 280–286. doi: 10.1016/j.ab.2009.01.035

Gurtler, J. B., Kornacki, J. L., and Beuchat, L. R. (2005). *Enterobacter sakazakii*: a coliform of increased concern to infant health. *Int. J. Food Microbiol.* 104, 1–34. doi: 10.1016/j.ijfoodmicro.2005.02.013

Hasan, F., Xess, I., Wang, X., Jain, N., and Fries, B. C. (2009). Biofilm formation in clinical Candida isolates and its association with virulence. *Microb. Infect.* 11, 753–761. doi: 10.1016/j.micinf.2009.04.018

Hecker, M., Becher, D., Fuchs, S., and Engelmann, S. (2010). A proteomic view of cell physiology and virulence of *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 300, 76–87. doi: 10.1016/j.ijmm.2009.10.006

Hochbaum, A. I., Kolodkin-Gal, I., Foulston, L., Kolter, R., Aizenberg, J., and Losick, R. (2011). Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. *J. Bacteriol.* 193, 5616–5622. doi: 10.1128/JB.05534-11

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

Huang, C. Z., Lin, X. M., Wu, L. N., Zhang, D. F., Liu, D., Wang, S. Y., et al. (2006). Systematic identification of the subproteome of *Escherichia coli* cell envelope reveals the interaction network of membrane proteins and membrane-associated peripheral proteins. *J. Proteome Res.* 5, 3268–3276. doi: 10.1021/pr060257h

Joseph, S., Cetinkaya, E., Drahovske, 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 novel 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., McClelland, 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

Kim, K., Kim, K.-P., Choi, J., Lim, J.-A., Lee, J., Hwang, S., et al. (2010). Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. *Appl. Environ. Microbiol.* 76, 5188–5198. doi: 10.1128/AEM.02498-09

Krachler, A. M., Ham, H., and Orth, K. (2011). Outer membrane adhesion factor multivalent adhesion molecule 7 initiates host cell binding during infection by gram-negative pathogens. *Proc. Natl. Aca. Sci. U.S.A.* 108, 11614–11619. doi: 10.1073/pnas.1102360108

Kothary, M. H., McCardell, B. A., Frazar, C. D., Deer, D., and Tall, B. D. (2007). Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for *Enterobacter sakazakii*. *Appl. Environ. Microbiol.* 73, 4142–4151. doi: 10.1128/AEM.02729-06

Krieg, R. C., Dong, Y., Schwamborn, K., and Knuechel, R. (2005). Protein quantification and its tolerance for different interfering reagents using the BCA-method with regard to 2D-SDS PAGE. *J. Biochem. Biophys. Methods* 65, 13–19. doi: 10.1016/j.jbbm.2005.08.005

Leblanc, S. K., Oates, C. W., and Raivio, T. L. (2011). Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. *J. Bacteriol.* 193, 3367–3375. doi: 10.1128/JB.01534-10

Lee, H. S., Gu, F., Ching, S. M., Lam, Y., and Chua, K. L. (2010). CdpA Is a *Burkholderia pseudomallei* cyclic di-GMP phosphodiesterase involved in autoaggregation, flagellum synthesis, motility, biofilm formation, cell invasion, and cytotoxicity. *Infect. Immun.* 78, 1832–1840. doi: 10.1128/IAI.00446-09

Liu, Q., Mittal, R., Emami, C. N., Iversen, C., Ford, H. R., and Prasadarao, N. V. (2012). Human isolates of *Cronobacter sakazakii* bind efficiently to intestinal epithelial cells in vitro to induce monolayer permeability and apoptosis. *J. Surg. Res.* 176, 437–447. doi: 10.1016/j.jss.2011.10.030

Mohan Nair, M. K., and Venkitanarayanan, K. (2007). Role of bacterial OmpA and host cytoskeleton in the invasion of human intestinal epithelial cells by *Enterobacter sakazakii*. *Pediatr. Res.* 62, 664–669. doi: 10.1203/PDR.0b013e3181587864

Mohan Nair, M. K., Venkitanarayanan, K., Silbart, L. K., and Kim, K. S. (2009). Outer membrane protein A (OmpA) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. *Foodborne Pathog. Dis.* 6, 495–501. doi: 10.1089/fpd.2008.0228

Monteville, M. R., Yoon, J. E., and Konkel, M. E. (2003). Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology* 149, 153–165. doi: 10.1099/mic.0.25820-0

Muytjens, H. L., Zanen, H. C., Sonderkamp, H. J., Kolee, L. A., Wachsmuth, I. K., and Farmer, J. J. (1983). Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. *J. Clin. Microbiol.* 18, 115–120.

Naves, P., Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., et al. (2008). Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains. *Microb. Pathog.* 45, 86–91. doi: 10.1016/j.micpath.2008.03.003

Niemann, G. S., Brown, R. N., Gustin, J. K., Stufkens, A., Shaikh-Kidwai, A. S., Li, J., et al. (2011). Discovery of novel secreted virulence factors from *Salmonella enterica* serovar Typhimurium by proteomic analysis of culture supernatants. *Infect. Immun.* 79, 33–43. doi: 10.1128/IAI.00771-10

Prasadarao, N. V., Wass, C. A., Weiser, J. N., Stins, M. F., Huang, S. H., and Kim, K. S. (1996). Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect. Immun.* 64, 146–153.

Raczkowska, A., Skorek, K., Bielecki, J., and Brzostek, K. (2011). OmpR controls *Yersinia enterocolitica* motility by positive regulation of flhDC expression. *Antonie Van Leeuwenhoek* 99, 381–394. doi: 10.1007/s10482-010-9503-8

Srinivasa Rao, P., Yamada, Y., Tan, Y. P., and Leung, K. Y. (2004). Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol. Microbiol.* 53, 573–586. doi: 10.1111/j.1365-2958.2004.04123.x

Sun, Y. M., Wang, M., Wang, Q., Cao, B. Y., He, X., Li, K., et al. (2012). 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

Tall, B. D., Chen, Y., Yan, Q., Gopinath, G. R., Grim, C. J., Jarvis, K. G., et al. (2014). *Cronobacter*: an emergent pathogen causing meningitis to neonates through their feeds. *Sci. Prog.* 97, 154–172. doi: 10.3184/003685014X13994743930498

Wood, T. K., Gonzalez Barrios, A. F., Herzberg, M., and Lee, J. (2006). Motility influences biofilm architecture in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 72, 361–367. doi: 10.1007/s00253-005-0263-8

Xu, X. K., Wu, Q. P., Zhang, J. M., Ye, Y. W., Yang, X. J., and Dong, X. H. (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

Ye, Y. W., Li, H., Ling, N., Han, Y. J., Wu, Q. P., Xu, X. K., et al. (2015). Identification of potential virulence factors of *Cronobacter sakazakii* isolates by comparative proteomic analysis. *Int. J. Food Microbiol.* doi: 10.1016/j.ijfoodmicro.2015.08.025

Zoetendal, E. G., Smith, A. H., Sundset, M. A., and Mackie, R. I. (2008). The BaeSR two-component regulatory system mediates resistance to condensed tannins in *Escherichia coli*. *Appl. Environ. Microbiol.* 74, 535–539. doi: 10.1128/AEM.02271-07

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.

frontiers in Microbiology

REVIEW
published: 03 November 2015
doi: 10.3389/fmicb.2015.01227

An insight into the isolation, enumeration, and molecular detection of *Listeria monocytogenes* in food

*Jodi Woan-Fei Law[1†], Nurul-Syakima Ab Mutalib[2†], Kok-Gan Chan[3] and Learn-Han Lee[1]**

[1] Biomedical Research Laboratory, Jeffrey Cheah School of Medicine and Health Sciences, Monash University, Bandar Sunway, Malaysia, [2] UKM Medical Molecular Biology Institute, UKM Medical Centre, Kuala Lumpur, Malaysia, [3] Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
Qiaobin Xiao, Cornell University, USA
Juan Aguirre, Food Safety Adviser Chile, Chile
Paula Teixeira, Universidade Católica Portuguesa, Portugal

***Correspondence:**
Learn-Han Lee
lee.learn.han@monash.edu; leelearnhan@yahoo.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: *20 August 2015*
Accepted: *20 October 2015*
Published: *03 November 2015*

Citation:
Law JW-F, 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

Listeria monocytogenes, a foodborne pathogen that can cause listeriosis through the consumption of food contaminated with this pathogen. The ability of *L. monocytogenes* to survive in extreme conditions and cause food contaminations have become a major concern. Hence, routine microbiological food testing is necessary to prevent food contamination and outbreaks of foodborne illness. This review provides insight into the methods for cultural detection, enumeration, and molecular identification of *L. monocytogenes* in various food samples. There are a number of enrichment and plating media that can be used for the isolation of *L. monocytogenes* from food samples. Enrichment media such as buffered *Listeria* enrichment broth, Fraser broth, and University of Vermont Medium (UVM) *Listeria* enrichment broth are recommended by regulatory agencies such as Food and Drug Administration-bacteriological and analytical method (FDA-BAM), US Department of Agriculture-Food and Safety (USDA-FSIS), and International Organization for Standardization (ISO). Many plating media are available for the isolation of *L. monocytogenes*, for instance, polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol, Oxford, and other chromogenic media. Besides, reference methods like FDA-BAM, ISO 11290 method, and USDA-FSIS method are usually applied for the cultural detection or enumeration of *L. monocytogenes*. most probable number technique is applied for the enumeration of *L. monocytogenes* in the case of low level contamination. Molecular methods including polymerase chain reaction, multiplex polymerase chain reaction, real-time/quantitative polymerase chain reaction, nucleic acid sequence-based amplification, loop-mediated isothermal amplification, DNA microarray, and next generation sequencing technology for the detection and identification of *L. monocytogenes* are discussed in this review. Overall, molecular methods are rapid, sensitive, specific, time- and labor-saving. In future, there are chances for the development of new techniques for the detection and identification of foodborne with improved features.

Keywords: ***Listeria* species, isolation, enumeration, molecular detection, foodborne pathogens**

INTRODUCTION

Listeria monocytogenes has become an important foodborne pathogen and it can be found in a variety of foods which include raw foods and processed foods (Gasanov et al., 2005; Janzten et al., 2006; Liu, 2006; Jeyaletchumi et al., 2010a; Välimaa et al., 2015). *L. monocytogenes* has been a serious threat to the food industry due to its ability to survive the most common food processing conditions such as extreme pH, high salt concentration, low water activity, and refrigeration temperatures (Liu, 2006; Zunabovic et al., 2011; Jadhav et al., 2012). It is known that *L. monocytogenes* can be eliminated or reduced by pasteurization process because it cannot survive pasteurization temperatures in food processing (Jadhav et al., 2012). For instance, a study conducted by Murphy et al. (2003) showed that hot water pasteurization and steam pasteurization resulted in a 7 $\log_{10}$ (CFU/g) reduction of *L. monocytogenes* in inoculated single packaged fully cooked chicken breast fillets, 227 g packaged fully cooked chicken strips and 454 g packaged fully cooked chicken strips when pasteurized at 90°C for 5, 25, and 35 min, respectively. However, post-processing or post-pasteurization contamination by *L. monocytogenes* may occur due to cross-contamination or biofilms (Jadhav et al., 2012). *L. monocytogenes* can attach to food contact surfaces such as stainless steel and polystyrene during food processing and form biofilms which is important for their survival in hostile environments (Jadhav et al., 2012; Da Silva and De Martinis, 2013; Välimaa et al., 2015). Biofilms may persist for a long period of time and they can tolerate high concentrations of disinfectants, sanitizers, and antimicrobials (Välimaa et al., 2015). Hence, this may result in the contamination of food contact surfaces which then lead to higher risk of food contamination during and/or after processing. Food contaminated with *L. monocytogenes* has posed a great concern to the food industry as it can cause serious infection known as listeriosis when ingested and it is also one of the causes of recalls which may result in large economic losses (Jemmi and Stephan, 2006). Thus, microbiological food testing is important to ensure the safety of food products (Dwivedi and Jaykus, 2011; Välimaa et al., 2015).

DETECTION AND IDENTIFICATION OF *Listeria monocytogenes*

Enrichment Media and Selectivity

There are various selective enrichment and plating media that have been developed and utilized for the isolation and detection of *L. monocytogenes* in food and environmental samples. As required by majority of the regulatory agencies, the isolation methods must be able to detect one *Listeria* organism per 25 g of food. In order to achieve this sensitivity, enrichment methods are required to allow the organism to grow and reach a detectable level of $\sim 10^4$–10^5 CFU ml^{-1}, before plating onto selective media and confirmation of cultures. Antimicrobial agents are employed in enrichment and plating media to suppress competing microflora as *Listeria* cells are slow growing and can be rapidly out-grown by competitors. The most common selective agents are acriflavine, nalidixic acid, and cycloheximide (Beumer and Hazeleger, 2003; Gasanov et al., 2005; Janzten et al., 2006; Jeyaletchumi et al., 2010a). The function of acriflavine is to inhibit the growth of other Gram-positive bacteria and it is often used in combination with other selective agents, for instance, polymyxin B-sulfate, cycloheximide, potassium thiocyanate, and nalidixic acid. Nalidixic acid is used for the inhibition of Gram-negative bacteria while cycloheximide is used for the inhibition of fungi (Beumer and Hazeleger, 2003; Janzten et al., 2006; Jeyaletchumi et al., 2010a). Besides, there are other antimicrobials that are usually added into the media such as broad-spectrum ceftazidime and moxalactam as well as lithium chloride (Janzten et al., 2006).

In addition, esculin is an important carbohydrate that is usually incorporated in *Listeria* enrichment and plating media (Bush and Donnelly, 1992; Janzten et al., 2006; Jeyaletchumi et al., 2010a). All *Listeria* sp. are capable of esculin hydrolysis and this process will result in the formation of an intense black color in the media. This is due to the presence of esculin and ferric iron in the media, in which the ferric iron forms complex with 6,7-dihidroxycoumarin, the product of esculin cleavage by β-D-glucosidase, resulting in a black precipitate (Fraser and Sperber, 1988; Janzten et al., 2006; Jeyaletchumi et al., 2010a). Hence, cultures that produce an intense black color indicate the presence of *Listeria*.

The regulatory agencies have recommended several selective enrichment media for *L. monocytogenes* such as buffered *Listeria* enrichment broth (BLEB), Fraser broth, and University of Vermont Medium (UVM) *Listeria* enrichment broth. BLEB is recommended in the US Food and Drug Administration (FDA) bacteriological and analytical method (BAM) for the isolation and identification of *L. monocytogenes*. BLEB is a modification of the formula by Lovett et al. (1987), in which disodium phosphate is added into the medium to increase the buffering capacity of the medium, thus, resulting in the improvement of enrichment properties. Selective agents which include acriflavine, cycloheximide, and nalidixic acid are added into the medium after an initial 4 h of non-selective pre-enrichment (Magalhães et al., 2014).

University of Vermont Medium *Listeria* enrichment broth is recommended in the US Department of Agriculture-Food and Safety (USDA-FSIS) method for the isolation and detection of *L. monocytogenes*. UVM is based on the formulation of Donnelly and Baigent (1986). This enrichment broth is suggested as the primary enrichment broth for the recovery of heat-injured *L. monocytogenes*. Nalidixic acid and acriflavine are the selective agents added into the medium (Magalhães et al., 2014).

International Organization for Standardization (ISO) 11290 method has suggested the use of Fraser broth for the selective enrichment of *L. monocytogenes* in food and environmental samples. Fraser broth is based on the formulation of Fraser and Sperber (1988) whereby it is a modification of USDA secondary enrichment broth by the addition of ferric ammonium citrate and lithium chloride. The selective agents added into Fraser broth are nalidixic acid and acriflavine (Magalhães et al., 2014).

Presumptive *L. monocytogenes* can be detected within 48 h by using Fraser broth (Fraser and Sperber, 1988).

The frequently recommended selective differential plating media by FDA-BAM, ISO, and USDA for the isolation of *Listeria* sp. are PALCAM (polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol) and Oxford (Zunabovic et al., 2011). PALCAM and Oxford are both useful for the isolation of *Listeria* sp. from food samples with injured *Listeria* cells and/or rich in competitive microflora (El Marrakchi et al., 2005). PALCAM agar was initially developed by Van Netten et al. (1989) for the detection and enumeration of *L. monocytogenes* and other *Listeria* sp. from food samples. PALCAM agar consists of Columbia Blood Agar with 23.0 g/L protease peptones, 0.5 g/L glucose, 1.0 g/L starch, 3.0 g/L yeast extract, and 5.0 g/L sodium chloride. The selectivity of this medium is achieved by the addition of 15 g/L lithium chloride, 0.01 g/L polymyxin B, 0.005 g/L acriflavine, and 0.02 g/L ceftazidime. The differentiation on PALCAM agar is based on (1) esculin hydrolysis, addition of 0.8 g/L esculin and 0.5 g/L ferric salt and (2) mannitol fermentation, addition of 10 g/L mannitol and 0.08 g/L phenol red (Van Netten et al., 1989; Magalhães et al., 2014). PALCAM agar plated with bacteria is usually incubated for 24–48 h at 37°C (Jamali et al., 2013; Ajay Kumar et al., 2014; Osman et al., 2014). Since all *Listeria* sp. can hydrolyze esculin, they are visually confirmed by a blackening of the medium and their colonies are about 2 mm diameter, gray-green in color with a black sunken center and a black halo. Occasionally, *Enterococcus* sp. or *Staphylococcus* sp. may grow on PALCAM agar. However, they can be distinguished from *Listeria* sp. via mannitol fermentation. Mannitol fermentation causes a color change in the colony and/or surrounding medium from gray or red to yellow due to the production of acids. Colonies of these mannitol fermenting organisms are yellow with a yellow halo or gray with a brown-green halo (Van Netten et al., 1989; Ajay Kumar et al., 2014; Osman et al., 2014).

Oxford agar was initially developed by Curtis et al. (1989) for the isolation of *L. monocytogenes* from clinical specimens. Oxford agar has been extensively used in many studies for the isolation and detection of *L. monocytogenes* from various food samples (Pinto et al., 2001; Rudol and Scherer, 2001; Gudbjörnsdóttir et al., 2004; Mena et al., 2004; Alessandria et al., 2010). Oxford consists of Columbia Blood Agar with 23 g/L protease peptones, 5.0 g/L sodium chloride, and 1.0 g/L starch. The selectivity of Oxford is achieved by the addition of 15 g/L lithium chloride, 0.005 g/L acriflavine, 0.02 g/L colistin sulfate, 0.4 g/L cycloheximide, 0.002 g/L cefotetan, and 0.01 g/L fosfomycin. The differentiation of *Listeria* sp. on Oxford agar is based on esculin hydrolysis which is aided by the addition of 1 g/L esculin and 0.5 g/L ferric ammonium citrate into the agar (Curtis et al., 1989; Janzten et al., 2006; Magalhães et al., 2014). Oxford agar is incubated for 24–48 h at 37°C after plating (Curtis et al., 1989; Alessandria et al., 2010). After 24 h of incubation, *L. monocytogenes* colonies are olive-green with a black halo. After 48 h of incubation, *L. monocytogenes* colonies are about 2–3 mm in diameter, the color turns darker with a black sunken center and surrounded by black zones. Other *Listeria* sp. colonies have a similar appearance to *L. monocytogenes* colonies. Colonies of other *Listeria* sp. are black with a black halo after 24 h of incubation and they remain the same after 48 h of incubation but with a sunken center (Curtis et al., 1989; Magalhães et al., 2014). *Staphylococcus* sp. may grow on Oxford agar occasionally and their colonies are yellow in color, irregular in size as well as shape (Curtis et al., 1989). A variation of Oxford agar has been developed and it is known as Modified Oxford Agar (MOX). MOX is recommended for the isolation and identification of *L. monocytogenes* from processed meat and poultry products whereas Oxford agar is recommended for the isolation of *L. monocytogenes* from enrichment broth cultures (Magalhães et al., 2014).

The main limitation of PALCAM and Oxford is the inability to distinguish between *L. monocytogenes* from non-pathogenic *Listeria* sp. (El Marrakchi et al., 2005; Zunabovic et al., 2011). Hence, these plating media are not able to provide a rapid detection of *L. monocytogenes* from foods. This has led to the development of chromogenic media which can improve the isolation of *L. monocytogenes* as they are able to differentiate *L. monocytogenes* and/or pathogenic *Listeria* sp. from other non-pathogenic *Listeria* sp. (Beumer and Hazeleger, 2003). Chromogenic media detect essential determinants of pathogenicity of *Listeria* sp. and majority of these media are commercially available as ready-to-use plates (Janzten et al., 2006; Zunabovic et al., 2011). Besides, presumptive *L. monocytogenes* can be identified after 24 h by using chromogenic media (Jeyaletchumi et al., 2010a). Several studies have demonstrated that chromogenic media such as Agar *Listeria* according to Ottaviani and Agosti (ALOA) and CHROMagar^{TM} *Listeria* are more sensitive, specific, time and cost saving in *L. monocytogenes* detection compared to non-chromogenic media such as PALCAM and Oxford (Vlaemynck et al., 2000; Hegde et al., 2007; Jamali et al., 2013).

Agar *Listeria* according to Ottaviani and Agosti is a chromogenic medium developed by Ottaviani et al. (1997) for the isolation of *Listeria* sp. and specific detection of *L. monocytogenes*. The selectivity of ALOA is achieved by the addition of lithium chloride and antimicrobials such as ceftazidime, polymyxin B, nalidixic acid, and cycloheximide (Beumer and Hazeleger, 2003; Magalhães et al., 2014). The differentiation of *Listeria* sp. on ALOA is achieved by the incorporation of chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, X-glucoside) in the medium for the detection of β-D-glucosidase activity, common to all *Listeria* sp. The differentiation of *L. monocytogenes* from other *Listeria* sp. is based on the production of phosphatidylinositol-specific phospholipase C (PI-PLC) which is encoded by the virulence gene *plc*A present in *L. monocytogenes*. ALOA detects PI-PLC that is present in *L. monocytogenes* and in some strains of *L. ivanovii* through the hydrolysis of L-α-phosphatidylinositol in the medium by PI-PLC. This results in the production of water insoluble fatty acids and the formation of an opaque halo around the colonies. In ALOA, all *Listeria* sp. produce blue-green colonies and pathogenic *Listeria* sp. such as *L. monocytogenes* and *L. ivanovii* produce blue-green colonies with an opaque halo (Ottaviani et al., 1997; Beumer and Hazeleger, 2003; Janzten et al.,

2006; Jeyaletchumi et al., 2010a; Zunabovic et al., 2011; Magalhães et al., 2014; Park et al., 2014).

CHROMagar™ *Listeria* (Becton Dickson Diagnostics) is one of the variations of ALOA that have been developed for the isolation and detection of *L. monocytogenes*. On CHROMagar™ *Listeria*, colonies of *L. monocytogenes* are blue with a white halo and colonies of other *Listeria* sp. are blue without halo. Some strains of *L. ivanovii* may grow on CHROMagar™ *Listeria* and they also produce blue colonies with a white halo (Magalhães et al., 2014). Additional variations of ALOA and commercially available media include Biosynth Chromogenic Medium (BCM) *L. monocytogenes* detection system (Biosynth), Compass L. mono (Biokar Diagnostics), Brilliance™ *Listeria* Agar (Oxoid) and chromID Ottaviani Agosti Agar (bioMérieux; Janzten et al., 2006; Zunabovic et al., 2011).

Rapid' *L. mono* agar is a chromogenic medium that operates differently than ALOA. Rapid' *L. mono* agar detects PI-PLC that is present in *L. monocytogenes* and *L. ivanovii* through the hydrolysis of a different substrate by PI-PLC, which is 5-bromo-4-chloro-3-indolyl-myo-inositol-1-phospjhate (X-IP). Cleavage of X-IP by PI-PLC results in the production of blue colonies. Hence, *L. monocytogenes* and *L. ivanovii* appear as blue colonies on Rapid' *L. mono* agar. Furthermore, the addition of xylose into the medium enables the differentiation of *L. monocytogenes* from *L. ivanovii*. The ability of *L. ivanovii* to metabolize xylose results in the production of blue colonies with a yellow halo. As for *L. monocytogenes*, the colonies produced are blue without halo due to the inability to metabolize xylose. Other *Listeria* sp. that grow on Rapid' *L. mono* agar will appear as white colonies with or without a yellow halo (Janzten et al., 2006; Zunabovic et al., 2011; Magalhães et al., 2014).

In general, chromogenic media are able to isolate and distinguish *L. monocytogenes* from other *Listeria* sp. and thus allowing a more rapid detection of *L. monocytogenes*. However, the sensitivity and specificity of the culture media may be affected by the types of food matrices (Andritsos et al., 2013). For instance, the study conducted by Aragon-Alegro et al. (2008) indicated that the sensitivity and specificity of CHROMagar™ *Listeria* for the detection of *L. monocytogenes* in sliced cooked ham (56.2% sensitivity and 73.6% specificity), minced beef meat (92.7% sensitivity and 76.8% specificity) and frankfurters (91.2% sensitivity and 84.2% specificity) were different. Hence, there are no particular medium which is perfect for the isolation of *L. monocytogenes* from various food samples (Churchill et al., 2006; Andritsos et al., 2013).

Cultural Detection and Enumeration of *Listeria monocytogenes*

Traditionally, the detection and identification of pathogens in foods involve the use of culture methods followed by phenotypic confirmation based on standard culture (e.g., haemolysis and phospholipase C), biochemical and immunological identification (Gasanov et al., 2005; Janzten et al., 2006). The conventional methods are simple, sensitive, inexpensive, and important when bacterial culture is required as the end result from positive samples (Churchill et al., 2006; Janzten et al., 2006; Law et al., 2015). Generally, the culture methods involve a two-stage enrichment process followed by plating on a selective differential agar (Beumer and Hazeleger, 2003; Janzten et al., 2006). The procedures may vary depending on the number of cells expected in a sample and/or the official culture reference methods used. The success of culture methods depends on several factors. For instance, the amount and state of the bacteria in the sample, the selectivity of the media (balance between inhibition of competitors and inhibition of the target bacteria), electivity of the isolation medium (difference between the target bacteria and competitive microflora) and the conditions of incubation (e.g., temperature, time, and oxygen; Beumer and Hazeleger, 2003).

The food samples are homogenized prior to the two-stage enrichment process which is divided into pre-enrichment stage and selective enrichment stage that involve incubation for ∼24–72 h at 30–37°C (Churchill et al., 2006; Janzten et al., 2006). Pre-enrichment is carried out in non- or half-selective enrichment medium in order to revive the injured target pathogen and to increase the amount of the target pathogen. In addition, pre-enrichment allows the dilution of inhibitory compounds present in foods such as preservatives and rehydration of bacterial cells sampled from dried or processed food matrices (Gasanov et al., 2005; Dwivedi and Jaykus, 2011; Jadhav et al., 2012; Välimaa et al., 2015). As for selective enrichment, it involves the use of selective medium that will increase the amount of target pathogen while suppress the growth of competing background microflora, thus, enabling the isolation and detection of the target pathogen (Dwivedi and Jaykus, 2011; Välimaa et al., 2015). Selective and differential plating is carried out after the two-stage enrichment process. The analysis is completed if there are no typical colonies can be observed on the selective differential agar and the results are reported as negative. If presumptive positive colonies are isolated, further tests are required to confirm the isolated pathogen such is described below (Jeyaletchumi et al., 2010a; Dwivedi and Jaykus, 2011; Välimaa et al., 2015).

The well-known culture reference methods for the isolation and detection of *L. monocytogenes* in foods are the FDA-BAM, ISO 11290 method, and USDA-FSIS method. These methods are recommended for the detection of *L. monocytogenes* from different food matrices and they utilize different enrichment media as well as plating media. Besides, the incubation time and temperature employed by each culture reference method are slightly different (Gasanov et al., 2005; Churchill et al., 2006; Janzten et al., 2006; Jeyaletchumi et al., 2010a; Välimaa et al., 2015). Numerous researchers have employed these culture reference methods for the investigation of *L. monocytogenes* in foods (Jeyaletchumi et al., 2010a; Goh et al., 2012; Lambertz et al., 2012; Jamali et al., 2013; Kramarenko et al., 2013; Wang et al., 2013; Osman et al., 2014). The culture reference methods are summarized in **Table 1**.

The qualitative information of the pathogen is provided by conventional methods. As for the quantitative information of the pathogen, it is required if the pathogen is detected in the food sample. The enumeration of the level of *L. monocytogenes* contamination in food sample can be done according to the ISO 11290-2 method (ISO, 2004b) and the protocols mentioned in FDA-BAM as well as USDA-FSIS method (**Table 1**). Besides,

TABLE 1 | Summary of each culture reference method for the isolation and detection of *L. monocytogenes* in foods and the detection limit of each method.

Method	Food matrices	Summary of method	Detection limit	Reference
FDA-BAM	Seafood, fruits, vegetables, and dairy products	(1) A 25 g of food sample stomached in 225 mL of BLEB and then incubate at 30°C for 4 h (2) After 4 h of incubation, add selective agents such as acriflavine, nalidixic acid, and cycloheximide into enrichment broth, incubate at 30°C for 48 h (3) Streak enrichment culture onto one of the prescribed selective differential agar plate (Oxford, MOX, or PALCAM) at 24 and 48 h (4) Incubate agar plate at 35°C for 24–48 h (5) Determine the presumptive colonies and then proceed to confirmation of *Listeria* sp. and *L. monocytogenes*	<1 CFU/mL	Hitchins and Jinneman, 2013; Välimaa et al., 2015
ISO 11290-1	All types of foods	(1) For primary enrichment, add *X* g or *X* mL of food sample to 9*X* mL of half Fraser broth, incubate at 30°C for 24 ± 2 h (2) Streak primary enrichment culture onto ALOA and second selective medium (Oxford or PALCAM), incubate at 37°C for 24 ± 2 h. If necessary, further 24 ± 2 h (3) For secondary enrichment, add 0.1 mL of primary enrichment culture to 10 mL of Fraser broth, incubate at 35 or 37°C for 48 ± 2 h (4) Streak secondary enrichment culture onto ALOA plate and second selective medium (Oxford or PALCAM), incubate at 37°C for 24 ± 2 h. If necessary, further 24 ± 2 h (5) Determine the presumptive colonies and then proceed to Confirmation of *Listeria* sp. and *L. monocytogenes*	<1 CFU/g in 25 g	ISO, 2004a; Zunabovic et al., 2011; Välimaa et al., 2015
USDA-FSIS	Red meat, poultry products, and egg products	(1) A 25 g of food sample stomached in 225 mL UVM broth and then incubate at 30 ± 2°C for 20–26 h (2) Streak primary enrichment culture onto MOX plate and then incubate at 35 ± 2°C for 26 ± 2 h. Determine the presumptive colonies from MOX plate. Proceed to confirmation of *Listeria* sp. and *L. monocytogenes* (3) For secondary enrichment, add 0.1 mL of primary enrichment culture to 10 mL of Fraser broth or MOPS-BLEB (4) For Fraser broth, incubate at 35 ± 2°C for 26 ± 2 h. After incubation, observe the broth for the presence of *L. monocytogenes* (darkening of medium due to esculin hydrolysis) (i) If positive, streak 0.1 mL of the Fraser broth onto MOX plate. Incubate MOX plate at 35 ± 2°C for 26 ± 2 h. Determine the presumptive colonies from MOX plate. Proceed to confirmation of *Listeria* sp. and *L. monocytogenes* (ii) If negative, reincubate Fraser broth for further 24 h. Re-examine the Fraser broth for confirmation of darkening. The sample is considered negative for *L. monocytogenes* if no darkening of Fraser broth and no suspected colonies on MOX are observed (5) For MOPS-BLEB, incubate at 35 ± 2°C for 18–24 h (i) After incubation, streak 0.1 mL of the MOPS-BLEB onto MOX plate. Incubate MOX plate at 35 ± 2°C for 26 ± 2 h (ii) Determine the presumptive colonies from MOX plate and proceed to confirmation of *Listeria* sp. and *L. monocytogenes*	<1 CFU/g	USDA-FSIS, 2013; Välimaa et al., 2015

the FDA and USDA have issued Compliance Policy Guides for food industry regarding the appropriate measures required to control *L. monocytogenes* in food and prevent contamination of food with *L. monocytogenes* (Kraiss, 2008). Enumeration of *L. monocytogenes* in food is important because an initial contamination as few as 1 CFU/100 g *L. monocytogenes* can cause the food unsafe in 32 days, while 10 CFU/g *L. monocytogenes* can cause the food unsafe in 8 days (Salvat and Fravalo, 2004). *L. monocytogenes* is able to grow over a wide range of temperatures, from around −0.4 to 45°C with an optimum temperature of 37°C (International Commission on Microbiological Specifications for Foods [ICMSF], 1996). Hence, this may cause the prevalence of *L. monocytogenes* in food to increase and reach unsafe levels during storage periods or long holding time before retailing. The infectious dose of *L. monocytogenes* for healthy or susceptible individuals has not been established, however, it is estimated to be $\sim 10^7$–10^9 CFU in healthy individuals and 10^5–10^7 CFU in susceptible individuals such as immunocompromised people or pregnant women (Farber et al., 1996; Smith et al., 2003). In order to enumerate the level of food sample contamination by presumptive *L. monocytogenes*, the primary enrichment broth is quantified prior to incubation, by direct spread plate count on chromogenic media (Hitchins and Jinneman, 2013). If the level of contamination is low, the enumeration of *L. monocytogenes* is done by the most probable number (MPN)

technique (Janzten et al., 2006; Jeyaletchumi et al., 2010a; Hitchins and Jinneman, 2013). Besides, some samples may contain particulate material that will interfere with plate count enumeration methods. Hence, MPN technique is applied for these types of samples (Sutton, 2010). MPN technique allows the estimation of population density of viable microorganisms in a sample through replicate liquid broth growth in 10-fold dilutions (Sutton, 2010; Letchumanan et al., 2014). The theoretical basis for MPN technique is to dilute the sample to some extent that inocula in the tubes will occasionally contain viable organisms. A reasonably accurate estimation of the most probable number of cells in the sample can thus be achieved by replicates and dilution series (Sutton, 2010). The FDA-BAM has described 10-fold serial dilution of sample in BLEB with the use of three or more tube MPN culture procedure on each dilution. The samples are incubated at 30°C for 48 h, followed by streaking on selective agar medium (Hitchins and Jinneman, 2013). *L. monocytogenes* can be directly enumerated if chromogenic media is used after MPN enrichment (Janzten et al., 2006; Jeyaletchumi et al., 2010a).

Most probable number technique is more sensitive as compared to direct plating, however, it is more labor intensive and it requires ∼7 days to complete the identification (Janzten et al., 2006; Jeyaletchumi et al., 2010a; Dwivedi and Jaykus, 2011). In MPN technique, the use of selective agar media or chromogenic media may not be selective enough as they may allow the growth of other competitive background microflora, thereby causing difficulties in determining presumptive *L. monocytogenes* (Jeyaletchumi et al., 2010a). MPN technique combined with polymerase chain reaction (PCR) technique is developed in order to overcome these limitations. MPN-PCR technique involves the detection of a particular gene in the target bacteria by PCR instead of isolation of the target bacteria for the enumeration of the bacteria in a sample (Letchumanan et al., 2014). Hence, this technique allows the direct enumeration of *L. monocytogenes* in food without interference of background microflora. The enumeration of *L. monocytogenes* by MPN-PCR technique can be completed in 2 days and this method has higher sensitivity than the standard MPN technique (Jeyaletchumi et al., 2010a). Several researchers have reported the success of MPN-PCR technique for the enumeration of *L. monocytogenes* in various food samples such as fermented sausages (Martín et al., 2004), salad vegetables (Jeyaletchumi et al., 2010b), and raw chicken (Goh et al., 2012).

MOLECULAR DETECTION OF *Listeria monocytogenes*

The detection of *L. monocytogenes* in food samples by conventional methods is simple, sensitive, and inexpensive if compared with molecular methods (Janzten et al., 2006; Law et al., 2015). However, conventional methods are laborious and time consuming as they require more than a week for the detection and confirmation of pathogen (Dwivedi and Jaykus, 2011; Law et al., 2015; Letchumanan et al., 2015b). Due to the recent advances in molecular technology, molecular methods have been used as an alternative to culture and serological methods for food testing (Gasanov et al., 2005). The detection of a pathogen present in food by nucleic-acid based molecular methods is based on the detection of specific DNA or RNA sequences in the target pathogen. Hence, these genetic methods can provide highly accurate and reliable results as compared to phenotypic methods. Nevertheless, molecular methods require specialized instruments and highly trained personnel (Gasanov et al., 2005; Jadhav et al., 2012; Law et al., 2015). There are various molecular methods available for the detection and identification of *L. monocytogenes*, for instance, PCR, multiplex polymerase chain reaction (mPCR), real-time/quantitative polymerase chain reaction (qPCR), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), DNA microarray as well as next generation sequencing (NGS) technology.

Polymerase chain reaction has been widely used for the detection of various foodborne bacterial pathogens. This method requires two single-stranded synthetic oligonucleotides or specific primers for the amplification of a specific target DNA sequence in a cyclic three steps process involving the use of a thermal cycler. The PCR amplification products are separated by agarose gel electrophoresis and visualized on the gel as bands with a DNA stain. The specific detection of the genus *Listeria* by PCR involves PCR primers based on the highly conserved 16S rRNA sequence present in all *Listeria* sp. with a resulting 938 bp amplification product (Levin, 2003; Burbano et al., 2006; Goh et al., 2012; Jamali et al., 2013). *L. monocytogenes* can be differentiated from other *Listeria* sp. by exploiting the molecular differences within the PCR amplified 16S rRNA gene, 23S rRNA gene and 16S–23S rRNA intergenic spacer regions (Wang et al., 1992; Graham et al., 1996, 1997; Sallen et al., 1996). In addition, PCR method also detects *L. monocytogenes* at the species level by targeting the virulence genes of the organism (Levin, 2003). Several virulence genes have been identified in *L. monocytogenes* and targeted for the PCR detection of the organism, for example, *hly* (*hlyA*) gene codes for listeriolysin O (LLO; Deneer and Boychuk, 1991; Johnson et al., 1992; Agersborg et al., 1997; Aznar and Alarcón, 2003; Amagliani et al., 2004; Burbano et al., 2006), *iap* gene codes for an invasion-associated protein known as p60 (Agersborg et al., 1997; Aznar and Alarcón, 2003; Swetha et al., 2012), *actA* gene codes for a surface protein known as ActA which is required for intracellular bacterial propulsion and cell to cell invasion (Moriishi et al., 1998; Levin, 2003), *lma*A gene codes for *L. monocytogenes* antigen (lmaA), which also known as *Dth*-18 gene codes for delayed-type hypersensitivity protein (DTH-18 factor; Wernars et al., 1991; Johnson et al., 1992; Levin, 2003), *inlA* gene codes for internalin A (Almeida and Almeida, 2000; Ingianni et al., 2001; Jung et al., 2003), *inlB* gene codes for internalin B (Pangallo et al., 2001; Jung et al., 2003), *prfA* gene codes for positive regulator factor A (PrFA; Simon et al., 1996), *pepC* codes for aminopeptidase C (Winters et al., 1999), *fbp* gene codes for fibronectin-binding protein (Gilot and Content, 2002) and *plcB* Phospholipase C protein (Volokhov et al., 2002). Among these targeted genes, the *hly*A gene is the most frequently chosen target gene for the PCR detection of *L. monocytogenes* (Aznar and Alarcón, 2003; Jadhav et al., 2012). The *hlyA* gene codes for a protein with pore forming activity,

which is known as listeriolysin O. This protein is found to be essential for the virulence of *L. monocytogenes* as it is responsible for the lysis of phagocyte vacuole and followed by the escape of *L. monocytogenes* from the vacuole (Kathariou et al., 1987; Cossart et al., 1989; Levin, 2003; Liu, 2006). Besides, it has been discovered that all clinical isolates of *L. monocytogenes* have hemolytic activity due to listeriolysin O and thus the *hlyA* gene is a relevant marker for the identification of *L. monocytogenes* (Groves and Welshimer, 1977; Golsteyn-Thomas et al., 1990).

Other than simple PCR, multiplex PCR (mPCR) is available for a more rapid detection of *L. monocytogenes*. Multiplex PCR is a variant of simple PCR in which multiple gene targets are simultaneously amplified by using several sets of specific primers in a single reaction (Liu et al., 2007). The primer design, concentration of primers, PCR buffer concentrations, quantities of DNA template, Taq DNA polymerase, balance between magnesium chloride and deoxynucleotide concentrations and cycling temperatures are very important for a successful mPCR assay (Markoulatos et al., 2002; Zhao et al., 2014; Law et al., 2015). Multiplex PCR is capable of detecting multiple virulence-associated genes of *L. monocytogenes* in a single PCR mixture. Hence, the possible failure in the detection of virulent *L. monocytogenes* can be prevented (Cooray et al., 1994). In the study conducted by Cooray et al. (1994), *L. monocytogenes* in milk samples was successfully detected by mPCR with primers targeting three virulence-associated genes, *prfA, hlyA,* and *plcB*. Liu et al. (2007) had developed an mPCR assay targeting *inlA*, *inlC,* and *inlJ* genes for the rapid species-specific and virulence-specific determination of *L. monocytogenes*. Besides, mPCR is employed for simultaneous detection of *L. monocytogenes*, *Listeria* sp. and other foodborne pathogens such as *Salmonella* sp., *Escherichia coli* O175:H7, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus* as well as *Campylobacter jejuni* in various food samples (Lawrence and Gilmour, 1994; Gilbert et al., 2003; Jofré et al., 2005; Germini et al., 2009; Kumar et al., 2009; Yuan et al., 2009; Zhang et al., 2009; Zarei et al., 2012; Yang et al., 2013). A novel mPCR assay that can simultaneously detect and discriminate six *Listeria* species including *L. monocytogenes, L. grayi, L. ivanovii, L. innocua, L. welshimeri,* and *L. seeligeri* was first developed by Ryu et al. (2013). A rapid mPCR assay for simultaneous detection of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, *Salmonella* Enteritidis, and *Shigella flexneri* in meat samples was developed by Chen et al. (2012). Lee et al. (2014) had carried out mPCR assay that can simultaneously detect *L. monocytogenes*, *B. cereus*, *E. coli* O157:H7, *V. parahaemolyticus*, *Salmonella* sp., and *S. aureus* in ready-to-eat food samples. Furthermore, the major *L. monocytogenes* serovars such as 1/2a, 1/2b, 1/2c, and 4b can be differentiated by mPCR targeting marker genes *Imo0730*, *Imo1118*, ORF2819, and ORF2110 (Doumith et al., 2004; Hamdi et al., 2007; Erol and Ayaz, 2011).

The development of real-time or quantitative PCR (qPCR) provides high-throughput analysis and low risk of cross-contamination since post-PCR processing for the detection of PCR products is not required (Fricker et al., 2007). Fluorescent dye such as SYBR green, hydrolysis probe such as TaqMan assays and oligonucleotide hybridization probes such as molecular beacons are used to monitor the PCR products in qPCR (Law et al., 2015). Recently, qPCR is widely used for the detection of foodborne pathogens and multiplex qPCR is also developed for this purpose. This method offers rapid and specific identification as well as quantification of *L. monocytogenes* in a variety of food samples such as soft cheese, fruit juice, fish, vegetables, salads, milk, meat, and crustaceans (Berrada et al., 2006; O'Grady et al., 2008; Kim and Cho, 2010; Garrido et al., 2013; Gianfranceschi et al., 2014). Oravcová et al. (2005) had developed a real-time 5′-nuclease PCR targeting a sequence of the gene *actA* for the identification and quantification of *L. monocytogenes*. In this study, TaqMan probe was used for the detection and quantification of qPCR products. Besides, Barbau-Piednoir et al. (2013) developed a combination of four qualitative SYBRgreen qPCR assays for the detection and discrimination of *Listeria* sp. and *L. monocytogenes* with high accuracy. In these assays, the *iap* and *prs* genes were targeted for detection of *Listeria* sp. and *hlyA* gene was targeted for detection of *L. monocytogenes*. The successful detection of *L. monocytogenes* in fresh produce using molecular beacon-qPCR targeting the *hlyA* gene was first reported by Liming et al. (2004). Furthermore, a novel 5′ exonuclease multiplex qPCR assay for the identification of six *Listeria* sp. including *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, *L. grayi,* and *L. innocua* was developed by Hage et al. (2014). In this study, two sets of triplex PCR were designed with one set identifying *L. seeligeri*, *L. welshimeri,* and *L. monocytogenes* and another set identifying *L. ivanovii*, *L. grayi,* and *L. innocua*. The *Listeria* species were differentiated by targeting their respective species-specific target genes and TaqMan probe was used to monitor the multiplex qPCR products.

Additionally, commercial qPCR kits for the detection of *L. monocytogenes* are available and this allows laboratories in food industry to adapt qPCR testing easily (Janzten et al., 2006). The examples of these commercial qPCR kits include BAX® System Real-time PCR Assay *Listeria monocytogenes* (DuPont-Qualicon), Probelia® *Listeria monocytogenes* PCR System (Bio-Rad), LightCycler® *Listeria monocytogenes* Detection Kit (Roche/Biotecon), TaqMan® *Listeria monocytogenes* Detection Kit (Applied Biosystems), GeneVision® Rapid Pathogen Detection System for *Listeria monocytogenes* (Warnex), ADIAFOOD rapid pathogen detection system for *Listeria monocytogenes* (AES Chemunex), CycleavePCR® *Listeria monocytogenes* (*inlA* gene) Detection Kit (TaKaRa Bio, Inc.) and iQ-Check *L. monocytogenes* kit (Bio-Rad Laboratories; Liming et al., 2004; Rodríguez-Lázaro et al., 2004; Becker et al., 2005; Janzten et al., 2006; Liu et al., 2012).

There is no doubt that PCR-based detection methods are rapid, highly sensitive, and specific. However, these methods require thermocycling system. Alternative methods have been developed for the amplification of nucleic acids under isothermal conditions. Two of the most commonly used isothermal nucleic acid amplification methods for the detection of foodborne pathogens are LAMP and NASBA.

Several types of LAMP assays such as multiplex LAMP, real-time LAMP, *in situ* LAMP and reverse-transcription LAMP have been developed and utilized for the detection of foodborne

pathogens (Ye et al., 2011; Law et al., 2015). Studies have shown that LAMP assay has high specificity and it exhibits higher sensitivity than PCR assays in the detection of *L. monocytogenes*. For example, Tang et al. (2011) conducted a sensitive and specific LAMP assay for the detection of *L. monocytogenes* with primers that target the *hlyA* gene region. In this study, the LAMP assay was evaluated against conventional PCR method for the detection of *L. monocytogenes* in food. The results indicated that LAMP assay was 100 times more sensitive than the conventional PCR assay. Besides, a real-time quantitative LAMP that amplifies the *hlyA* gene of *L. monocytogenes* was designed by Shan et al. (2012). This LAMP assay was then used to detect *L. monocytogenes* in four different types of retail food samples such as raw meat, vegetables, deli, and seafood. The study also proven that LAMP assay was more sensitive than PCR in the detection of *L. monocytogenes*. A double LAMP (dLAMP) assay was first conducted by Wu et al. (2014) for the detection of *L. monocytogenes* in food samples including pork, beef, chicken, mutton, shrimp, fish, and quick-frozen rice flour products. LAMP primers targeting the *hlyA* and *iap* genes of *L. monocytogenes* were used to ensure the dLAMP assay is more rapid, sensitive and specific. The results of this study showed that dLAMP assay was more sensitive and less time consuming as compared to normal LAMP assay. Recently, LAMP has been commercialized as kits for the detection of *L. monocytogenes*, for instance, Loopamp® *Listeria monocytogenes* Detection Kit (Eiken Chemical, Co., Ltd.) and Isothermal Master Mix (OptiGene; Wang et al., 2015).

In general, NASBA often involves in the amplification of mRNA targets under isothermal conditions (Leone et al., 1998). NASBA selectively amplifies the mRNA targets even in the presence of genomic DNA and it has been used to detect various foodborne pathogens (Simpkins et al., 2000). The main advantage of NASBA over other molecular detection methods is its ability to detect viable bacterial cells that are present in environmental samples and food samples (Simpkins et al., 2000; Cook, 2003). A highly specific NASBA system was developed by Blais et al. (1997) for the detection of *L. monocytogenes* with primers targeting the *hlyA* mRNA sequences. The NASBA system was capable of detecting low numbers of *L. monocytogenes* (<10 CFU/g) in artificially contaminated dairy and egg products after 48 h enrichment period. Nevertheless, false-positive results were reported and the researchers suggested that the reason for this could be due to cross-contamination of NASBA reactions with amplicons from previous amplifications performed at the same site. The post-NASBA product detection steps involving agarose gel electrophoresis, enzyme-linked gel assay, enzymatic bead-based detection and numerous probing and/or blotting techniques can be laborious. Hence, homogenous real-time NASBA that

TABLE 2 | Application of molecular methods for the detection and identification of *L. monocytogenes* in various food samples.

Detection method	Gene target	Food matrix	Reference
Simple PCR	*hlyA, iap*	Naturally contaminated fish samples	Swetha et al., 2012
	hlyA	Naturally contaminated raw meat (chicken, beef, and fish), milk and milk products (raw milk, cheese, and curd)	Khan et al., 2013
	actA	Artificially contaminated milk, pork, and water	Zhou and Jiao, 2005
Multiplex PCR	*iap,hly*	Artificially contaminated milk	Zeng et al., 2006
	plcA, *hlyA*, *actA*, *iap*	Artificially contaminated milk	Rawool et al., 2007
	16S rRNA, *iap*	Naturally contaminated deli meat samples: pork and chicken products	Liu et al., 2015
Real-time/quantitative PCR	*prfA*	Artificially contaminated raw milk, salmon, pâté, and green-veined cheese Naturally contaminated fish, meat, meat products, and dairy products	Rossmanith et al., 2006
	Iap	Artificially contaminated milk	Hein et al., 2001
	Hly	Artificially contaminated pork meat	Gattuso et al., 2014
	16S-23S rRNA intergenic spacer regions	Artificially contaminated soft cheese, fermented sausage, cured ham, and ready-to-eat salad Naturally contaminated fresh meat, fresh sausages, fermented sausages, fresh cheeses, and ripened cheeses	Rantsiou et al., 2008
	16S rRNA	Leafy vegetables: collard green, cabbage, lettuce, mixed parsley, chinese cabbage, spring onion bunches, spinach, wild chicory, arugula, and watercress	De Oliveira et al., 2010
	inlA	Artificially contaminated chicken meat	Navas et al., 2006
LAMP	*prfA*	Artificially contaminated milk	Cho et al., 2014
	hlyA	Artificially contaminated chicken, pork, ground beef, and milk powder	Wan et al., 2012
	iap	Artificially and naturally contaminated raw milk	Wang et al., 2011
NASBA	16S rRNA	Artificially contaminated chicken breast meat, soft cheese, shrimps, dry sausage, minced meat (pork and beef), radish and mushrooms	Uyttendaele et al., 1995
DNA microarray	Genomic DNA of *L. monocytogenes*	Artificially contaminated milk	Bang et al., 2013
NGS	Whole genome of *L. monocytogenes*	Deli turkey meat Ready to eat meat Quargel cheese	Orsi et al., 2008 Gilmour et al., 2010 Rychli et al., 2014

utilizes fluorescently labeled probes (e.g., molecular beacon) to monitor the amplicons is developed in order to overcome this problem (Leone et al., 1998). A molecular beacon-based real time NASBA assay for the detection of *L. monocytogenes* in cooked ham and smoked salmon slices was first described by Nadal et al. (2007). Sequence from the mRNA transcript of *hly* gene was used as a target for this assay and the detection limit of this assay for *L. monocytogenes* was 400 CFU/mL. This study also involved the use of a commercial NASBA kit, which was NucliSens® Basic Kit (bioMérieux) for the detection of *L. monocytogenes*.

DNA microarrays, which were initially being applied for the study of gene expression, could be used for the investigation of microbial evolution and epidemiology as well as for the detection of foodborne pathogens (Gasanov et al., 2005; Severgnini et al., 2011). DNA microarrays comprise multiple specific oligonucleotide probes (with sequence length ranges from 25 to 80 bp) or PCR probes which are coated on to glass slides or chips. The target nucleic acid which can be either DNA, mRNA or cDNA is labeled with fluorescent dye and then applied to the DNA microarray. The target nucleic acid will bind to its corresponding oligonucleotide probe and the hybridization is detected by production of fluorescent signal from probe-sample complex. DNA microarrays are capable of detecting multiple foodborne pathogens simultaneously and thus suitable for high-throughput analysis (De Boer and López, 2012; Law et al., 2015). An oligonucleotide DNA microarray assay that can simultaneously detect and discriminate six *Listeria* sp. including *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. grayi,* and *L. welshimeri* was performed by Volokhov et al. (2002). The microarray assay was based on *iap*, *hly*, *inlB*, *plcA*, *plcB,* and *clpE* genes for the identification of *Listeria* species. Oligonucleotide DNA microarray was also used for the simultaneous detection of different foodborne bacterial pathogens which include *L. monocytogenes*, *E. coli* O157:H7, *Salmonella enterica* and *C. jejuni* in food samples (Suo et al., 2010). DNA microarrays provide high-throughput analysis but the shortcoming is that large amounts of target DNA or RNA are needed for these methods (Gasanov et al., 2005).

The majority of bacterial genome sequences available today have been generated using the Sanger chain termination sequencing chemistries. Despite being very instrumental in the rise of the field of genomics, it is time consuming as well as resource intensive (Sanger et al., 1977; Medini et al., 2008). The post-Sanger era sequencing technologies, the NGS technologies, have been developed since 2005 to permit extremely rapid high-throughput whole genome sequencing (WGS) hence providing a broader application of comparative genomics (Medini et al., 2008; Shendure and Ji, 2008; Letchumanan et al., 2015a). Due

TABLE 3 | Advantages and limitations of molecular methods for the detection and identification of *L. monocytogenes.*

Molecular methods	Advantages	Limitations	Reference
Simple PCR	• High sensitivity and specificity • Accurate and reliable results	• Sensitivity may be affected by non-optimized protocols and PCR inhibitors • Requires DNA purification step	Mandal et al., 2011; Letchumanan et al., 2014; Law et al., 2015
Multiplex PCR	• High sensitivity and specificity • Enables simultaneous detection of multiple foodborne pathogens	• Sensitivity may affected by non-optimized protocols and PCR inhibitors • Primer design and other mPCR conditions (e.g., primer concentration, PCR buffer concentration, and quantities of DNA template) are important	Markoulatos et al., 2002; Mandal et al., 2011; Law et al., 2015
Real-time/ quantitative PCR	• Higher sensitivity and specificity than simple PCR • More rapid than simple PCR and mPCR as post-amplification products processing is not required • Assay can be multiplexed • Allows high-throughput analysis	• Costly • Sensitivity may affected by PCR inhibitors • Trained personnel is needed	Oravcová et al., 2005; Mandal et al., 2011; Letchumanan et al., 2014; Law et al., 2015
LAMP	• Higher sensitivity and specificity than PCR • Cost-effective • Simple • Operates without thermal cycling system	• Complicated primer design	Letchumanan et al., 2014; Zhao et al., 2014; Law et al., 2015
NASBA	• Sensitive and specific • Cost-effective • Operates without thermal cycling system • Enables the detection of viable bacteria	• Requires viable bacteria • Might not be easy to handle RNA	Lauri and Mariani, 2009; Zhao et al., 2014; Law et al., 2015
DNA microarray	• High sensitivity and specificity • Enables simultaneous detection of multiple foodborne pathogens • Allows high-throughput analysis	• Costly • Trained personnel is needed • Requires large amount of target DNA or RNA	Gasanov et al., 2005; Lauri and Mariani, 2009; Law et al., 2015
NGS	• High sensitivity and specificity • Enables simultaneous detection of multiple foodborne pathogens • Allows high-throughput analysis • Enable the analysis of whole genome of the pathogens	• Costly • Trained personnel is needed • Requires Bioinformatics skills for analysis and interpretation • Computationally intensive	Sabat et al., 2013; Fournier et al., 2014

to rapid decreasing costs for sequencers and reagents, a bacterial genome sequence can be obtained within a few days for less than US$500 (Didelot et al., 2012), and more than 36,000 bacterial genome sequences are available in public databases (Reddy et al., 2015). Other than serving as a detection tool, WGS is also a feasible tool for retrospective epidemiological analyses and is frequently used for the latter purpose. Genome sequencing of several *L. monocytogenes* strains have revealed serotype- and strain-specific characteristics of *L. monocytogenes* (Orsi et al., 2008; Fretz et al., 2010; Gilmour et al., 2010) and provided novel insights into the genomic causes underlying pathogenicity and survival in food and food processing settings (Buchrieser and Glaser, 2011).

A listeriosis outbreak in Oklahoma, USA in the year 1988 was linked to the consumption of turkey franks contaminated with *L. monocytogenes* produced in a food processing facility in Texas, USA (Centers for Disease Control, and Prevention [CDC], 1989). In 2000, 11 states in the US faced listeriosis outbreak affecting 29 individuals including four fatalities, and it was linked to consumption of deli turkey meat produced in the same facility (Stone and Shoenberger, 2001; Olsen et al., 2005). Using NGS, Orsi et al. (2008) revealed that the human listeriosis outbreak in 2000 in the USA was caused by a *L. monocytogenes* strain that persisted in that food processing plant for over 12 years in which the same strain has also been responsible for a sporadic case in 1988.

In 2008, *L. monocytogenes* serotype 1/2a caused an outbreak of listeriosis associated with ready to eat meat products in Canada, resulting in 22 deaths and at least 57 illnesses (Gilmour et al., 2010). The authors reported the first real-time application of WGS during an active listeriosis outbreak investigation using the high-throughput to characterize two outbreak-associated isolates of *L. monocytogenes*. In 2009 and 2010, another large listeriosis outbreak occurred in Austria, Germany, and the Czech Republic due to intake of a traditional Austrian cheese called "Quargel," an acid curd cheese with a red smear made from skimmed pasteurized milk (Fretz et al., 2010). Molecular typing via PFGE revealed that two different *L. monocytogenes* strains, both serotype 1/2a (Pichler et al., 2011). From June 2009 to January 2010 Quargel outbreak clone 1 (QOC1) was the culprit in 14 cases, including five with a lethal outcome (Fretz et al., 2010). Whereas between December 2009 and February 2010, Quargel outbreak clone 2 (QOC2) accounted for 20 cases, which resulted in three deaths (Fretz et al., 2010). Rychli et al. (2014) sequenced and analyzed the genomes of both outbreak strains in order to retrospectively investigate the extent of genetic diversity between the two strains. WGS analysis revealed that these two strains have distinct *in vitro* virulence potential despite originating from similar serovar (Rychli et al., 2014).

The development of benchtop sequencers using NGS technology such as 454 or GS FLX^{TM} (Roche), MiSeq (Illumina) and Ion Torrent Personal Genome Sequencer (PGM; Life Technologies) will enable bacterial WGS even in small research and clinical laboratories (Didelot et al., 2012). WGS has already been actively used for the characterization of bacterial isolates in several large outbreaks in the world (Gilmour et al., 2010; Reuter et al., 2013) and also being used as a tool for retrospective epidemiological analyses (Orsi et al., 2008; Rychli et al., 2014). In the near future this technology is likely to substitute currently used typing methodologies due to its ultimate resolution and sensitivity (Sabat et al., 2013). However, NGS also has its limitation. Until now, it is still too laborious and time-consuming to obtain useful data for routine surveillance (Fournier et al., 2014). Library preparation and sequencing protocol requires adept and skillful technician; however, this limitations are likely to be overcome due to higher level of automation which will lead to a more streamline processed. Bioinformatics analysis and interpretation, as well as computational hardware are also another challenges to be solved especially by small laboratories (Fournier et al., 2014). In addition, a fundamentally unsolved question is how the sequences should be examined for epidemiological characterization (Sabat et al., 2013). More examples of studies that involved the application of molecular methods for the detection and identification of *L. monocytogenes* in food samples are listed in **Table 2**.

There are many advantages of using molecular methods for the detection and identification of *L. monocytogenes*. For instance, the main advantages of molecular detection methods are due to their high sensitivity and specificity. Nonetheless, limitations can be found in these methods such as some molecular methods can be costly, complex and require trained personnel. The advantages and limitations of molecular methods are summarized and listed in **Table 3**.

CONCLUSION

Early detection of *L. monocytogenes* contaminated food is crucial as it can prevent the outbreaks of foodborne illness. Till date, the culture reference methods mentioned in this review are still applicable and being used in many studies. Simple PCR and mPCR have been used routinely for rapid, sensitive, and specific screening as well as confirmation of *L. monocytogenes*. The introduction of LAMP and NASBA allow accurate and cost-effective screening of *L. monocytogenes*. For large number of samples, high-throughput assays such as qPCR and DNA microarray are often used for the detection of *L. monocytogenes*. Cultural and molecular techniques are continuously being developed and improved in order to provide higher sensitivity and specificity of *L. monocytogenes* detection. The advancements of molecular methods allow the rapid detection of *L. monocytogenes* in food samples with high sensitivity and specificity whilst substituting the lengthy and laborious conventional detection methods. Molecular methods have provide many advantages, nonetheless, there are still some limitations in these methods such as the need to use highly advance technology that are costly compared to conventional methods. The combined use of two or more detection methods is also possible and may improve the accuracy of detecting *L. monocytogenes*. Still, there are great potential for the development and application of new techniques for foodborne pathogens detection and analysis.

ACKNOWLEDGMENTS

This work was supported by a University of Malaya for High Impact Research Grant (UM-MOHE HIR Nature Microbiome Grant No. H-50001-A000027 and No. A000001-50001) awarded to CK-G and External Industry Grants from Biotek Abadi Sdn Bhd (vote no. GBA-808138 and GBA-808813) awarded to LL-H.

REFERENCES

Agersborg, A., Dahl, R., and Martinez, I. (1997). Sample preparation and DNA extraction procedures for polymerae chain reaction identification of *Listeria monocytogenes* in seafoods. *Int. J. Food Microbiol.* 35, 275–280. doi: 10.1016/S0168-1605(97)01245-2

Ajay Kumar, V. J., Latha, C., Sunil, B., and Dhanya, V. R. (2014). Comparison of different cultural techniques in isolation of *Listeria monocytogenes* from various samples. *J. Foodborne Zoonotic Dis.* 2, 10–14.

Alessandria, V., Rantsiou, K., Dolci, P., and Cocolin, L. (2010). Molecular methods to asses *Listeria monocytogenes* route of contamination in a dairy processing plant. *Int. J. Food Microbiol.* 141, S156–S162. doi: 10.1016/j.ijfoodmicro.2010.02.001

Almeida, P. F., and Almeida, R. C. C. (2000). A PCR protocol using inl gene as a target for specific detection of *Listeria monocytogenes*. *Food Control* 11, 97–101. doi: 10.1016/S0956-7135(99)00067-5

Amagliani, G., Brandi, G., Omiccioli, E., Casiere, A., Bruce, I. J., and Magnani, M. (2004). Direct detection of *Listeria monocytogenes* from milk by magnetic based DNA isolation and PCR. *Food Microbiol.* 21, 597–603. doi: 10.1016/j.fm.2003.10.008

Andritsos, N. D., Mataragas, M., Paramithiotis, S., and Drosinos, E. H. (2013). Quantifying *Listeria monocytogenes* prevalence and concentration in minced pork meat and estimating performance of three culture media from presence/absence microbiological testing using a deterministic and stochastic approach. *Food Microbiol.* 36, 395–405. doi: 10.1016/j.fm.2013.06.020

Aragon-Alegro, L. C., Aragon, D. C., Martinez, E. Z., Landgraf, M., De Melo Franco, B. D. G., and Destro, M. T. (2008). Performance of a chromogenic medium for the isolation of *Listeria monocytogenes* in food. *Food Control* 19, 483–486. doi: 10.1016/j.foodcont.2007.05.013

Aznar, R., and Alarcón, B. (2003). PCR detection of *Listeria monocytogenes*: a study of multiple factors affecting sensitivity. *J. Appl. Microbiol.* 95, 958–966. doi: 10.1046/j.1365-2672.2003.02066.x

Bang, J., Beuchat, L. R., Song, H., Gu, M. B., Chang, H. I., Kim, H. S., et al. (2013). Development of random genomic DNA microarray for the detection, and identification of *Listeria monocytogenes* in milk. *Int. J. Food Microbiol.* 161, 134–141. doi: 10.1016/j.ijfoodmicro.2012.11.023

Barbau-Piednoir, E., Botteldoorn, N., Yde, M., Mahillon, J., and Roosens, N. H. (2013). Development and validation of qualitative SYBR® Green real-time PCR for detection and discrimination of *Listeria* spp. and *Listeria monocytogenes*. *Appl. Microbiol. Biotechnol.* 97, 4021–4037. doi: 10.1007/s00253-012-4477-2

Becker, B., Jordan, S., and Holzapfel, W. H. (2005). Rapid and specific detection of *Listeria monocytogenes* in smoked salmon with BAX®-PCR. *Food Control* 16, 717–721. doi: 10.1016/j.foodcont.2004.06.009

Berrada, H., Soriano, J. M., Picó, Y., and Mañes, J. (2006). Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. *Int. J. Food Microbiol.* 107, 202–206. doi: 10.1016/j.ijfoodmicro.2005.07.006

Beumer, R. R., and Hazeleger, W. C. (2003). *Listeria monocytogenes*: diagnostic problems. *FEMS Immunol. Med. Microbiol.* 35, 191–197. doi: 10.1016/S0928-8244(02)00444-3

Blais, B. W., Turner, G., Sooknanan, R., and Malek, L. T. (1997). A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes* hlyA sequences. *Appl. Environ. Microbiol.* 63, 310–313. doi: 10.1007/BF00158944

Buchrieser, C., and Glaser, P. (2011). *Genomics of Listeria monocytogenes and Other Members of the Genus Listeria. Genomes of Foodborne and Waterborne Pathogens*. Washington DC: ASM Press, 125–145.

Burbano, E., Sierra, S., Torres, K., Mercado, M., Carrascal, A., and Poutou, R. (2006). Rapid DNA extraction and PCR validation for direct detection of *Listeria monocytogenes* in raw milk. *Rev. MVZ Cordoba* 11, 715–724.

Bush, S. V., and Donnelly, C. W. (1992). Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria* innocua. *Appl. Environ. Microbiol.* 58, 14–20.

Centers for Disease Control, and Prevention [CDC] (1989). Epidemiological (Notes) and reports listeriosis associated with consumption of turkey franks. *Morbidity Mortailty Wkly. Rep.* 38, 267–268.

Chen, J., Tang, J., Liu, J., Cai, Z., and Bai, X. (2012). Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *J. Appl. Microbiol.* 112, 823–830. doi: 10.1111/j.1365-2672.2012.05240.x

Cho, A. R., Dong, H. J., Seo, K. H., and Cho, S. (2014). Development of a loop-mediated isothermal amplification assay for detecting *Listeria monocytogenes* prfA in milk. *Food Sci. Biotechnol.* 23, 467–474. doi: 10.1007/s10068-014-0064-x

Churchill, R. L. T., Lee, H., and Hall, J. C. (2006). Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food. *J. Microbiol. Methods* 64, 141–170. doi: 10.1016/j.mimet.2005.10.007

Cook, N. (2003). The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J. Microbiol. Methods* 53, 165–174. doi: 10.1016/S0167-7012(03)00022-8

Cooray, K. J., Nishibori, T., Xiong, H., Matsuyama, T., Fujita, M., and Mitsuyama, M. (1994). Detection of multiple virulence associated-genes of *Listeria monocytogenes* by PCR in artificially contaminated milk samples. *Appl. Environ. Microbiol.* 60, 3023–3026.

Cossart, P., Vicente, M. F., Mengaud, J., Baquero, F., Perez-Diaz, J. C., and Berche, P. (1989). Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun.* 57, 3629–3636.

Curtis, G. D. W., Mitchell, R. G., King, A. F., and Griffin, E. J. (1989). A selective differential medium for the isolation of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* 8, 95–98. doi: 10.1111/j.1472-765X.1989.tb00231.x

Da Silva, E. P., and De Martinis, E. C. P. (2013). Current knowledge and perspective on biofilm formation: the case of *Listeria monocytogenes*. *Appl. Microbiol. Biotechnol.* 97, 957–968. doi: 10.1007/s00253-012-4611-1

De Boer, S. H., and López, M. M. (2012). New grower-friendly methodsfor plant pathogen monitoring. *Annu. Rev. Phytopathol.* 50, 197–218. doi: 10.1146/annurev-phyto-081211-172942

Deneer, H. G., and Boychuk, I. (1991). Species-specific detection of *Listeria monocytogenes* by DNA amplification. *Appl. Environ. Microbiol.* 57, 606–609.

De Oliveira, M. A., Ribeiro, E. G. A., Bergamini, A. M. M., and De Martinis, E. C. P. (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiol.* 27, 19–23. doi: 10.1016/j.fm.2009.07.003

Didelot, X., Bowden, R., Wilson, D. J., Peto, T. E., and Crook, D. W. (2012). Transforming clinical microbiology with bacterial genome sequencing. *Nat. Rev. Genet.* 13, 601–612. doi: 10.1038/nrg3226

Donnelly, C. W., and Baigent, G. J. (1986). Method for flow cytometric detection of *Listeria monocytogenes* in milk. *Appl. Environ. Microbiol.* 52, 689–695.

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

Dwivedi, H. P., and Jaykus, L. A. (2011). Detection of pathogens in foods: the current state-of-the-art and future directions. *Crit. Rev. Microbiol.* 37, 40–63. doi: 10.3109/1040841X.2010.506430

El Marrakchi, A., Boum'handi, N., and Hamama, A. (2005). Performance of a new chromogenic plating medium for the isolation of *Listeria monocytogenes* from marine environments. *Lett. Appl. Mircrobiol.* 40, 87–91. doi: 10.1111/j.1472-765X.2004.01651.x

Erol, I., and Ayaz, N. D. (2011). Serotype distribution of *Listeria monocytogenes* isolated from Turkey meat by multiplex PCR in Turkey. *J. Food Saf.* 31, 149–153. doi: 10.1111/j.1745-4565.2010.00278.x

Farber, J. M., Ross, W. H., and Harwig, J. (1996). Health risk assessment of *Listeria monocytogenes* in Canada. *Int. J. Food Microbiol.* 30, 145–156. doi: 10.1016/0168-1605(96)01107-5

Fournier, P. E., Dubourg, G., and Raoult, D. (2014). Clinical detection and characterization of bacterial pathogens in the genomics era. *Genome Med.* 6:114. doi: 10.1186/s13073-014-0114-2

Fraser, J. A., and Sperber, W. H. (1988). Rapid detection of *Listeria* spp. in food and environmental samples by esculin hydrolysis. *J. Food Prot.* 51, 762–765.

Fretz, R., Sagel, U., Ruppitsch, W., Pietzka, A., Stoger, A., Huhulescu, S., et al. (2010). Listeriosis outbreak caused by acid curd cheese Quargel. Austria and Germany 2009. *Euro. Surveill.* 15:pii:19477.

Fricker, M., Messelhäußer, U., Busch, U., Scherer, S., and Ehling-Schulz, M. (2007). Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Appl. Environ. Microbiol.* 73, 1892–1898. doi: 10.1128/AEM.02219-06

Garrido, A., Chapela, M. J., Román, B., Fajardo, P., Vieites, J. M., and Cabado, A. G. (2013). In-house validation of a multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 164, 92–98. doi: 10.1016/j.ijfoodmicro.2013.03.024

Gasanov, U., Hughes, D., and Mansbro, P. M. (2005). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiol. Rev.* 29, 851–875. doi: 10.1016/j.femsre.2004.12.002

Gattuso, A., Gianfranceschi, M. V., Sonnessa, M., Delibato, E., Marchesan, M., Hernandez, M., et al. (2014). Optimization of real time PCR based method for the detection of *Listeria monocytogenes* in pork meat. *Int. J. Food Microbiol.* 184, 106–108. doi: 10.1016/j.ijfoodmicro.2014.04.015

Germini, A., Masola, A., Carnevali, P., and Marchelli, R. (2009). Simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control* 20, 733–738. doi: 10.1016/j.foodcont.2008.09.010

Gianfranceschi, M. V., Rodriguez-Lazaro, D., Hernandez, M., González-García, P., Comin, D., Gattuso, A., et al. (2014). European validation of a real-time PCR-based method for detection of *Listeria monocytogenes* in soft cheese. *Int. J. Food Microbiol.* 184, 128–133. doi: 10.1016/j.ijfoodmicro.2013.12.021

Gilbert, C., O'Leary, A., Winters, D., and Slavik, M. (2003). Development of a multiplex PCR assay for the specific detection of *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. *J. Rapid Methods Autom. Microbiol.* 11, 61–74. doi: 10.1111/j.1745-4581.2003.tb00409.x

Gilmour, M. W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K. M., et al. (2010). High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics* 11:120. doi: 10.1186/1471-2164-11-120

Gilot, P., and Content, J. (2002). Specific identification of *Listeria welshimeri* and *Listeria monocytogenes* by PCR assays targeting a gene encoding a fibronectin-binding protein. *J. Clin. Microbiol.* 40, 698–703. doi: 10.1128/JCM.40.2.698-703.2002

Goh, S. G., Kuan, C. H., Loo, Y. Y., Chang, W. S., Lye, Y. L., Soopna, P., et al. (2012). *Listeria monocytogenes* in retailed raw chicken meat in Malaysia. *Poult. Sci.* 91, 2686–2690. doi: 10.3382/ps.2012-02349

Golsteyn-Thomas, E. J., King, R. K., Burchak, J., and Gannon, V. P. J. (1990). Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Appl. Environ. Microbiol.* 57, 2576–2580.

Graham, T. A., Golsteyn-Thomas, E. J., Gannon, V. P. J., and Thomas, J. E. (1996). Genus- and species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. *Can. J. Microbiol.* 42, 1155–1162. doi: 10.1139/m96-147

Graham, T. A., Golsteyn-Thomas, E. J., Thomas, J. E., and Gannon, V. P. J. (1997). Inter- and intraspecies comparison of the 16S-23S rRNA operon intergenic spacer regions of six *Listeria* spp. *Int. J. Syst. Bacteriol.* 47, 863–869. doi: 10.1099/00207713-47-3-863

Groves, R. D., and Welshimer, H. J. (1977). Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. *J. Clin. Microbiol.* 5, 559–563.

Gudbjörnsdóttir, B., Suihko, M. L., Gustavsson, P., Thorkelsson, G., Salo, S., Sjöberg, A. M., et al. (2004). The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. *Food Microbiol.* 21, 217–225. doi: 10.1016/S0740-0020(03)00012-1

Hage, E., Mpamugo, O., Ohai, C., Sapkota, S., Swift, C., Wooldridge, D., et al. (2014). Identification of six *Listeria* species by real-time PCR assay. *Lett. Appl. Microbiol.* 58, 535–540. doi: 10.1111/lam.12223

Hamdi, T. H., Naïm, M., Martin, P., and Jacquet, C. (2007). Identification and molecular characterization of *Listeria monocytogenes* isolated in raw milk in the region of Algiers (Algeria). *Int. J. Food Microbiol.* 116, 190–193. doi: 10.1016/j.ijfoodmicro.2006.12.038

Hegde, V., Leon-Velarde, C. G., Stam, C. M., Jaykus, L. A., and Odumeru, J. A. (2007). Evaluation of BBL CHROMagar *Listeria* agar for the isolation and identification of *Listeria monocytogenes* from food and environmental samples. *J. Microbiol. Methods* 68, 82–87. doi: 10.1016/j.mimet.2006.06.011

Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl, E., and Wagner, M. (2001). Detection and quantification of the iap gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. *Res. Microbiol.* 152, 37–46. doi: 10.1016/S0923-2508(00)01166-9

Hitchins, A. D., and Jinneman, K. (2013). *Bacteriological Analytical Manual (BAM) Chapter 10: Detection and Enumeration of Listeria monocytogenes in Foods*. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm [accessed May 16, 2015].

Ingianni, A., Floris, M., Palomba, P., Madeddu, M. A., Quartuccio, M., and Pompei, R. (2001). Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Mol. Cell. Probes* 15, 275–280. doi: 10.1006/mcpr.2001.0372

International Commission on Microbiological Specifications for Foods [ICMSF] (1996). *Microorganisms in Foods 5 – Characteristics of Microbial Pathogens*. London: Blackie Academic & Professional, 148.

ISO (2004a). *EN ISO 11290-1:1996/Amd 1:2004 Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Listeria monocytogenes – Detection Method*, Part I. Geneva: International Organization for Standardization.

ISO (2004b). *EN ISO 11290-2:1998/Amd 1:2004 Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Listeria monocytogenes – Enumeration Method*, Part II. Geneva: International Organization for Standardization.

Jadhav, S., Bhave, M., and Palombo, E. A. (2012). Methods used for the detection and subtyping of *Listeria monocytogenes*. *J. Microbiol. Methods* 88, 327–341. doi: 10.1016/j.mimet.2012.01.002

Jamali, H., Chai, L. C., and Thong, K. L. (2013). Detection and isolation of *Listeria* spp. and *Listeria monocytogenes* in ready-to-eat foods with various selective culture media. *Food Control* 32, 19–24. doi: 10.1016/j.foodcont.2012.11.033

Janzten, M. M., Navas, J., Corujo, A., Moreno, R., López, V., and Martínez-Suárez, J. V. (2006). Review. Specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR. *Span. J. Agric. Res.* 4, 235–247. doi: 10.5424/sjar/2006043-198

Jemmi, T., and Stephan, R. (2006). *Listeria monocytogenes*: food-borne pathogen and hygiene indicator. *Rev. Sci. Tech.* 25, 571–580.

Jeyaletchumi, P., Tunung, R., Margaret, S. P., Son, P., Farinazleen, M. G., and Cheah, Y. K. (2010a). Detection of *Listeria monocytogenes* in foods. *Int. Food Res. J.* 17, 1–11.

Jeyaletchumi, P., Tunung, R., Margaret, S. P., Son, R., Ghazali, F. M., Cheah, Y. K., et al. (2010b). Quantification of *Listeria monocytogenes* in salad vegetables by MPN-PCR. *Int. Food Res. J.* 17, 281–286. doi: 10.1155/2014/608296

Jofré, A., Martin, B., Garriga, M., Hugas, M., Pla, M., and Rodríguez-Lázaro, D. (2005). Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. *Food Microbiol.* 22, 109–115. doi: 10.1016/j.fm.2004.04.009

Johnson, W. M., Tyler, S. D., Ewan, E. P., Ashton, F. E., Wang, G., and Rozee, K. R. (1992). Detection of genes coding for listeriolysin and *Listeria monocytogenes* antigen A (lmaA) in *Listeria* spp. by the polymerase chain reaction. *Microb. Pathog.* 12, 79–86. doi: 10.1016/0882-4010(92)90068-Y

Jung, Y. S., Frank, J. F., Brackett, R. E., and Chen, J. (2003). Polymerase chain reaction detection of *Listeria monocytogenes* in frankfurters using

oligonucleotide primers targeting the genes encoding internalin AB. *J. Food Prot.* 66, 237–241.

Kathariou, S., Metz, P., Hof, H., and Goebel, W. (1987). Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *J. Bacteriol.* 169, 1291–1297.

Khan, J. A., Rathore, R. S., Khan, S., and Ahmad, I. (2013). In vitro detection of pathogenic *Listeria monocytogenes* from food sources by conventional, molecular and cell culture method. *Braz. J. Microbiol.* 44, 751–758. doi: 10.1590/S1517-83822013000300013

Kim, H. J., and Cho, J. C. (2010). Simple and rapid detection of *Listeria monocytogenes* in fruit juice by real-time PCR without enrichment culture. *Food Control* 21, 1419–1423. doi: 10.1016/j.foodcont.2010.04.006

Kraiss, J. B. R. K. (2008). *Listeria Regulations in the fda and usda: Implications for Dual-Jurisdiction facilitites. Food regulation in the United States, 1-18*. Available at: http://www.iflr.msu.edu/uploads/files/Student%20Papers/Janet%20B.%20Rowat%20KraissListeriaRegulationsFDAandUSDA.pdf [accessed September 23, 2015].

Kramarenko, T., Roasto, M., Meremäe, K., Kuningas, M., Põltsama, P., and Elias, T. (2013). *Listeria monocytogenes* prevalence and serotype diversity in various foods. *Food Control* 30, 24–29. doi: 10.1016/j.foodcont.2012.06.047

Kumar, T. D. K., Murali, H. S., and Batra, H. V. (2009). Simultaneous detection of pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* by multiplex PCR. *Indian J. Microbiol.* 49, 283–289. doi: 10.1007/s12088-009-0032-y

Lambertz, S. T., Nilsson, C., Brådenmark, A., Sylvén, S., Johansson, A., Jansson, L. M., et al. (2012). Prevalence and level of *Listeria monocytogenes* in ready-to-eat foods in Sweden 2010. *Int. J. Food Microbiol.* 160, 24–31. doi: 10.1016/j.ijfoodmicro.2012.09.010

Lauri, A., and Mariani, P. O. (2009). Potentials and limitations of molecular diagnostic methods in food safety. *Genes Nutr.* 4, 1–12. doi: 10.1007/s12263-008-0106-1

Law, J. W. F., Ab 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

Lawrence, L. M., and Gilmour, A. (1994). Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. *Appl. Eviron. Microbiol.* 60, 4600–4604.

Lee, N., Kwon, K. Y., Oh, S. K., Chang, H. J., Chun, H. S., and Choi, S. W. (2014). A multiplex PCR assay for simultaneous detection of *Escherichia coli* O157:H7, *Bacillus cereus*, *Vibrio parahaemolyticus*, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in Korean ready-to-eat food. *Foodborne Pathog. Dis.* 11, 574–580. doi: 10.1089/fpd.2013.1638

Leone, G., van Schijndel, H., van Gemen, B., Kramer, F. R., and Schoen, C. D. (1998). Molecular beacon probes combined with amplification by NASBA enable homogenous, real-time detection of RNA. *Nucleic Acids Res.* 26, 2150–2155. doi: 10.1093/nar/26.9.2150

Letchumanan, V., Chan, K. G., and Lee, L. H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front. Microbiol.* 5:705. doi: 10.3389/fmicb.2014.00705

Letchumanan, V., Chan, K. G., and Lee, L. H. (2015a). An insight of traditional plasmid curing in *Vibrio* species. *Front. Microbiol.* 6:735. doi: 10.3389/fmicb.2015.00735

Letchumanan, V., Yin, W. F., Lee, L. H., and Chan, K. G. (2015b). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Front. Microbiol.* 6:33. doi: 10.3389/fmicb.2015.00033

Levin, R. E. (2003). Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: a review of methodology. *Food Biotechnol.* 17, 99–116. doi: 10.1081/FBT-120023074

Liming, S. H., Zhang, Y., Meng, J., and Bhagwat, A. A. (2004). Detection of *Listeria monocytogenes* in fresh produce using molecular beacon-real-time PCR technology. *J. Food Sci.* 69, M240–M245. doi: 10.1111/j.1750-3841.2004.tb18020.x

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

Liu, D., Lawrence, M. L., Austin, F. W., and Ainsworth, A. J. (2007). A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *J. Microbiol. Methods* 71, 133–140. doi: 10.1016/j.mimet.2007.08.007

Liu, H., Lu, L., Pan, Y., Sun, X., Hwang, C. A., Zhao, Y., et al. (2015). Rapid detection and differentiation of *Listeria monocytogenes* and *Listeria* species in deli meats by a new multiplex PCR method. *Food Control* 52, 78–84. doi: 10.1016/j.foodcont.2014.12.017

Liu, P., Mizue, H., Fujihara, K., Kobayashi, H., Kamikado, H., Tanaka, T., et al. (2012). A new rapid real-time PCR method for detection of *Listeria monocytogenes* targeting the hlyA gene. *Food Sci. Technol. Res.* 18, 47–57. doi: 10.3136/fstr.18.47

Lovett, J., Francis, D. W., and Hunt, J. M. (1987). *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. *J. Food Prot.* 50, 188–192. doi: 10.1128/AEM.00219-13

Magalhães, R., Mena, C., Ferreira, V., Almeida, G., Silva, J., and Teixeira, P. (2014). "Traditional methods for isolation of *Listeria monocytogenes*," in *Listeria monocytogenes: Methods and Protocols*, eds K. Jordan, E. M. Fox, and M. Wagner (New York, NY: Springer Science), 15–30.

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.* 6, 87–102. doi: 10.3923/ajft.2011.87.102

Markoulatos, P., Siafakas, N., and Moncany, M. (2002). Multiplex polymerase chain reaction: a practical approach. *J. Clin. Lab. Anal.* 16, 47–51. doi: 10.1002/jcla.2058

Martín, B., Jofré, A., Garriga, M., Hugas, M., and Aymerich, T. (2004). Quantification of *Listeria monocytogenes* in fermented sausages by MPN-PCR method. *Lett. Appl. Microbiol.* 39, 290–295. doi: 10.1111/j.1472-765X.2004.01580.x

Medini, D., Serruto, D., Parkhill, J., Relman, D. A., Donati, C., Moxon, R., et al. (2008). Microbiology in the post-genomic era. *Nat. Rev. Microbiol.* 6, 419–430. doi: 10.1038/nrmicro1901

Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Hogg, T., and Gibbs, P. A. (2004). Incidence of *Listeria monocytogenes* in different food products commercialized in Portugal. *Food Microbiol.* 21, 213–216. doi: 10.1016/S0740-0020(03)00057-1

Moriishi, K., Terao, M., Koura, M., and Inoue, S. (1998). Sequence analysis of the actA gene of *Listeria monocytogenes* isolated from human. *Microbiol. Immunol.* 42, 129–132. doi: 10.1111/j.1348-0421.1998.tb02261.x

Murphy, R. Y., Duncan, L. K., Driscoll, K. H., Beard, B. L., Berrang, M. B., and Marcy, J. A. (2003). Determination of thermal lethality of *Listeria monocytogenes* in fully cooked chicken breast fillets and strips during postcook in-package pasteurization. *J. Food. Prot.* 66, 578–583.

Nadal, A., Coll, A., Cook, N., and Pla, M. (2007). A molecular beacon-based real time NASBA assay for detection of *Listeria monocytogenes* in food products: role of target mRNA secondary structure on NASBA design. *J. Microbiol. Methods* 68, 623–632. doi: 10.1016/j.mimet.2006.11.011

Navas, J., Ortiz, S., Lopez, P., Jantzen, M. M., Lopez, V., and Martinez-Suarez, J. V. (2006). Evaluation of effects of primary and secondary enrichment for the detection of *Listeria monocytogenes* by real-time PCR in retail ground chicken meat. *Foodborne Pathog. Dis.* 3, 347–354. doi: 10.1089/fpd.2006.3.347

O'Grady, J., Sedano-Balbás, S., Maher, M., Smith, T., and Barry, T. (2008). Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the ssrA gene, a novel diagnostic target. *Food Microbiol.* 25, 75–84. doi: 10.1016/j.fm.2007.07.007

Olsen, S. J., Patrick, M., Hunter, S. B., Reddy, V., Kornstein, L., MacKenzie, W. R., et al. (2005). Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clin. Infect. Dis.* 40, 962–967. doi: 10.1086/428575

Oravcová, K., Kaclíková, E., Krascsenicsová, K., Pangallo, D., Brežná, B., Siekel, P., et al. (2005). Detection and quantification of *Listeria monocytogenes* by 5'-nuclease polymerase chain reaction targeting the actA gene. *Lett. Appl. Microbiol.* 42, 15–18. doi: 10.1111/j.1472-765X.2005.01793.x

Orsi, R. H., Borowsky, M. L., Lauer, P., Young, S. K., Nusbaum, C., Galagan, J. E., et al. (2008). Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. *BMC Genomics* 9:539. doi: 10.1186/1471-2164-9-539

Osman, K. M., Samir, A., Orabi, A., and Zolnikov, T. R. (2014). Confirmed low prevalence of *Listeria monocytogenes* in she-camel milk delivers a safe, alternative milk for human consumption. *Acta Trop.* 130, 1–6. doi: 10.1016/j.actatropica.2013.10.001

Ottaviani, F., Ottaviani, M., and Agosti, M. (1997). Esperienza su un agar selettivo e differentiale per *Listeria monocytogenes*. *Ind. Alimentarius* 36, 1–3.

Pangallo, D., Kaclikova, E., Kuchta, T., and Drahovska, H. (2001). Detection of *Listeria monocytogenes* by polymerase chain reaction oriented to inlB gene. *New Microbiol.* 24, 333–339.

Park, S. H., Chang, P. S., Ryu, S., and Kang, D. H. (2014). Development of a novel selective and differential medium for the isolation of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 80, 1020–1025. doi: 10.1128/AEM.02840-13

Pichler, J., Appl, G., Pietzka, A., and Allerberger, F. (2011). Lessons to be learned from an outbreak of foodborne Listeriosis, Austria 2009–2010. *Food Prot. Trends* 31, 268–273.

Pinto, M., Burri, S., Mena, C., Almeida, G., Carneiro, L., Teixeira, P., et al. (2001). Comparison of Oxford Agar, PALCAM and *Listeria monocytogenes* Blood Agar for the recovery of *L. monocytogenes* from foods and environmental samples. *Food Control* 12, 511–514. doi: 10.1016/S0956-7135(01)00052-4

Rantsiou, K., Alessandria, V., Urso, R., Dolci, P., and Cocolin, L. (2008). Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *Int. J. Food Microbiol.* 121, 99–105. doi: 10.1016/j.ijfoodmicro.2007.11.006

Rawool, D. B., Malik, S. V. S., Barbuddhe, S. B., Shakuntala, I., and Aurora, R. (2007). A multiplex PCR for detection of virulence associated genes in *Listeria monocytogenes*. *Int. J. Food Saf.* 9, 56–62.

Reddy, T. B., Thomas, A. D., Stamatis, D., Bertsch, J., Isbandi, M., Jansson, J., et al. (2015). The Genomes OnLine Database (GOLD) v.5: a metadata management system based on a four level (meta) genome project classification. *Nucleic Acids Res.* 43:D1099-106. doi: 10.1093/nar/gku950

Reuter, S., Ellington, M. J., Cartwright, E. J., Köser, C. U., Török, M. E., Gouliouris, T., et al. (2013). Rapid bacterial whole-genome sequencing to enhance diagnostic and public health microbiology. *JAMA Intern. Med.* 173, 1397–1404. doi: 10.1001/jamainternmed.2013.7734

Rodríguez-Lázaro, D., Hernández, M., and Pla, M. (2004). Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using a duplex real-time PCR-based assay. *FEMS Microbiol. Lett.* 233, 257–267. doi: 10.1111/j.1574-6968.2004.tb09490.x

Rossmanith, P., Krassnig, M., Wagner, M., and Hein, I. (2006). Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the prfA gene. *Res. Microbiol.* 157, 763–771. doi: 10.1016/j.resmic.2006.03.003

Rudol, M., and Scherer, S. (2001). High incidence of *Listeria monocytogenes* in European red smear cheese. *Int. J. Food Microbiol.* 63, 91–98. doi: 10.1016/S0168-1605(00)00413-X

Rychli, K., Müller, A., Zaiser, A., Schoder, D., Allerberger, F., Wagner, M., et al. (2014). Genome sequencing of *Listeria monocytogenes* "Quargel" listeriosis outbreak strains reveals two different strains with distinct in vitro virulence potential. *PLoS ONE* 9:e89964. doi: 10.1371/journal.pone.0089964

Ryu, J., Park, S. H., Yeom, Y. S., Shrivastav, A., Lee, S. H., Kim, Y. R., et al. (2013). Simultaneous detection of *Listeria* species isolated from meat processed foods using multiplex PCR. *Food Control* 32, 659–664. doi: 10.1016/j.foodcont.2013.01.048

Sabat, A. J., Budimir, A., Nashev, D., Sá-Leão, R., Van Dijl, J. M., Laurent, F., et al. (2013). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro. Surveill.* 18:20380.

Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F., and Mabilat, C. (1996). Comparative analysis of 16S and 23S rRNA sequences of *Listeria* species. *Int. J. Syst. Bacteriol.* 46, 669–674. doi: 10.1099/00207713-46-3-669

Salvat, G., and Fravalo, P. (2004). Risk assessment strategies for Europe: integrated safety or final product control: example of *Listeria monocytogenes* in processed products from pork meat industry. *Dtsch. Tierarztl. Wochenschr.* 111, 331–334.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467. doi: 10.1073/pnas.74.12.5463

Severgnini, M., Cremonesi, P., Consolandi, C., De Bellis, G., and Castiglioni, B. (2011). Advances in DNA microarray technology for the detection of foodborne pathogens. *Food Bioprocess. Technol.* 4, 936–953. doi: 10.4061/2011/310135

Shan, X., Zhang, Y., Zhang, Z., Chen, M., Su, Y., Yuan, Y., et al. (2012). Rapid detection of food-borne *Listeria monocytogenes* by real-time quantitative loop-mediated isothermal amplification. *Food Sci. Biotechnol.* 21, 101–106. doi: 10.1007/s10068-012-0012-6

Shendure, J., and Ji, H. (2008). Next-generation DNA sequencing. *Nat. Biotechnol.* 26, 1135–1145. doi: 10.1038/nbt1486

Simon, M. C., Gray, D. I., and Cook, N. (1996). DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. *Appl. Environ. Microbiol.* 62, 822–824.

Simpkins, S. A., Chan, A. B., Hays, J., Pöpping, B., and Cook, N. (2000). An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Lett. Appl. Microbiol.* 30, 75–79. doi: 10.1046/j.1472-765x.2000.00670.x

Smith, M. A., Takeuchi, K., Brackett, R. E., McClure, H. M., Raybourne, R. B., Williams, K. M., et al. (2003). Nonhuman primate model for *Listeria monocytogenes*-induced stillbirths. *Infect. Immun.* 71, 1574–1579. doi: 10.1128/IAI.71.3.1574-1579.2003

Stone, S. C., and Shoenberger, J. (2001). Update: multistate outbreak of listeriosis – United States, 2000. *Ann. Emerg. Med.* 38, 339–341. doi: 10.1067/mem.2001.117781

Suo, B., He, Y., Paoli, G., Gehring, A., Tu, S. I., and Shi, X. (2010). Development of an oligonucleotide-based microarray to detect multiple foodborne pathogens. *Mol. Cell. Probes* 24, 77–86. doi: 10.1016/j.mcp.2009.10.005

Sutton, S. (2010). The most probable number method and its uses in enumeration, qualification, and validation. *Microbiol. Top.* 16, 35–38.

Swetha, C. S., Madhava Rao, T., Krishnaiah, N., and Vijaya Kumar, A. (2012). Detection of *Listeria monocytogenes* in fish samples by PCR assay. *Ann. Biol. Res.* 3, 1880–1884.

Tang, M. J., Zhou, S., Zhang, X. Y., Pu, J. H., Ge, Q. L., Tang, X. J., et al. (2011). Rapid and sensitive detection of *Listeria monocytogenes* by loop-mediated isothermal amplification. *Curr. Microbiol.* 63, 511–516. doi: 10.1007/s00284-011-0013-3

USDA-FSIS (2013). *Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry and Egg Products, and Environmental Samples. Effective January, 2013 MLG 8.09*. Available at: http://www.fsis.usda.gov/wps/wcm/connect/1710bee8-76b9-4e6c-92fc-fdc290dbfa92/MLG-8.pdf?MOD=AJPERES [accessed May 16, 2015].

Uyttendaele, M., Schukkink, R., van Gemen, B., and Debevere, J. (1995). Development of NASBA®, a nucleic acid amplification system, for identification of *Listeria monocytogenes* and comparison to ELISA and a modified FDA method. *Int. J. Food Microbiol.* 27, 77–89. doi: 10.1016/0168-1605(95)00166-H

Välimaa, A. L., Tilsala-Timisjärvi, A., and Virtanen, E. (2015). Rapid detection and identification methods for *Listeria monocytogenes* in the food chain – a review. *Food Control*55, 103–114. doi: 10.1016/j.foodcont.2015.02.037

Van Netten, P., Perales, I., van de Moosdijk, A., Curtis, G. D. W., and Mossel, D. A. A. (1989). Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. *Int. J. Food Microbiol.* 8, 299–316. doi: 10.1016/0168-1605(89)90001-9

Vlaemynck, G., Lafarge, V., and Scotter, S. (2000). Improvement of the detection of *Listeria monocytogenes* by the application of ALOA, a diagnostic, chromogenic isolation medium. *J. Appl. Microbiol.* 88, 430–441. doi: 10.1046/j.1365-2672.2000.00978.x

Volokhov, D., Rasooly, A., Chumakov, K., and Chizhikov, V. (2002). Identification of *Listeria* species by microarray-based assay. *J. Clin. Microbiol.* 40, 4720–4728. doi: 10.1128/JCM.40.12.4720-4728.2002

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, D. G., Brewster, J. D., Paul, M., and Tomasula, P. M. (2015). Two methods for increased specificity and sensitivity in loop-mediated isothermal amplification. *Molecules* 20, 6048–6059. doi: 10.3390/molecules20046048

Wang, D., Zhang, G., Lu, C., Deng, R., Zhi, A., Guo, J., et al. (2011). Rapid detection of *Listeria monocytogenes* in raw milk with loop-mediated isothermal amplification and chemosensor. *J. Food Sci.* 76, M611–M615. doi: 10.1111/j.1750-3841.2011.02383.x

Wang, R. F., Cao, W. W., and Johnson, M. G. (1992). 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Appl. Environ. Microbiol.* 58, 2827–2831.

Wang, X. M., Lü, X. F., Yin, L., Liu, H. F., Zhang, W. J., Si, W., et al. (2013). Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. *Food Control* 32, 153–158. doi: 10.1016/j.foodcont.2012.11.032

Wernars, K., Heuvelman, C. J., Chakraborty, T., and Notermans, S. H. W. (1991). Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. *J. Appl. Bacteriol.* 70, 121–126. doi: 10.1111/j.1365-2672.1991.tb04437.x

Winters, D. K., Maloney, T. P., and Johnson, M. G. (1999). Rapid detection of *Listeria monocytogenes* by a PCR assay specific for an aminopeptidase. *Mol. Cell. Probes* 13, 127–131. doi: 10.1006/mcpr.1999.0224

Wu, R., Liu, X., Guo, B., Chen, F., and Wang, X. (2014). Development of double loop-mediated isothermal amplification to detect *Listeria monocytogenes* in food. *Curr. Microbiol.* 69, 839–845. doi: 10.1007/s00284-014-0661-1

Yang, Y., Xu, F., Xu, H., Aguilar, Z. P., Niu, R., Yuan, Y., et al. (2013). Magnetic nano-beads based separation combined with propidium monoazide treatment and multiplex PCR assay for simultaneous detection of viable *Salmonella* Typhimurium. *Escherichia coli* O157:H7 and *Listeria monocytogenes* in food products. *Food Microbiol.* 34, 418–424. doi: 10.1016/j.fm.2013.01.004

Ye, Y., Wang, B., Huang, F., Song, Y., Yan, H., Alam, M. J., et al. (2011). Application of in situ loop-mediated isothermal amplification method for detection of *Salmonella* in foods. *Food Control* 22, 438–444. doi: 10.1016/j.foodcont.2010.09.023

Yuan, Y., Xu, W., Zhai, Z., Shi, H., Luo, Y., Chen, Z., et al. (2009). Universal primer-multiplex PCR approach for simultaneous detection of *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. in food samples. *J. Food Sci.* 74, M446–M452. doi: 10.1111/j.1750-3841.2009.01321.x

Zarei, M., Maktabi, S., and Ghorbanpour, M. (2012). Prevalence of *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. in seafood products using multiplex polymerase chain reaction. *Foodborne Pathog. Dis.* 9, 108–112. doi: 10.1089/fpd.2011.0989

Zeng, H., Zhang, X., Sun, Z., and Fang, W. (2006). Multiplex PCR identification of *Listeria monocytogenes* isolates from milk and milk-processing environments. *J. Sci. Food Agric.* 86, 367–371. doi: 10.1002/jsfa.2352

Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X., and Feng, Y. (2009). Simultaneous detection of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* O157:H7 in food samples using multiplex PCR method. *J. Food Saf.* 29, 348–363. doi: 10.1111/j.1745-4565.2009.00161.x

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

Zhou, X., and Jiao, X. (2005). Polymerase chain reaction detection of *Listeria monocytogenes* using oligonucleotide primers targeting actA gene. *Food Control* 16, 125–130. doi: 10.1016/j.foodcont.2004.01.001

Zunabovic, M., Domig, K. J., and Kneifel, W. (2011). Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments – a review. *LWT-Food Sci. Technol.* 44, 351–362. doi: 10.1016/j.lwt.2010.08.005

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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 29 September 2015
doi: 10.3389/fmicb.2015.01026

Prevalence, enumeration, and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from raw foods in South China

Moutong Chen[1,2], Qingping Wu[1], Jumei Zhang[1], Shi Wu[1,2] and Weipeng Guo[1]*

[1] Guangdong Institute of Microbiology, State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangzhou, China, [2] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China

OPEN ACCESS

Edited by:
Giovanna Suzzi, Università degli Studi di Teramo, Italy

Reviewed by:
Alexandra Lianou, Agricultural University of Athens, Greece
Graziella Bourdin, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail, France

***Correspondence:**
Qingping Wu, Guangdong Institute of Microbiology, State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Xianlie Zhong Road 100 #, 58th Building, Guangzhou 510070, China
wuqp203@163.com

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

Received: *06 July 2015*
Accepted: *09 September 2015*
Published: *29 September 2015*

Citation:
Chen M, Wu Q, Zhang J, Wu S and Guo W (2015) Prevalence, enumeration, and pheno- and genotypic characteristics of Listeria monocytogenes isolated from raw foods in South China. Front. Microbiol. 6:1026. doi: 10.3389/fmicb.2015.01026

Listeria monocytogenes is an important foodborne pathogen that can cause serious illness in immunocompromised individuals, pregnant women, the elderly, and newborns. The aim of this study was to: (i) evaluate the prevalence and contamination level [most probable number (MPN)] of *L. monocytogenes* in 567 retail raw foods (fishery products, $n = 154$; raw/fresh meat, $n = 123$; frozen foods, $n = 110$; edible fungi, $n = 108$; vegetables, $n = 72$) collected from South China and (ii) to gain further knowledge on the phenotype and genotype distributions of this important foodborne pathogen. Approximately 22% of the samples were positive for *L. monocytogenes*. The contamination levels were between 0.3 and 10 MPN/g in 75.0%, between 10 and 100 MPN/g in 11.0% and less than 100 MPN/g in 14.0% of the countable samples. Five serogroups were identified among the 177 foodborne *L. monocytogenes* isolates, with 1/2a-3a (42.4%) and 1/2b-3b (26.0%) serogroups being the most dominant. Serogroups I.1 and II.2 were only found in the edible mushrooms, while serogroup III was dominant in the fishery products, suggesting that specific serogroups of *L. monocytogenes* may have distinct ecological niches. Ten (5.6%) *L. monocytogenes* isolates exhibited multidrug resistance. Genetic relatedness analysis revealed the absence of distinct associations between specific food types, antibiotic resistance, serogroups, and genetic diversity. The present study provided the first baseline data on the prevalence, contamination level, and characteristics of *L. monocytogenes* isolated from raw foods in South China. Some multidrug resistant strains belonged to the epidemiologically important serogroups (I.1 and II.1), implying a potential public health risk. In addition, these findings also provide basic information for the Chinese food safety associated authorities to draft appropriate standards to control *L. monocytogenes* contamination and improve microbiological safety of raw foods.

Keywords: *Listeria monocytogenes*, most probable number, enterobacterial repetitive intergenic consensus PCR, random amplified polymorphic DNA, antimicrobial susceptibility profile

Introduction

Listeria monocytogenes, a facultative intracellular foodborne pathogen, is capable of causing serious disease in humans, especially in immunocompromised individuals, pregnant women, the elderly, and newborns. It has the ability to inhabit a wide range of environments and is commonly found in food, nature, and the food processing environment (Abadias et al., 2008). Listeriosis is a severe invasive disease and the manifestations include neurological infections like encephalitis, meningitis, septicemia, and abortion, with a mortality rate of up to 20–30% (Vazquez-Boland et al., 2001; Kang et al., 2013).

Foodborne outbreaks of listeriosis have been documented in Asia, Europe, and the USA (Makino et al., 2005; de Castro et al., 2012; Lomonaco et al., 2013). In recent years, several sporadic cases of listeriosis have been reported in China (Wu et al., 2008; Hsieh et al., 2009; Feng et al., 2011). *L. monocytogenes* isolates associated with outbreaks or sporadic cases of listeriosis have been detected in different kinds of foods including dairy products, raw meat, vegetables, and fishery products (Schlech, 2000; Bell and Kyriakides, 2005; Yücel et al., 2005). Identifying the source of infection is essential to undertake preventive measures and control the exposure to such infection (Franciosa et al., 1998). Thus, determining the prevalence and contamination level of *L. monocytogenes* in retail foods are of utmost importance to control and track the definite source of *L. monocytogenes*. In China, previous studies have focused on the prevalence of *L. monocytogenes* in retail foods. However, these studies only focused their investigation to limited regions, such as Heilongjiang Province, Guangzhou City, and Gansu Province (Hu et al., 2013; Shi et al., 2013; Wang et al., 2013). In this context, it is notable that South China is located in a subtropical region where the climate is suitable for *L. monocytogenes* growth. Several studies have focused on the occurrence of *L. monocytogenes* in ready-to-eat foods (Huang et al., 2005; Mei et al., 2006; Lianou and Sofos, 2007; Jin et al., 2009; Sauders et al., 2009). Additionally, numerous previous studies have reported the contamination level of *L. monocytogenes* in ready-to-eat products in other countries (Lianou and Sofos, 2007). Wang et al. (2013) reported occurrence and counts of *L. monocytogenes* in retail raw foods in Heilongjiang Province (Northeast of China) from 2008 to 2009. However, there are limited studies that focused on the contamination level of *L. monocytogenes* in retail raw foods in South China, especially the contamination level in edible fungi and vegetables, thus, hampering the potential risk analysis of *L. monocytogenes* in retail raw foods. It is necessary to monitor the occurrence of *L. monocytogenes* in retail raw foods because of possible chances of cross-contamination during food processing and food storage.

Since the first multiresistant *L. monocytogenes* strain was isolated from a patient with meningoencephalitis in France in 1988 (Poyart-Salmeron et al., 1990), antimicrobial resistant strains have been commonly recovered from food, natural environment, and clinical cases of listeriosis (Rodas-Suárez et al., 2006; Zhang et al., 2007; Conter et al., 2009; Morvan et al., 2010; Granier et al., 2011).The resistance profiles are varied and may be influence by overuse of antimicrobials in humans and livestock animals, as well as by geographical differences. In China, the rates of antibiotic resistance in 467 foodborne *L. monocytogenes* isolates were 4.5% in 2005, and the antimicrobial resistance was most frequently observed for ciprofloxacin and tetracycline (Yang et al., 2008); similar results were also reported by Yan et al. (2010). Zhao et al. (2012) reported that the rates of antibiotic resistance in 1069 foodborne *L. monocytogenes* isolates were 6.92% in China. The resistance was most prevalently observed for antibiotics such as tetracycline, doxycycline, erythromycin, chloramphenicol, and ciprofloxacin. Based on this trend of antibiotic resistance, it is advisable to monitor the patterns of antibiotic resistance of *L. monocytogenes* in food sources from different regions in China.

Listeria monocytogenes has 13 serotypes that can be divided into five serogroups, such as I.1 (1/2a-3a), I.2 (1/2c-3c), II.1 (4b-4d-4e), II.2 (1/2b-3b-7), and III (4a-4c; Doumith et al., 2004). As 95% of the strains responsible for human listeriosis and found in food samples belong to serotype 1/2a, 1/2b, 1/2c, and 4b (Pontello et al., 2012; Althaus et al., 2014), serotype analysis is not very efficient method to differentiate *L. monocytogenes* strains isolated from foods and clinical samples. Recently, numerous molecular subtyping techniques have been developed for the surveillance or tracing the sources of *L. monocytogenes*; these include pulsed field gel electrophoresis (PFGE; Gerner-Smidt et al., 2006), amplified fragment length polymorphism (AFLP; Parisi et al., 2010), random amplified polymorphic DNA (RAPD; Farber and Addison, 1994), multi-locus sequence typing (MLST; Parisi et al., 2010), and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR; Chen et al., 2010). Of these techniques, ERIC-PCR is a relatively simple, highly reliable, and cost-effective method that has been demonstrated to generate distinct DNA fingerprints within a single bacterial species (Jersek et al., 1999).

The objective of the present study was to: (i) determine the prevalence and contamination level of *L. monocytogenes* in retail raw foods in South China and (ii) to determine the genetic variation and phenotypic characteristics of *L. monocytogenes* isolates.

Materials and Methods

Sampling Procedure

From July 2011 to August 2012, a total of 567 retail raw food samples were collected from rural markets, open-air markets, and large supermarkets in South China, including five districts of Guangzhou City and 11 cities in Guangdong, Fujian, Guangxi, and Hainan Province. Samples comprised of fishery products ($n = 154$, including prawn, Grass carp, fresh squid, speckled fish, yellow croaker, catfish, oyster, *Tilapia mossambica*), raw/fresh meat ($n = 123$, including fresh pork, fresh beef, chicken, duck, Minced pork, fresh pork ribs, fresh pork ball, mutton), frozen foods ($n = 110$, including Chinese dumpling, frozen mutton, frozen chicken wing, frozen han sausage, frozen drumstick, wonton, frozen salted pork), fresh edible fungi ($n = 108$, including *Flammulina velutipes*, *Pleurotus eryngii*, *Hypsizygus marmoreus*, *Coriandrum sativum*, *Lentinus edodes*, oyster mushroom, *Pleurotus geesteranus*), and vegetables ($n = 72$,

including Lettuce, cucumber, coriander). Samples were stored in insulated shipping coolers containing frozen gel packs placed on the sides, middle, and the tops of the samples. All the samples were kept below 4°C during transportation and test was initiated within 4 h after receipt.

Qualitative and Quantitative Analysis for Food Samples

An enrichment method according to the National Food Safety Standard of China – Food microbiological examination: *L. monocytogenes* (GB 4789.30-2010; National Standard of the People's Republic of China, 2010) with minor modifications was used for qualitative detection. In brief, samples were analyzed for the presence of *L. monocytogenes* by homogenizing 25 g samples in 225 mL *Listeria* enrichment broth I (LB1; Huankai, Co. Ltd., China). Homogenates were incubated at 30°C for 24 h. Thereafter, 0.1 mL LB1 enrichment culture was transferred to 10 mL *Listeria* enrichment broth II (LB2) at 30°C for 24 h. A loopful of the LB2 enrichment culture was streaked onto Chromagar®*Listeria* selective agar plates (CHROM-agar, Paris, France) and incubated at 37°C for 48 h. Three to five presumptive colonies that were typically blue in color with a white halo were selected for identification of *L. monocytogenes* by Microgen ID *Listeria* identification system (Microgen, Camberley, UK) according to the manufacturer's instructions.

To determine the most probable number (MPN), the method was adapted from a previous study conducted by Gombas et al. (2003). Briefly, a nine-tube MPN method was used. The nine tubes were divided into three sets of three tubes each. The second and third sets of tubes contained 10 ml of Fraser broth medium. Three aliquots (10, 1, and 0.1 mL) of the sample homogenate were dispensed into three sets, representing 1.0, 0.1, and 0.01 g of the original sample, respectively. The tubes were incubated at 30 ± 2°C for 24 ± 2 h, and subsequently 0.1 mL of content from each tube was transferred to a new tube containing 10 ml of fresh Fraser broth. The tubes were incubated at 30 ± 2°C for 26 ± 2 h. Darkened Fraser tubes were subjected to confirmation. If a Fraser broth failed to darken, it was examined again after an additional 26 ± 2 h of incubation. The MPN values were determined based on the number of positive tube(s) in each of the three sets and the MPN table (U. S. Department of Agriculture, 1998; Hitchins, 1998).

Serogroup Analysis using Multiplex PCR

Genomic DNA was extracted from *L. monocytogenes* using Bacterial Genomic DNA Purification Kit (Dongsheng Biotech. Inc., Guangzhou, China) according to the manufacturer's instruction. DNA concentration was determined at O.D. 260 nm using Nano Drop®ND-1000UVeVis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Serogroup analysis of 177 isolates was performed using a multiplex PCR as previously described by Doumith et al. (2004),13 serotypes of *L. monocytogenes* were categorized into five distinct phylogenetic groups, viz. I.1 (1/2a-3a), I.2 (1/2c-3c), II.1 (4b-4d-4e), II.2 (1/2b-3b-7), and III (4a-4c). The PCR mixture (50-μL) contained 1.5 unit GoTaq®Hotstart polymerase (Promega, Madison, WI, USA), 1 μM for *lmo0737*, *ORF2819*, and *ORF2110*; 1.5 μM for *lmo1118*; and 0.2 μM for *prs*, 2.5 mM MgCl2, 0.2 mM each dNTP, and 40 ng of template genomic DNA. PCR was performed with the following thermal cycle: initial denaturation step at 94°C for 3 min; 35 cycles of 94°C for 35 s, 53°C for 50 s, and 72°C for 60 s; and a final cycle of 72°C for 7 min in a thermocycler (Applied Biosystems, Foster City, CA, USA). Five microliters of the reaction mixture was mixed with 5 μL of loading buffer and separated on a 2% agarose gel in TAE buffer. The PCR product was visualized by Goldview®staining (0.005%, v/v).The primers are shown in Supplementary Table S1.

Enterobacterial Repetitive Intergenic Consensus Sequence Polymerase Chain Reaction (ERIC-PCR)

Enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) typing was carried out on the *L. monocytogenes* isolates and five reference strains using the protocol described by Chen et al. (2014). The ERIC primers was described by Versalovic et al. (1991), ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′, and ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′. The PCR mixture (25-μL) contained one unit GoTaq®Hotstart polymerase (Promega, Madison, WI, USA), 0.6 μM of each primer, 2.5 mM MgCl2, 0.2 mM each dNTP, and 40 ng of template genomic DNA. Amplifications were performed in a DNA thermocycler (Applied Biosystems, Foster City, CA, USA) with the following temperature profile: an initial denaturation at 94°C for 3 min; 35 cycles each consisting of 30 s at 94°C, 30 s at 46°C, 30 s at 49°C, and 3 min at 72°C; and a final extension at 72°C for 10 min. The ERIC-PCR products were separated by electrophoresis on a 1.5% agarose gel with Goldview®staining (0.005%, v/v), and photographed using an UV Imaging System (GE Healthcare, Waukesha, WI, USA). The images were captured in TIFF file format for further analysis.

Cluster Analysis

The observed bands in the gels were evaluated based on the presence (coded 1) or absence (coded 0) of polymorphic fragments for the ERIC and RAPD primers. Analysis of TIFF images was carried out using Gel Pro Analyzer (Version 4.0) and NTSYS-pc (Version 2.10), a numerical taxonomy and multivariate analysis software package (Rohlf, 2000). Similarity between fingerprints was determined by the Dice's similarity coefficient at 1% band position tolerance and dendrograms were generated by unweighted pair group method using arithmetic average (UPGMA).The Simpson's indexes of discrimination (DI) of ERIC-PCR and RAPD were calculated as described by Hunter and Gaston to determine the ability of each typing method (Hunter and Gaston, 1988).

Random Amplified Polymorphic DNA

According to a previous study conducted by Chen et al. (2014), the 10-mer primer UBC155 (5′-CTGGCGGCTG-3′) was chosen for typing the *L. monocytogenes* isolates in this study (Farber and Addison, 1994). The PCR reaction condition used for the selected *L. monocytogenes* isolates and five reference strains was similar to the protocol described by Chen et al. (2014). The reaction

TABLE 1 | Antimicrobial resistance of foodborne *L. monocytogenes* isolates collected in South China.

Antimicrobial class	Antimicrobial agents (concentrations, μg or U)	Breakpoints (mm)			No. of isolates (%)		
		Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Aminoglycosides	Kanamycin (30)	≥18	14–17	≤13	167 (94.3)	7(4.0)	3(1.7)
	Gentamycin (10)	≥15	13–14	≤12	172 (97.2)	1(0.6)	4(2.2)
	Vancomycin (30)*	≥17	15–16	≤14	172 (97.2)	5(2.8)	0(0)
Potentiated sulfonamide	Sulfamethoxazole with trimethoprim (23.75/1.25)	≥16	11–15	≤10	168 (94.4)	0(0)	9(5.6)
Tetracyclines	Doxycycline (30)	≥16	13–15	≤12	171 (96.6)	6(3.4)	0(0)
	Tetracycline (30)	≥19	15–18	≤14	164 (92.7)	7(4.0)	6(3.3)
Chloramphenicols	Chloramphenicol (30)	≥18	13–17	≤12	173 (97.8)	2(1.1)	2(1.1)
β-Lactam	Penicillin (10)	≥29	–	≤28	136 (76.8)	11(6.2)	30(16.9)
	Ampicillin (10)	≥29	–	≤28	140 (79.1)	7(4.0)	30(16.9)
	Sulbactam/ampicillin (10/10)	≥15	12–14	≤11	177 (100.0)	0(0)	0(0)
Fluoroquinolones	Levofloxacin (5)	≥19	16–18	≤15	158 (89.3)	17(9.6)	2(1.1)
	Ciprofloxacin (5)	≥21	16–20	≤15	134 (75.7)	40(22.6)	3(1.7)
Macrolides	Erythromycin (15)	≥23	14–22	≤13	174 (98.3)	2(1.1)	1(0.6)
Cephalosporins	Cephalothin (30)	≥18	15–17	≤14	172 (97.2)	5(2.8)	0(0)
Ansamycin	Rifampin (5)	≥20	17–19	≤16	172 (97.2)	1(0.6)	4(2.2)

**Breakpoint for Entercococcus spp.*

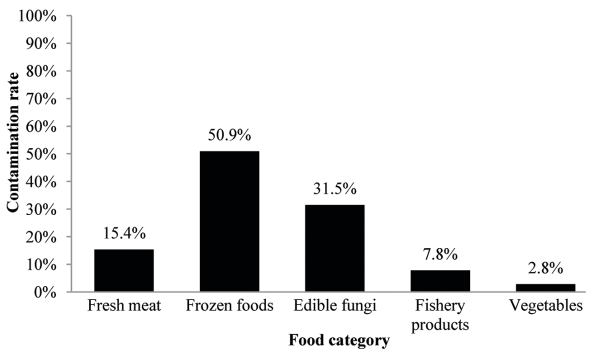


FIGURE 1 | Contamination rate of *Listeria monocytogenes* in different food products in South China.

TABLE 2 | Distribution of *L. monocytogenes* in retail raw food samples collected from South China.

Food category	MPN value (MPN/g)		
	0.3 ≤ MPN < 10 (%)	10 ≤ MPN < 100 (%)	MPN ≥ 100 (%)
Fresh meat	12/14 (85.7)	2/14 (14.3)	0 (0)
Frozen foods	45/47 (95.8)	1/47 (2.1)	1/47 (2.1)
Edible fungi	12/31 (38.7)	7/31 (22.6)	12/31 (38.7)
Fishery products	5/7 (71.4)	1/7 (14.3)	1/7 (14.3)
Vegetables	1/1 (100.0)	0 (0)	0 (0)
Total	75/100 (75.0)	11/100 (11.0)	14/100 (14.0)

mixtures were placed in a hot-lid cycler (Applied Biosystems, Foster City, CA, USA) and subjected to the following temperature profile: an initial five cycles at 94°C for 5 min, 35°C for 5 min, 72°C for 5 min; then 30 cycles each consisting of 1 min at 94°C, 2 min at 35°C, and 2 min at 72°C; and a final extension at 72°C for 10 min. The amplicons were electrophoresed on 1.5% agarose gel and photographed that were saved as TIFF file format for genotype analysis.

Strain Library Construction

Strain library construction was performed as described in a previous study with minor modifications (Casarez et al., 2007). In brief, results of ERIC-PCR fingerprinting and multiplex PCR-based serogroups were used to screen isolates from the same sample in order to identify clonal isolates and assure as diverse a known source library as possible. Three to five *L. monocytogenes* isolates from each positive sample were fingerprinted by ERIC-PCR and serogroup analysis using multiplex PCR, and subsequently compared with each other. Isolates from the same sample with >90% similarity were considered as clonal. If the ERIC-PCR fingerprint of one isolate with >90% similarity from the same sample but has different serogroup determined by PCR, the isolate was included in the strain library. Only the isolate with >90% similarity ERIC-PCR fingerprint and the same serogroup from the same sample source were considered clonal. Clonal isolates from individual sample were excluded. At least one *L. monocytogenes* isolate from each known source sample was included in the library.

Antimicrobial (AM) Susceptibility Test

Since no resistance criteria exist for *Listeria* antibacterial susceptibility test in Clinical and Laboratory Standards Institute guidelines for the tested AMs, criteria for *Staphylococcus aureus* were used except for where noted (**Table 1**; Clinical and Laboratory Standards Institute, 2014). A panel of 15 antimicrobials at the specific concentration per disk (Oxoid, Boston, MA, USA) was tested in this study (**Table 1**).

TABLE 3 | Results of serogroup analysis carried out for the foodborne *L. monocytogenes* isolates collected in South China[#].

Food category	Serogroup				
	I.1 (%)	I.2 (%)	II.1 (%)	II.2 (%)	III (%)
Fresh meat	13/26 (50.0)	3/26 (11.5)	7/26 (26.9)	1/26 (3.8)	2/26 (7.7)
Frozen foods	28/82 (34.1)	28/82 (34.1)	4/82 (4.9)	21/82 (25.6)	1/82 (1.2)
Edible fungi	31/51 (60.8)	0 (0)	0 (0)	20/51 (39.2)	0 (0)
Fishery products	3/15 (20.0)	1/15 (6.7)	1/15 (6.7)	2/15 (13.3)	8/15 (53.3)
Vegetables	0 (0)	0 (0)	1/3 (66.7)	2/3 (33.3)	0 (0)
Total	75/177 (42.4)	32/177 (18.1)	13/177 (7.3)	46/177 (26.0)	11/177 (6.2)

#The 177 L. monocytogenes isolates included in the strain library were used for statistical analysis.

Antimicrobial susceptibility test was performed as described in a previous study (Kovacevic et al., 2012). *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as quality control strains for this study. Zones of inhibition were measured with a precision caliper to the nearest 0.01 mm. Isolates exhibiting resistance to three or more classes of antibiotics were considered as multidrug-resistant strains (Magiorakos et al., 2012).

Results

Prevalence and Quantitative Analysis

In this study, 123 (21.7%) samples were positive for *L. monocytogenes* out of 567 collected samples. Depending on the food category, the prevalence of *L. monocytogenes* in frozen food samples was 50.9% (56/110) and 31.5% in fresh edible fungus (34/108, including 29 *F. velutipes* samples and five other edible fungus samples), 15.4% in fresh meat (19/123), 7.8% in fishery products (12/154), and 2.8% in vegetables (2/72; **Figure 1**). Based on quantitative analysis, 75.0% (75/100) of the positive samples were contaminated at levels ranging between 0.3 and 10 MPN/g, that included 45 frozen foods, 12 fresh meat, 12 edible fungi, five fishery products, and one vegetable sample. Only in 14.0% of the positive samples the contamination level exceeded 100 MPN/g, which included 12 edible fungi samples, one frozen food, and one fishery product. The contamination level of *L. monocytogenes* in frozen food samples was low; in 95.8% (45/47) of the positive samples the levels were less than 10 MPN/g, only in one sample the levels exceeded 100 MPN/g. Surprisingly, 38.7% (12/31) of edible fungus samples were more than 100 MPN/g (**Table 2**).

Serogroup Analysis using Multiplex PCR

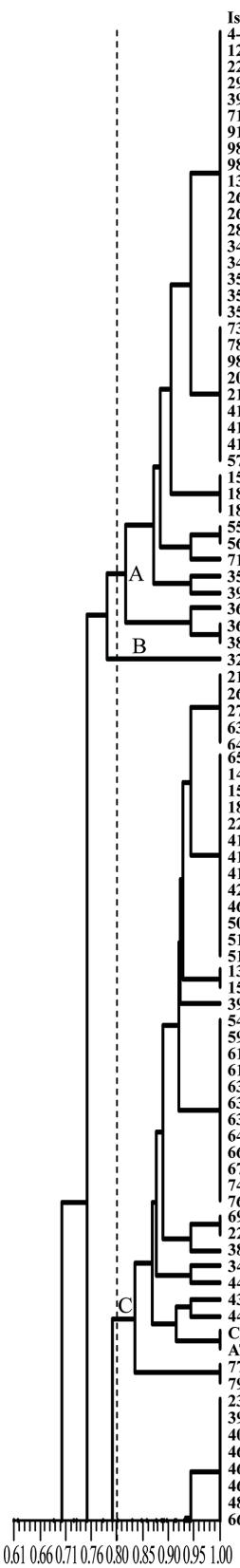
One hundred and seventy-seven *L. monocytogenes* isolates included in the strain library and five reference strains were used for serogroup analysis using multiplex PCR (Supplementary Table S2). As shown in **Table 3**, 42.4% (75/177) of the 177 *L. monocytogenes* isolates were recognized as serogroup I.1, of which isolates mainly isolated from fresh meat (13 isolates), frozen products (28 isolates), and edible fungi samples (31 isolates); 18.1% (32/177) as serogroup I.2, of which 28 isolates were recovered from frozen products samples; 7.3% as serogroup II.1, 26.0% (46/177) as serogroup II.2, which comprised of 20 isolates from edible fungi samples and 21 isolates from frozen products samples; and 6.2% as serogroup III. Additionally, serogroup I.1 (60.8%) and II.2 (39.2%) were identified in *L. monocytogenes* strains that were isolated from fresh edible fungi samples. Serogroup III (53.3%) was predominant in fishery product samples.

ERIC-PCR Typing

Using ERIC-PCR, genomic DNA of *L. monocytogenes* isolates displayed DNA bands in sizes of between 200 and 2200 bp, most of isolates from the same sample generated similar fingerprints. Sixty ERIC-types (DI = 0.960) were classified among 177 foodborne isolates. Based on cluster analysis of genetic profiles obtained from ERIC-PCR typing (**Figure 2**), there were three singletons and eight clusters at a relative similarity coefficient of 80%; isolates belonging to a distinct serogroup clustered together, such as in cluster D that included the strains 283-4, 298-1, 302-4, 547-4, and 648-1. It is noteworthy that 23 isolates, from *F. velutipes* samples, belonging to serogroup I.1 fell into cluster D, which included strains 298-1, 318-1, 518-1, 548-3, 618-1, 633-2, 733-1, 283-4, 648-2, 668-4, 398-1, 483-1, 418-4, 718-1, 798-1, 283-6, 283-7, 418-1, 243-2, 648-1, 698-4, 698-1, and 748-1. For fishery products, four isolates (556-1, 557-1, 588-1, and 589-1) shared the same ERIC-type under cluster F; three isolates (55-1, 85-1, and 189-1) shared similar genetic fingerprints as in cluster K, and all these seven isolates belonged to serogroup III.

RAPD Genotyping Analysis

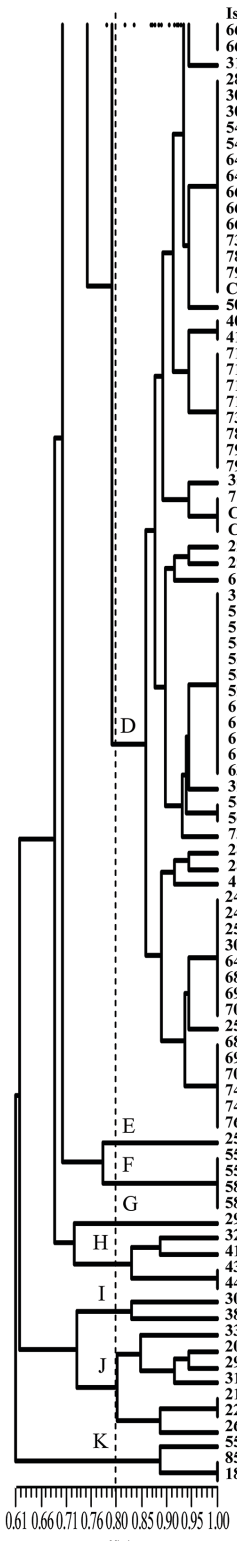
Using UBC-155 primer, it was determined that at a relative similarity coefficient of 80%, 177 isolates and five reference strains fell into 14 clusters and five singletons (designated as I to XIX; **Figure 3**). The discriminatory index of RAPD typing for the 177 isolates was 0.972 based on Simpson's Index of diversity (Hunter and Gaston, 1988). The strong correlation between RAPD-type and serogroup was observed using RAPD typing (**Figure 3**), cluster I included serogroup II.2 and II.1, which accounted for 78.3% (36/46) and 17.4% (8/46), respectively. Most of the isolates in cluster VIII belonged to serogroup III (87.5%); serogroup I.2 (27/36) dominated in cluster IX and serogroup I.1 dominated in cluster X (77.8%), XI (93.1%), and XII (100%). As shown in **Figure 2**, when comparing the isolates included in the clusters of ERIC-PCR and RAPD, an agreement was observed with ERIC-PCR and RAPD subtyping, i.e., isolates in cluster XI and IX typed using RAPD belonged to cluster I of ERIC-PCR).



Isolates	Serogroup	UBC-155	Resistant profile[a]	Source[b]	Date[c]
4-1	I.1	XI	/	Chinese dumpling	2011.7.28
12-1	I.1	XI	/	Grass carp	2011.7.28
22-1	I.1	XI	/	Chicken	2011.7.28
29-1	I.2	IX	/	Fresh pork ribs	2011.8.07
39-1	I.2	IX	/	Frozen drumstick	2011.8.07
71-1	I.1	XI	t-SXT	Minced pork	2011.8.16
91/0.1	I.1	VIII	/	*Flammulina velutipes*	2011.8.16
98PAL	II.2	I	/	Frozen drumstick	2011.8.16
98/1	I.1	IX	/	Frozen drumstick	2011.8.16
132	I.1	XI	/	*Flammulina velutipes*	2011.9.10
267-1	I.1	IX	P-cip-A-l	Frozen mutton	2011.12.20
268-1	I.1	XIV	p-cip	*Flammulina velutipes*	2011.12.20
282-1	I.1	XIV	p	Fresh pork ribs	2011.12.20
344-1	I.1	X	p	Frozen drumstick	2012.3.12
348-1	I.1	XVII	/	*Flammulina velutipes*	2012.3.12
351-1	I.1	XV	/	Fresh pork ball	2012.1.10
353-1	I.1	X	p	Beef	2012.1.10
353-3	I.2	IX	p	Beef	2012.1.10
73-1	I.2	IX	/	Frozen drumstick	2011.8.16
78-1	I.2	IX	/	Frozen drumstick	2011.8.28
98-1	I.2	XI	/	Frozen drumstick	2011.10.6
202-2	I.1	XI	/	Chinese dumpling	2011.10.6
219-1	I.1	XI	/	Beef	2012.2.23
415-4	I.2	IX	/	Chinese dumpling	2012.2.23
416-1	I.2	IX	/	Chinese dumpling	2012.2.23
417-1	I.2	IX	/	Chinese dumpling	2012.5.22
570-1	I.1	VIII	/	*Lentinus edodes*	2011.9.17
153-3	I.1	XI	/	Chinese dumpling	2011.9.26
180-1	I.1	XI	/	*Flammulina velutipes*	2011.9.26
187-3	I.1	XI	/	Frozen drumstick	2012.5.22
553-1	I.2	IX	cip-a-l	Frozen beef	2012.5.22
567-1	I.2	IX	/	Frozen mutton	2012.7.24
715-1	I.2	IX	cip	Chinese dumpling	2012.1.10
351-4	I.1	XVII	T-P-cip-k-A-CN-e-d-SXT-v-r-l-K	Fresh pork	2012.1.10
396-1	I.1	IX	cip	Fresh pork	2012.1.10
367-1	I.2	IX	/	Frozen mutton	2012.1.10
368-1	I.1	X	/	*Flammulina velutipes*	2012.1.10
385-1	I.1	X	P-cip-A-l	oyster mushroom	2012.1.10
323-3	I.1	XV	t	Fresh squid	2011.12.28
21-1	II.2	I	/	Frozen chicken wing	2011.7.28
26-1	II.2	I	/	Wonton	2011.8.07
27-1	II.2	I	/	*Flammulina velutipes*	2011.8.07
63-1	II.2	I	/	Chinese dumpling	2011.8.16
64-1	II.2	I	/	Frozen chicken wing	2011.8.16
65-1	II.1	I	/	Minced pork	2011.8.16
144-1	II.2	I	/	Frozen drumstick	2011.9.10
152-1	II.1	IV	/	Frozen drumstick	2011.9.17
187-1	II.2	I	/	Frozen drumstick	2011.9.17
222-3	II.1	I	/	Frozen drumstick	2011.10.6
413-2	II.1	I	/	Lettuce	2012.2.23
415-1	II.1	I	t-p-SXT	Chinese dumpling	2012.2.23
418-2	II.2	I	/	*Flammulina velutipes*	2012.2.23
421-1	II.1	I	/	Beef	2012.2.23
468-1	II.2	I	/	*Flammulina velutipes*	2012.3.19
507-1	II.1	V	/	Speckled fish	2012.5.08
518-2	II.2	I	p-CIP-a	*Flammulina velutipes*	2012.5.08
519-1	II.2	I	cip	*Pleurotus geesteranus*	2012.5.08
133-1	II.2	I	/	*Coriandrum sativum*	2011.9.10
153-1	II.2	I	/	Chinese dumpling	2011.9.17
398-2	II.2	IV	P-cip-A	*Flammulina velutipes*	2012.1.10
548-1	II.2	I	/	*Flammulina velutipes*	2012.5.08
598-1	II.2	I	/	*Flammulina velutipes*	2012.5.22
615-1	II.2	I	/	Chinese dumpling	2012.6.17
618-2	II.2	I	A-P	*Flammulina velutipes*	2012.6.17
632-1	II.1	I	cip-A	Frozen chicken wing	2012.6.17
632-3	II.2	I	A-k	Frozen chicken wing	2012.6.17
633-1	II.2	I	P-A-k	*Flammulina velutipes*	2012.6.17
647-4	II.2	I	A-P	Frozen chicken wing	2012.6.17
668-1	II.2	I	/	*Flammulina velutipes*	2012.7.11
671-1	II.1	I	/	Beef	2012.7.11
747-1	II.2	I	p-A-cn	Frozen chicken wing	2012.7.24
767-1	II.2	I	/	Frozen salted pork	2012.8.12
69-1	II.2	I	/	Fresh pork ribs	2011.8.16
221-1	II.2	I	/	Frozen drumstick	2011.10.06
383-1	II.2	IV	A-P	*Flammulina velutipes*	2012.1.10
348-2	II.2	IV	P-cip-A	*Flammulina velutipes*	2012.3.12
448-3	I.1	XI	/	*Flammulina velutipes*	2012.2.23
431-1	II.2	I	p-l	Frozen chiecken wing	2012.2.23
448-1	II.2	I	/	*Flammulina velutipes*	2012.2.23
CMCC54007	II.1	VI	ND	CMCC	\
ATCC19115	II.1	VI	ND	ATCC	\
771-1	II.2	I	p-A	Fresh beef	2012.8.12
797-1	II.2	I	/	Frozen drumstick	2012.8.12
230-3	I.1	IX	/	Frozen chicken wing	2011.10.14
398-1	I.1	X	P-CIP-A	*Flammulina velutipes*	2012.1.10
401-1	I.1	XI	P-cip-A	Fresh pork	2012.2.23
465-1	I.1	I	/	Chinese dumpling	2012.3.19
466-1	I.2	IX	/	Frozen ham sausage	2012.2.23
466-3	I.1	XI	t-C-e-SXT-k	Frozen ham sausage	2012.2.23
483-1	I.1	XIV	/	*Flammulina velutipes*	2012.3.24
665-1	I.2	IX	/	Chinese dumpling	2012.7.11

FIGURE 2 | Characterization of foodborne L. monocytogenes isolates obtained in food products from South China.

(Continued)



Isolates	Serogroup	UBC-155	Resistant profile[a]	Source[b]	Date[c]
666-1	I.1	XI	cip-a	Chinese dumpling	2012.7.11
666-4	I.2	IX	/	Chinese dumpling	2012.7.11
317-1	I.1	XIV	/	Frozen mutton	2012.12.28
283-4	I.1	IX	/	*Flammulina velutipes*	2011.12.12
302-1	I.1	XIV	P-cip-A-l	Minced pork	2011.12.28
302-4	III	XVII	P-cip-A	Minced pork	2011.12.28
547-1	I.1	XI	/	Frozen drumstick	2012.5.08
547-4	I.2	IX	P-cip	Frozen drumstick	2012.5.08
647-1	I.1	XII	/	Frozen chicken wing	2012.6.17
648-2	I.1	XII	cip-l	*Flammulina velutipes*	2012.6.17
667-1	I.2	IX	cip	Frozen chicken wing	2012.7.11
667-2	I.1	XII	/	Frozen chicken wing	2012.7.11
668-4	I.1	XII	a	*Flammulina velutipes*	2012.7.11
731-1	I.1	XII	cip	Frozen chicken wing	2012.7.24
781-1	I.1	XI	/	Frozen chicken wing	2012.8.12
797-4	I.1	XI	/	Frozen drumstick	2012.8.12
CMCC54002	I.1	XVIII	ND	CMCC	\
507-2	I.1	XIX	/	Speckled fish	2012.5.08
403-1	I.1	XIII	cip-l	Fresh pork	2012.2.23
418-4	I.1	XI	/	*Flammulina velutipes*	2012.2.23
716-1	I.2	IX	/	Wonton	2012.7.24
717-1	I.1	XII	/	Frozen beef	2012.7.24
717-2	I.2	IX	cip	Frozen beef	2012.7.24
718-1	I.1	XII	cip	*Flammulina velutipes*	2012.7.24
732-1	I.2	IX	cip	Frozen mutton	2012.7.24
782-1	I.1	X	T-A	Frozen Chicken	2012.8.12
797-3	I.2	VII	/	Frozen drumstick	2012.8.12
798-1	I.1	XI	/	*Flammulina velutipes*	2012.8.12
351-3	I.1	XVII	T-P-cip-k-A-c-e-d-SXT-v-R-l-K	Fresh pork	2012.1.10
715-2	I.2	IX	T-cip-k-A-C-CN-E-d-SXT-v-R-l-K	Chinese dumpling	2012.7.24
CMCC54003	I.2	XVIII	ND	CMCC	\
CMCC54004	I.2	XVIII	ND	CMCC	\
297-1	I.1	IX	P-cip-A	Frozen chicken wing	2011.12.12
298-1	II.2	III	P-cip-A	*Flammulina velutipes*	2011.12.12
617-3	III	XVI	/	Frozen chicken wing	2012.6.17
318-1	I.1	XVII	k	*Flammulina velutipes*	2011.12.28
517-1	I.2	IX	/	Frozen mutton	2012.5.08
518-1	I.1	XI	/	*Flammulina velutipes*	2012.5.08
548-3	I.1	XI	/	*Flammulina velutipes*	2012.5.08
552-1	I.1	XI	/	Minced pork	2012.5.22
581-1	I.1	XI	/	Frozen chicken wing	2012.5.22
597-1	I.2	IX	/	Frozen drumstick	2012.5.22
616-1	I.1	XII	/	Chinese dumpling	2012.6.17
617-1	I.2	IX	/	Frozen mutton	2012.6.17
617-2	I.1	XI	/	Frozen mutton	2012.6.17
618-1	I.1	XII	cip-A-l	*Flammulina velutipes*	2012.6.17
633-2	I.1	IX	p-cip-a-l	*Flammulina velutipes*	2012.6.17
396-3	I.1	IX	/	Pork ball	2012.1.10
547-2	I.2	XI	/	Frozen drumstick	2012.5.08
567-3	I.2	IX	T-P-CIP-k-A-C-CN-E-d-SXT-v-R-l-K	Frozen mutton	2012.5.22
733-1	I.1	XII	cip	*Flammulina velutipes*	2012.7.24
283-6	II.2	III	p	*Flammulina velutipes*	2011.12.12
283-7	I.1	III	p	*Flammulina velutipes*	2011.12.12
418-1	I.1	XI	/	*Flammulina velutipes*	2012.2.23
242-2	II.2	I	/	Frozen drumstick	2011.10.14
243-2	II.2	I	/	*Flammulina velutipes*	2011.10.14
253-1	II.2	III	P-A	Minced pork	2011.12.20
303-3	II.1	IV	P-cip-A-c-e-l-k	Minced pork	2011.12.28
648-1	II.2	I	a-r	*Flammulina velutipes*	2012.6.17
685-5	II.2	I	/	*Hypsizygus marmoreus*	2012.7.11
698-4	II.2	I	p-a	*Flammulina velutipes*	2012.7.11
704-1	II.2	I	cip-A	Prawn	2011.7.24
251-1	II.1	III	/	Fresh pork	2011.12.20
685-1	I.1	XII	cip-l	*Hypsizygus marmoreus*	2011.7.11
698-1	I.1	XI	/	*Flammulina velutipes*	2011.7.11
704-3	I.2	IX	cip	Prawn	2012.7.24
748-1	I.1	XI	/	*Flammulina velutipes*	2012.7.24
749-1	I.1	XII	/	*pleurotus eryngii*	2012.7.24
765-1	I.1	XII	t-d-SXT	Chinese dumpling	2012.8.01
251-3	III	X	/	Fresh pork	2011.12.20
556-1	III	VIII	cip-l	Catfish	2012.5.22
557-1	III	VIII	/	Frozen mutton	2012.5.22
588-1	III	VIII	/	Grass carp	2012.5.22
589-1	III	VIII	cip	*Tilapia mossambica*	2012.5.22
297-4	II.2	III	P-a	Frozen chicken wing	2011.12.12
323-1	II.2	IV	t-p	Fresh squid	2011.12.28
413-4	II.2	I	/	Lettuce	2012.2.23
431-3	II.2	I	P-cip	Frozen chicken wing	2012.2.23
446-1	I.2	I	cip-CN	Frozen ham sausage	2012.2.23
303-4	II.1	IV	P-cip-A-k	Minced pork	2011.12.28
389-1	III	X	/	*Tilapia mossambica*	2012.1.10
33-1	I.1	XI	/	Frozen chicken wing	2011.8.07
202-1	I.2	IX	/	Chinese dumpling	2011.10.06
297-3	I.1	III	T-P-cip-k-A-CN-e-s-SXT-v-R-l	Frozen chicken wing	2011.12.12
318-2	I.1	X	/	*Flammulina velutipes*	2011.12.28
219-2	I.1	XI	/	Beef	2011.10.06
222-1	II.2	IX	/	Frozen drumstick	2011.10.06
268-2	I.1	XIV	p	*Flammulina velutipes*	2011.12.20
55-1	III	VIII	/	Yellow croaker	2011.8.16
85-1	III	VIII	/	*Tilapia mossambica*	2011.8.28
189-1	III	VIII	/	Grass carp	2011.9.26

FIGURE 2 | Continued

The dendrogram was constructed based on ERIC-PCR analysis; a: T (t), tetracycline; P (p), penicillin; CIP (cip), ciprofloxacin; K (k), kanamycin, A (a), ampicillin; C (c), cephalothin; CN (cn), gentamycin, E (e), erythromycin, D (d), doxycycline; SXT (sxt), sulfamethoxazole with trimethoprim; V (v): vancomycin; R (r), rifampin; L (l), levofloxacin; /, no resistance; ND, not detected; Name of antibiotics with capital letters implies resistance; Name of antibiotics with lowercase letters implies intermediate resistance. b: -, no gene absent; genes listed mean absent. c: CMCC, China Medical Culture Collection; ATCC, American Type Culture Collection. d: \, unknown.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility of 177 *L. monocytogenes* isolates were evaluated using the disk diffusion method. Based on the breakpoint criteria for *S. aureus* or *Enterococcus* spp., 42 antimicrobial susceptibility profiles were identified in this study (**Figure 2**). Penicillin (23.1%), ampicillin (20.9%), and ciprofloxacin (24.3%) were the three most frequent antimicrobial resistance profiles among the *L. monocytogenes* isolates, while the resistance rate of tetracycline and doxycycline was 7.3 and 3.4%, respectively. For the other 12 antibacterial agents, all 177 isolates of *L. monocytogenes* displayed 89.3–100% susceptibility (**Table 1**). Considering the fact that an intermediate resistant isolate may become a resistant strain under certain circumstances (Ruiz-Bolivar et al., 2011), all 177 isolates were grouped into 42 antibacterial resistance patterns, including 106 (59.9%) isolates of *L. monocytogenes* that were susceptible to the 15 tested antibacterial agents and 10 (5.6%) isolates that were multidrug resistant (**Figure 2**). Notably, 83.3% of the isolates (10/12) that were resistant to up to four antimicrobials were isolated from the livestock and poultry meat and were associated food products (**Figure 2**).

Discussion

In this study, we collected 567 food samples including raw meat, frozen foods, edible fungi, fishery products, and vegetables from16 cities or districts in South China. The contamination rate of *L. monocytogenes* (21.7%) in food samples from South China was in consistent with that in Southeastern China as shown by Wang et al. (2013). Over 15% of fresh meat samples were positive for *L. monocytogenes* in the present study, however, raw meat and poultry are not considered at high risk for causing foodborne listeriosis provided adequate cooking precedes consumption and that cross-contamination is avoided. Nevertheless, raw or insufficiently cooked meats may serve as sources of cross-contamination for products that are intended to be consumed without heat treatment, and along with inadequate cleaning and sanitation, these foods have been recognized as the main sources of post-processing contamination of RTE meat products (Lianou and Sofos, 2007; Sauders et al., 2009). Quantitative analysis revealed that over 75% of *L. monocytogenes*-positive samples had counts below 10 MPN/g, which was consistent with the results of the retail raw foods collected in Northeast of China (Wang et al., 2013). In the present study, the contamination rate of frozen foods was up to 50.9%, while the MPN value of positive samples were mostly below 10 MPN/g, these results may associated with the tolerance ability of *L. monocytogenes* in low temperature environments (Vazquez-Boland et al., 2001). Surprisingly, 76.3% (29/38) of *F. velutipes* samples were positive for *L. monocytogenes* and the MPN value of 12 positive samples exceeded 100 MPN/g, while the contamination rates of other edible fungi samples were low (data not shown), these data were significantly higher than that reported in other countries as reviewed by Lianou and Sofos (2007). To date, limited data have been available about the prevalence of *L. monocytogenes* in edible fungi products in China. In addition, there are no specific qualitative and quantitative standards to determine the presence of *L. monocytogenes* in the mushroom products. However, some countries have formulated a zero-tolerance policy for *L. monocytogenes* in mushroom products, such as in the United States and Canada (Canadian Food Inspection Agency, 2012; U. S. Food and Drug Administration, 2012). It is necessary to draft a corresponding microbiological standard to ensure the quality of mushroom products in China.

Previous studies reported over 95% of the isolates in human listeriosis and food samples belonged to serotype 1/2a, 1/2b, 1/2c, and 4b (Pontello et al., 2012; Althaus et al., 2014). In this study, 42.4% of *L. monocytogenes* isolates belonged to serogroup I.1 (serotype 1/2a-3a), followed by serogroup II.2 (26.0%), serogroup I.2 (18.1%), serogroup II.1 (7.3%), and serogroup III (6.2%). Serogroup I.1 (serotype 1/2a-3a) predominant among the foodborne isolates, the serogroup compositions of these foodborne isolates was in agreement with that previously reported for other foodborne isolates (Chen et al., 2009; Korsak et al., 2012). Interestingly, isolates recovered from edible fungi samples only belonged to serogroup I.1 and II.2, while Viswanath et al. (2013) reported only serotype 4a contamination in a small-scale mushroom production facility. Serogroup III was rarely isolated from foods (Orsi et al., 2011), however, in this study 11 isolates belonging to serogroup III were isolated from food samples; 72.7% (8/11) isolates were from fishery products, Korsak et al. (2012) reported that serogroup III was mainly isolated from vegetables in Poland. These results indicated that some specific serogroups of *L. monocytogenes* may have distinct ecological niches; Ochiai et al. (2010) also reported this for *L. monocytogenes* isolated from retail meat in Japan. Continuous and comprehensive investigation should be carried out for better understanding of the ecology of *L. monocytogenes*.

As contaminated foods are considered as the transmission source for human clinical listeriosis, we decided to carry out our investigation using *L. monocytogenes* strains that were isolated from food samples in South China, and examining the diversity of their antimicrobial profiles. In the present study, all of the *L. monocytogenes* isolates were susceptible to sulbactam/ampicillin (**Table 1**) and over 90% of isolates were susceptible to 10 antibacterial agents, namely kanamycin, gentamycin, sulfamethoxazole with trimethoprim, doxycycline, chloramphenicol, erythromycin, cephalothin, rifampin, tetracycline, and vancomycin, which indicated that these antibacterial agents are still effective for the treatment of listeriosis. The tetracycline and ciprofloxacin resistance rates were significantly different than that of the *L. monocytogenes* isolated in Northern China as described by Yan et al. (2010), which is possibly due to the influence of geographic differences. Surprisingly, 20% of *L. monocytogenes* isolates exhibited resistance toward the first-choice drug penicillin and ampicillin (**Table 1**), although no penicillin-resistant strain was found in the previous studies conducted in China (Chao et al., 2007; Yang et al., 2008; Zhao et al., 2012). Therefore, more attention is required while monitoring the variation in the trend of resistance toward penicillin and

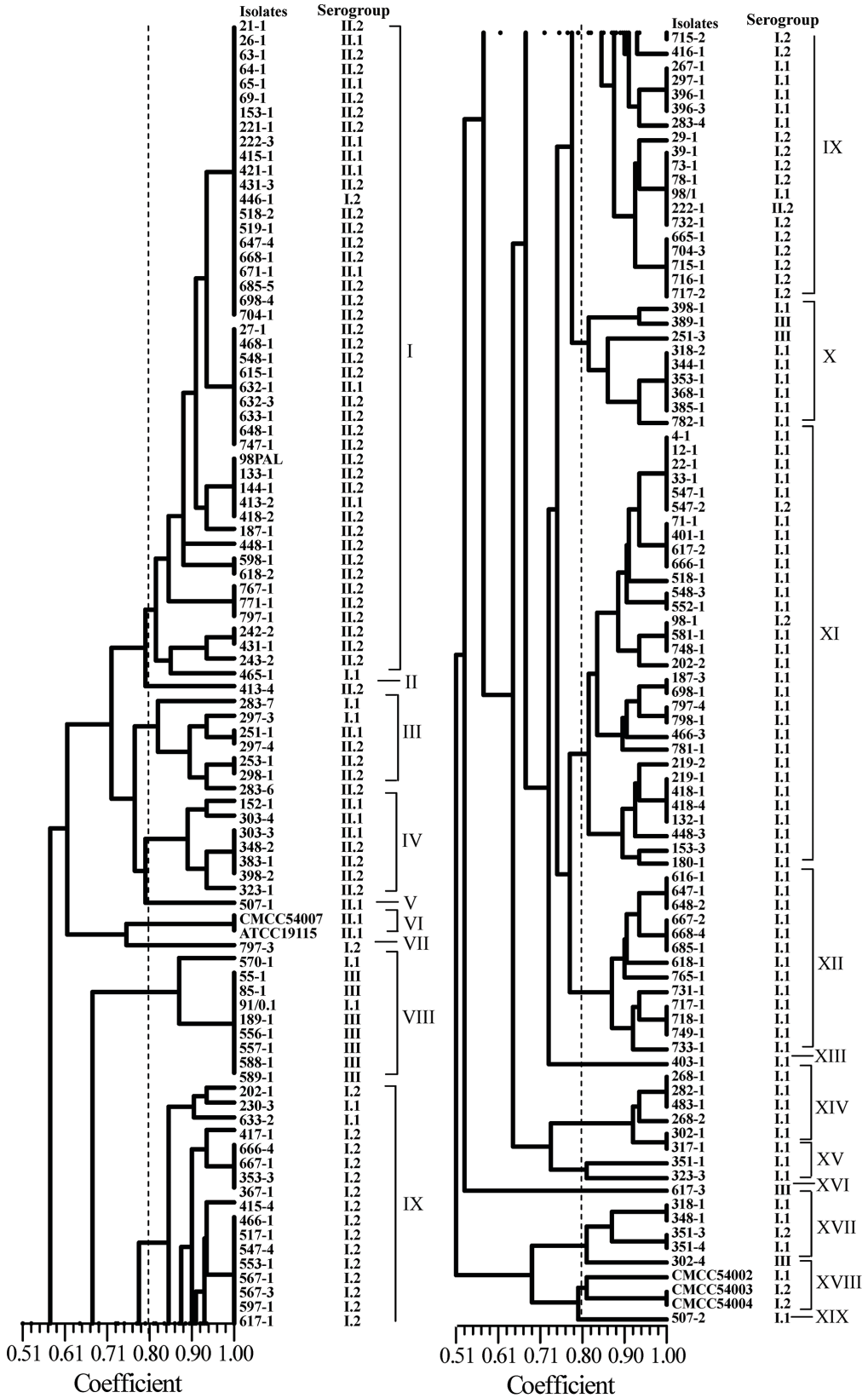


FIGURE 3 | Random amplified polymorphic DNA (RAPD) analyses of foodborne *L. monocytogenes* isolated from food products in South China.

ampicillin. These findings indicated that *L. monocytogenes* is slowly becoming resistant to antibiotics in South China. This emphasized the need to continuously conduct surveillance for antibacterial susceptibility of foodborne *L. monocytogenes* in China.

In the present study, 5.6% (10/177) of *L. monocytogenes* isolates were determined as multidrug resistant (**Figure 2**) and ten isolates that were resistant to four antibiotics were obtained from animal foods and its associated products. These results support that the use of antibiotics in poultry and livestock as growth promoter has led to the emergence of antimicrobial-resistant bacteria in the food and associated environment (Harakeh et al., 2009; Wieczorek et al., 2012). These results also indicated that foods from both poultry and livestock are possibly the reservoir of multidrug resistant *L. monocytogenes* strains. However, the results of multidrug resistance were inconsistent with that previously reported (Yan et al., 2010; Zhao et al., 2012), which can be attributed to the different source-composition of foodborne *L. monocytogenes* as well as different criteria used to determine multidrug resistance. In addition, it should be noted that, in our study 31.4% (16/51) isolates recovered from edible fungi samples exhibited resistance to more than two antibiotics. To the best of our knowledge, the processes involved in the production of edible fungi in scale-level plants would not use any antibiotics to prevent edible fungi contaminating other harmful microbes. This is the first report that *L. monocytogenes* isolates recovered from edible fungi samples demonstrated a high frequency of multidrug resistance, indicating that mushrooms may serve as the potential reservoirs of multidrug-resistant strains of *L. monocytogenes*.

Recently, high discriminatory molecular typing methods, i.e., PFGE, RAPD, and ERIC-PCR, have been developed for the differentiation of pathogenic bacteria. ERIC-PCR and RAPD techniques have been extensively used to determine genetic lineages of *L. monocytogenes* (Zhou and Jiao, 2004; Aurora et al., 2009; Chen et al., 2010). In the present study, ERIC-PCR and RAPD also yielded comparable results for the typing of *L. monocytogenes* strains, the discriminatory ability of ERIC-PCR and RAPD were 0.960 and 0.972, respectively. Genetic relatedness analysis revealed that there were no prominent associations between specific food types, antibiotic resistance, serogroups, and genetic diversity. The clustering results generated were different depending on the typing methods used (**Figures 2** and **3**), because the typing methods possibly target different genetic markers (Marshall et al., 1999). Seven isolates (418-1, 418-4, 518-1, 548-3, 698-1, 748-1, and 798-1) belonging to serogroup I.1 were grouped into ERIC-D and RAPD-XI; ten isolates (27-1, 398-2, 418-2, 468-1, 518-2, 548-1, 598-1, 618-1, 633-1, 668-1) belonging to serogroup II.2 were clustered into ERIC-C and RAPD-I. These isolates were obtained from *F. velutipes* samples, indicating that these isolates share similar genetic information and can better tolerate the disinfectants and sanitizers used in the mushroom production facility. Further studies are likely to elucidate the characteristics of these specific subtypes of *L. monocytogenes* colonizing the processing environments that in turn will aid in exploring better cleaning and sanitation measures. Previous studies demonstrated that the antibacterial resistance in *L. monocytogenes* was possibly acquired *via* self-transferable plasmids (Poyart-Salmeron et al., 1990), conjugative mobilization (Charpentier et al., 1999; Toomey et al., 2009), and efflux pumps (Rakic-Martinez et al., 2011; Jiang et al., 2012). Interestingly, two multidrug-resistant isolates (351-3, 715-2) were clustered together as revealed by ERIC-PCR, while isolates 351-3, 351-4 recovered from the fresh pork samples displayed unique fingerprints based on RAPD typing, suggesting that the multidrug-resistant isolates may acquire resistance by vertical and horizontal gene transfer. Further studies are required to elucidate the underlying molecular mechanisms for the acquisition of antimicrobial resistance by *L. monocytogenes*.

Conclusion

Overall, the findings of this study demonstrated high contamination rate of *L. monocytogenes* in raw foods in South China, while the MPN values were relative low. Serogroup I.1 and II.2 were dominant among the foodborne *L. monocytogenes* strains, indicating that some specific serogroups of *L. monocytogenes* may have distinct ecological niches. Approximately 59.9% of the strains were susceptible to 15 antibiotics. All 177 isolates were grouped into 42 antibacterial susceptibility profiles and 5.6% of the isolates were multidrug resistant. Both ERIC-PCR and RAPD displayed excellent discriminatory ability for typing *L. monocytogenes*, the discriminatory index of ERIC-PCR and RAPD were 0.960 and 0.972, respectively. The present study provided the first baseline data on the prevalence, contamination level, and characteristics of *L. monocytogenes* isolated from raw foods in South China. Some multidrug resistant strains belonged to an epidemiologically important serogroup (I.1 and II.1), implying a potential public health risk. In addition, Chinese food safety authorities should draft appropriate standards to control *L. monocytogenes* contamination for the improvement of microbiological safety of retail raw foods.

Funding

The authors would like to acknowledge the financial support of National Natural Science Foundation of China (U1031003, 31471664); Science and Technology Program of Guangdong Province (2013B050800026); Science and Technology Program of Guangzhou, China (201504010036); Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2013BAD16B05).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01026

References

Abadias, M., Usall, J., Anguera, M., Solsona, C., and Vinas, I. (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *Int. J. Food. Microbiol.* 123, 121–129. doi: 10.1016/j.ijfoodmicro.2007.12.013

Althaus, D., Lehner, A., Brisse, S., Maury, M., Tasara, T., and Stephan, R. (2014). Characterization of *Listeria monocytogenes* strains isolated during 2011-2013 from human infections in Switzerland. *Foodborne. Pathog. Dis.* 11, 753–758. doi: 10.1089/fpd.2014.1747

Aurora, R., Prakash, A., and Prakash, S. (2009). Genotypic characterization of *Listeria monocytogenes* isolated from milk and ready-to-eat indigenous milk products. *Food. Control.* 20, 835–839. doi: 10.1016/j.foodcont.2008.10.017

Bell, C., and Kyriakides, A. (2005). *Listeria, a Practical Approach to the Organism and its Control in Foods*, 2nd Edn. London: Blackwell Pub.

Canadian Food Inspection Agency. (2012). *Sliced Crimini Mushrooms May Contain Listeria monocytogenes*. Available at: http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recalls-and-allergy-alerts/complete-listing/2012-08-20/eng/1355956870478/1355956870509. [assessed 23 August 2015].

Casarez, E. A., Pillai, S. D., Mott, J. B., Vargas, M., Dean, K. E., and Di Giovanni, G. D. (2007). Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol.* 103, 350–364. doi: 10.1111/j.1365-2672.2006.03246.x

Chao, G., Zhou, X., Jiao, X., Qian, X., and Xu, L. (2007). Prevalence and antimicrobial resistance of foodborne pathogens isolated from food products in China. *Foodborne. Pathog. Dis.* 4, 277–284. doi: 10.1089/fpd.2007.0088

Charpentier, E., Gerbaud, G., and Courvalin, P. (1999). Conjugative mobilization of the rolling-circle plasmid pIP823 from *Listeria monocytogenes* BM4293 among gram-positive and gram-negative bacteria. *J. Bacteriol.* 181, 3368–3374.

Chen, B. Y., Pyla, R., Kim, T.-J., Silva, J. L., and Jung, Y. S. (2010). Prevalence and contamination patterns of *Listeria monocytogenes* in catfish processing environment and fresh fillets. *Food. Microbiol.* 27, 645–652. doi: 10.1016/j.fm.2010.02.007

Chen, J., Luo, X., Jiang, L., Jin, P., Wei, W., Liu, D., et al. (2009). Molecular characteristics and virulence potential of *Listeria monocytogenes* isolates from Chinese food systems. *Food. Microbiol.* 26, 103–111. doi: 10.1016/j.fm.2008.08.003

Chen, M. T., Wu, Q. P., Zhang, J. M., Yan, Z. A., and Wang, J. (2014). Prevalence and characterization of *Listeria monocytogenes* isolated from retail-level ready-to-eat foods in South China. *Food. Control.* 38, 1–7. doi: 10.1016/j.foodcont.2013.09.061

Clinical and Laboratory Standards Institute (2014). *Performance Standards for Antimicrobial Susceptibility Testing: 24th Informational Supplement (M100-S24)*. Wayne, PA: Clinical and Laboratory Standards Institute.

Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A., and Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. *Int. J. Food. Microbiol.* 128, 497–500. doi: 10.1016/j.ijfoodmicro.2008.10.018

de Castro, V., Escudero, J., Rodriguez, J., Muniozguren, N., Uribarri, J., Saez, D., et al. (2012). Listeriosis outbreak caused by Latin-style fresh cheese, Bizkaia, Spain, August 2012. *Euro. Surveill* 17, pii: 20298.

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

Farber, J. M., and Addison, C. J. (1994). RAPD typing for distinguishing species and strains in the genus *Listeria*. *J. Appl. Bacteriol.* 77, 242–250. doi: 10.1111/j.1365-2672.1994.tb03070.x

Feng, Y. F., Ran, L., and Zhang, L. S. (2011). Listeriosis cases reported in medical literatures in China, 2000–2009. *Dis. Surveill.* 26, 654–659.

Franciosa, G., Pourshaban, M., Gianfranceschi, M., and Aureli, P. (1998). Genetic typing of human and food isolates of *Listeria monocytogenes* from episodes of listeriosis. *Eur. J. Epidemiol.* 14, 205–210. doi: 10.1023/A:1007448210169

Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytiä-Trees, E., et al. (2006). PulseNet USA: a five-year update. *Foodborne. Pathog. Dis.* 3, 9–19. doi: 10.1089/fpd.2006.3.9

Gombas, D. E., Chen, Y., Clavero, R. S., and Scott, V. N. (2003). Survey of *Listeria monocytogenes* in ready-to-eat foods. *J. Food. Protect.* 66, 559–569.

Granier, S. A., Moubareck, C., Colaneri, C., Lemire, A., Roussel, S., Dao, T. T., et al. (2011). Antimicrobial resistance of *Listeria monocytogenes* isolates from food and the environment in France over a 10-year period. *Appl. Environ. Microbiol.* 77, 2788–2790. doi: 10.1128/AEM.01381-10

Harakeh, S., Saleh, I., Zouhairi, O., Baydoun, E., Barbour, E., and Alwan, N. (2009). Antimicrobial resistance of *Listeria monocytogenes* isolated from dairy-based food products. *Sci. Total. Environ.* 407, 4022–4027. doi: 10.1016/j.scitotenv.2009.04.010

Hitchins, A. D. (1998). "Listeria monocytogenes," in *FDA Bacteriological Analytical Manual*, 8th Edn, Chap. 10, ed. G. Jackson (Gaithersburg, MD: AOAC International).

Hsieh, W. S., Tsai, L. Y., Jeng, S. F., Hsu, C. H., Lin, H. C., Hsueh, P. R., et al. (2009). Neonatal listeriosis in Taiwan, 1990–2007. *Int. J. Infect. Dis.* 13, 193–195. doi: 10.1016/j.ijid.2008.06.006

Hu, X. N., Su, C. Y., and Quan, Y. L. (2013). Detection and analysis of *L. monocytogenes* from instant food in Gansu Province from 2007 to 2011. *Bull. Dis. Cont. Prevent. (China).* 28, 50–52.

Huang, Y. L., He, H., Li, X. Z., He, J. Y., Xiao, Y., and Cong, L. D. (2005). Investigation on contamination caused by *Listeria* in foods. *Dis. Surveil.* 20, 359–361.

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.

Jiang, X., Zhou, L., Gao, D., Wang, Y., Wang, D., Zhang, Z., et al. (2012). Expression of efflux pump gene lde in ciprofloxacin-resistant foodborne isolates of *Listeria monocytogenes*. *Microbiol. Immunol.* 56, 843–846. doi: 10.1111/j.1348-0421.2012.00506.x

Jersek, B., Gilot, P., Gubina, M., Klun, N., Mehle, J., Tcherneva, E., et al. (1999). Typing of *Listeria monocytogenes* strains by repetitive element sequence-based PCR. *J. Clin. Microbiol.* 37, 103–109.

Jin, X. Y., Han, J., Yu, H. W., and Jia, Y. M. (2009). The survey on the contamination situation of *Listeria monocytogenes* in foods. *J. Chin. Institute. Food. Sci. Technol.* 9, 226–231.

Kang, Q. H., Guo, Y., Hong, S. Q., and Jiang, L. (2013). *Listeria monocytogenes* meningitis in infants: report of two cases. *Zhongguo Dang Dai Er Ke Za Zhi.* 15, 1150–1152. doi: 10.7499/j.issn.1008-8830.2013.12.029

Korsak, D., Borek, A., Daniluk, S., Grabowska, A., and Pappelbaum, K. (2012). Antimicrobial susceptibilities of *Listeria monocytogenes* strains isolated from food and food processing environment in Poland. *Int. J. Food. Microbiol.* 158, 203–208. doi: 10.1016/j.ijfoodmicro.2012.07.016

Kovacevic, J., Mesak, L. R., and Allen, K. J. (2012). Occurrence and characterization of *Listeria* spp. in ready-to-eat retail foods from Vancouver. British Columbia. *Food. Microbiol.* 30, 372–378. doi: 10.1016/j.fm.2011.12.015

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 Protoc.* 70, 2172–2198.

Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., et al. (2013). Novel epidemic clones of *Listeria monocytogenes*, United States, 2011. *Emerg. Infect. Dis.* 19, 147–150. doi: 10.3201/eid1901.121167

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., 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

Makino, S. I., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S., et al. (2005). An outbreak of foodborne listeriosis due to cheese in Japan, during 2001. *Int. J. Food. Microbiol.* 104, 189–196. doi: 10.1016/j.ijfoodmicro.2005.02.009

Marshall, S., Clark, C. G., Wang, G., Mulvey, M., Kelly, M. T., and Johnson, W. M. (1999). Comparison of molecular methods for typing *Vibrio parahaemolyticus*. *J. Clin. Microbiol.* 37, 2473–2478.

Mei, L. L., Cheng, S. Y., Zhu, M., and Wang, Z. X. (2006). Investigation on Listeria *monocytogenes* contamination in food of Zhejiang Province during 2000 ~2004. *Chin. J. Health. Lab. Technol.* 16, 815.

Morvan, A., Moubareck, C., Leclercq, A., Hervé-Bazin, M., Bremont, S., Lecuit, M., et al. (2010). Antimicrobial resistance of *Listeria monocytogenes* strains isolated from humans in France. *Antimicrob. Agents. Chemother.* 54, 2728–2731. doi: 10.1128/AAC.01557-09

National Standard of the People's Republic of China (2010). *National Food Safety Standard Food Microbiological Examination: Listeria monocytogenes* (GB 4789.30Ű2010). Beijing: Ministry of Health of People's Republic of China.

Ochiai, Y., Yamada, F., Batmunkh, O., Mochizuki, M., and Takano, T. (2010). Prevalence of *Listeria monocytogenes* in retailed meat in the Tokyo metropolitan area. *J. Food. Protect.* 73, 1688–1693.

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

Parisi, A., Latorre, L., Normanno, G., Miccolupo, A., and Fraccalvieri, R. (2010). Amplified fragment length polymorphism and multi-locus sequence typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. *Food. Microbiol.* 27, 101–108. doi: 10.1016/j.fm.2009.09.001

Pontello, M., Guaita, A., Sala, G., Cipolla, M., Gattuso, A., Sonnessa, M., et al. (2012). *Listeria monocytogenes* serotypes in human infections (Italy, 2000-2010). *Ann. Ist. Super. Sanita.* 48, 146–150. doi: 10.4415/ANN_12_02_07

Poyart-Salmeron, C., Carlier, C., Trieu-Cuot, P., Courtieu, A. L., and Courvalin, P. (1990). Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes*. *Lancet.* 335, 1422–1426.

Rakic-Martinez, M., Drevets, D. A., Dutta, V., Katic, V., and Kathariou, S. (2011). *Listeria monocytogenes* strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. *Appl. Environ. Microbiol.* 77, 8714–8721. doi: 10.1128/AEM.05941-11

Rodas-Suárez, O. R., Flores-Pedroche, J. F., Betancourt-Rule, J. M., Quiñones-Ramírez, E. I., and Vázquez-Salinas, C. (2006). Occurrence and antibiotic sensitivity of *Listeria monocytogenes* strains isolated from oysters, fish, and estuarine water. *Appl. Environ. Microbiol.* 72, 7410–7412. doi: 10.1128/AEM.00956-06

Rohlf, F. J. (2000). *NTSYS-pc. Numerical Taxonomy and Multi-Variate Analysis System, Version* 2.1. Setauket, NY: Exeter Software.

Ruiz-Bolivar, Z., Neuque-Rico, M. C., Poutou-Pinales, R. A., Carrascal-Camacho, A. K., and Mattar, S. (2011). Antimicrobial susceptibility of *Listeria monocytogenes* food isolates from different cities in Colombia. *Foodborne. Pathog. Dis.* 8, 913–919. doi: 10.1089/fpd.2010.0813

Sauders, B. D., Sanchez, M. D., Rice, D. H., Corby, J., Stich, S., Fortes, E. D., et al. (2009). Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. *J. Food. Prot.* 72, 2337–2349.

Schlech, W. F. (2000). Foodborne listeriosis. *Clin. Infect. Dis.* 31, 770–775. doi: 10.1086/314008

Shi, L., Wang, W. Y., and Yan, H. (2013). Isolation and antimicrobial susceptibilities of *Listeria monocytogenes* in Pork. *Modern. Food. Sci. Technol.* 29, 2826–2829.

Toomey, N., Monaghan, A., Fanning, S., and Bolton, D. J. (2009). Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using in vitro methods and mating in a food matrix. *Foodborne. Pathog. Dis.* 6, 925–933. doi: 10.1089/fpd.2009.0278

U. S. Department of Agriculture (1998). "Isolation and identification of Listeria monocytogenes from meat, poultry and egg products," in *Microbiology Guide Book*, 3rd Edn, Chap. 8, eds B. P. Dey and C. P. Lattuada (Washington, DC: Government Printing Office).

U. S. Food and Drug Administration (2012). *Health Hazard Alert – Certain Sliced White Mushrooms May Contain Listeria monocytogenes*. Available at: http://www.fda.gov/Safety/Recalls/ucm316052.htm. [assessed 23 August, 2015].

Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., and Dominguez-Bernal, G. (2001). Listeria pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14, 584–640. doi: 10.1128/CMR.14.3.584-640.2001

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

Viswanath, P., Murugesan, L., Knabel, S. J., Verghese, B., Chikthimmah, N., and Laborde, L. F. (2013). Incidence of *Listeria monocytogenes* and *Listeria* spp. in a small-scale mushroom production facility. *J. Food. Protect.* 76, 608–615. doi: 10.4315/0362-028X.JFP-12-292

Wang, X. M., Lü, X. F., Yin, L., Liu, H. F, Zhang, W. J., Si, W., et al. (2013). Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. *Food. Control.* 32, 153–158. doi: 10.1016/j.foodcont.2012.11.032

Wieczorek, K., Dmowska, K., and Osek, J. (2012). Characterization and antimicrobial resistance of *Listeria monocytogenes* isolated from retail beef meat in Poland. *Foodborne. Pathog. Dis.* 9, 681–685. doi: 10.1089/fpd.2012.1137

Wu, L., Zhang, X. H., Chen, H., and Yin, X. L. (2008). Neonatal septicemia caused by *Listeria monocytogenes*: report of 6 cases. *Zhonghua Er Ke Za Zhi* 46, 22–25.

Yan, H., Neogi, S. B., Ziyao, M., Guan, W., and Shen, Z. (2010). Prevalence and characterization of antimicrobial resistance of foodborne *Listeria monocytogenes* isolates in Hebei province of Northern China, 2005–2007. *Int. J. Food. Microbiol.* 144, 310–316. doi: 10.1016/j.ijfoodmicro.2010.10.015

Yang, Y., Fu, P., Guo, Y., and Liu, X. (2008). Analysis of antibiotic susceptibility of foodborne *Listeria monocytogenes* in China. *Wei. Sheng. Yan. Jiu.* 37, 183–186. doi: 10.1089/fpd.2013.1528

Yücel, N., Çitak, S., and Önder, M. (2005). Prevalence and antibiotic resistance of *Listeria* species in meat products in Ankara. *Turkey. Food. Microbiol.* 22, 241–245. doi: 10.1016/j.fm.2004.03.007

Zhang, Y., Yeh, E., Hall, G., Cripe, J., Bhagwat, A. A., and Meng, J. (2007). Characterization of *Listeria monocytogenes* isolated from retail foods. *Int. J. Food. Microbiol.* 113, 47–53. doi: 10.1016/j.ijfoodmicro.2006.07.010

Zhao, Y., Fu, P., Pei, X. Y., Wang, G., and Guo, Y. C. (2012). Characteristic analysis of antimicrobial resistance of *Listeria monocytogenes* in foods. *Chin. J. Food Hygiene* 24, 5–8.

Zhou, X. H., and Jiao, X. A. (2004). Investigation of *Listeria monocytogenes* contamination pattern in local Chinese food market and the tracing of two clinical isolates by RAPD analysis. *Food. Microbiol.* 21, 695–702. doi: 10.1016/j.fm.2004.02.007

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.

frontiers in Microbiology

MINI REVIEW
published: 28 August 2015
doi: 10.3389/fmicb.2015.00873

Presence of toxic microbial metabolites in table olives

*Eduardo Medina-Pradas and Francisco Noé Arroyo-López**

Food Biotechnology Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Seville, Spain

Table olives have an enormous importance in the diet and culture of many Mediterranean countries. Albeit there are different ways to produce this fermented vegetable, brining/salting, fermentation, and acidification are common practices for all of them. Preservation methods such as pasteurization or sterilization are frequently used to guarantee the stability and safety of fermented olives. However, final products are not always subjected to a heat treatment. Thus, microbiota is not always removed and appropriate levels of acidity and salt must be obtained before commercialization. Despite the physicochemical conditions not being favorable for the growth of foodborne pathogens, some illness outbreaks have been reported in the literature. Street markets, inappropriate manipulation and storage conditions were the origin of many of the samples in which foodborne pathogens or their metabolites were detected. Many authors have also studied the survival of pathogens in different styles of table olive elaboration, finding in general that olive environment is not appropriate for their presence. Inhibitory compounds such as polyphenols, low availability of nutrients, high salt content, low pH levels, bacteriocins, or the addition of preservatives act as hurdles against undesirable microorganisms, which contribute to obtaining a safe and good quality product.

Keywords: microbial risk, foodborne pathogens, table olives, mycotoxins, *Clostridium*, biogenic amines

OPEN ACCESS

Edited by:
Maria Schirone, University of Teramo, Italy

Reviewed by:
George-John Nychas, Agricultural University of Athens, Greece
Rosalba Lanciotti, University of Bologna, Italy

****Correspondence:***
Francisco Noé Arroyo-López, Food Biotechnology Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas – Pablo de Olavide University, Building 46, Ctra. Utrera, Km 1, 41013 Seville, Spain
fnarroyo@cica.es

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

Received: *03 June 2015*
Accepted: *10 August 2015*
Published: *28 August 2015*

Citation:
Medina-Pradas E and Arroyo-López FN (2015) Presence of toxic microbial metabolites in table olives. Front. Microbiol. 6:873. doi: 10.3389/fmicb.2015.00873

Production of Table Olives

The fermentation of olive fruit has many centuries of history, particularly in the Mediterranean basin, where this fermented vegetable has had a great influence on the culture and diet of many countries. According to the last consolidated statistics of the International Olive Council, worldwide production currently exceeds 2.4 million tons per year. Spain, Turkey, Egypt, Syria, Algeria, Greece, and Morocco are among the main producers, albeit Argentina, Peru, and USA are also important contributors (International Olive Council [IOC], 2015). Thus, table olive processing is spread worldwide and represents an important economic source for olive-growing countries.

Olive fruit cannot be consumed directly from the tree due to its peculiar characteristics (presence of the bitter glucoside compound oleuropein, high fat, and low sugar content). For this reason, diverse methods were developed to make them palatable. Although many of them share the general process of brining/salting, fermentation, and acidification, they can differ slightly between areas of production. The Trade Standard Applying to Table Olives (International Olive Council [IOC], 2004) defines table olives as: '*the product obtained from suitable olive cultivars, processed to remove their natural bitterness, and preserved (by natural fermentation, heat treatment or preservatives) with or without brine until consumption.*' Among the most important table olive

industrial processing methods we can find: (i) the so-called Spanish-style (alkali treated green olives), which represent about 50–60% of production, (ii) the so-called Californian-style (ripening of olives by alkaline oxidation), and (iii) directly brined olives (green, changing color or naturally black fruits) (Garrido-Fernández et al., 1997).

In all table olive processing methods described above, microorganisms have an important role, determining the safety, quality and flavor of the final product. Lactic acid bacteria (LAB) and yeasts are considered beneficial microorganisms, opposite to the role played by *Enterobacteriaceae* and *Propionibacteriaceae* (Garrido-Fernández et al., 1997; Arroyo-López et al., 2012; Hurtado et al., 2012). Traditionally, olive fermentation occurred spontaneously, but the process is not fully predictable and sometimes can lead to product spoilage or sanitary risks (Lanza, 2013). The present mini-review deals with the biological hazards posed by microorganisms in table olives, as well as the diverse hurdles that olive fermentation environments offer against growth of undesirable microorganisms.

Sanitary Risks Caused by Microorganisms or Their Metabolites in Table Olives

Despite fermented table olives having a long history of microbial safety, diverse biological hazards may be present in the finished product (**Table 1**). Among the most relevant, we can mention:

(i) Biogenic Amines

The consumption of foods containing high amounts of toxic biogenic amines may cause food intoxication and intolerance, with diverse associated symptoms such as migraines, headaches, depression, diarrhea, insomnia, etc., indicating the need for a better hygiene process. These compounds can be formed in table olives by spoilage microorganisms with amino acid decarboxylase activity. Hornero-Mendez and Garrido-Fernández (1994) reported the presence of biogenic amines (putrescine, cadaverine, and tyramine) in fermented green table olives with "zapatera" spoilage. The concentration of biogenic amines can increase during olive storage but the levels found in the final products are usually low and should not represent a health concern (García-García et al., 2001). Recently, Tofalo et al. (2012) also detected in naturally fermented olives a low quantity of biogenic amines, as well as the presence by RT-qPCR of biogenic amines producing bacteria.

(ii) Mycotoxins

These compounds are secondary toxic metabolites produced by some species of mold (mainly *Aspergillus*, *Penicillium*, and *Fusarium* genera) under aerobic and humidity conditions (El Adlouni et al., 2006). Mycotoxins in foods can be of concern for consumers, causing disease in human and other vertebrates with symptoms such as skin irritation, immunosuppression, neurotoxicity, etc. Contamination of table olives with various types of mycotoxins (Ochratoxin, Aflatoxin B, and Citrinin) have been documented in cracked olives (Franzetti et al., 2011), but Greek-style black olives are the most affected (Gourama and Bullerman, 1988; Ghitakou et al., 2006). Fortunately, mycotoxin levels usually found in table olives are too low to cause disease.

(iii) Foodborne Pathogenic Bacteria

Diverse works have reported the presence of *Listeria monocytogenes* (Caggia et al., 2004; RASFF Portal, 2012a), *Staphylococcus aureus* (Asehraou et al., 1992; Pereira et al., 2008), and *Enterobacteriaceae* species such as *Yersinia enterocolitica* and *Escherichia coli* (Asehraou et al., 1992; Franzetti et al., 2011; Lucena-Padrós et al., 2014) in table olives. However, there are no reports of illness outbreaks caused by these microorganisms in table olives. Botulism, associated with *Clostridium botulinum* growth, is certainly the most relevant biohazard in table olives. Diverse outbreaks associated with homemade table olives and recalls of suspected products have been reported (Debord et al., 1920; Fenicia et al., 1992; Cawthorne et al., 2005; Jalava et al., 2011; Pingeon et al., 2011; RASFF Portal, 2012b). It should be emphasized that artisanal productions or inadequate storage ($pH \geq 4.5$ units) were often the origin of these outbreaks. **Table 2** shows the epidemiological cases of botulism reported in table olives.

(iv) Degradation of Organic Acids

Spoilage microorganisms associated with fermented vegetables such as *Lactobacillus buchneri* are able to produce acetic acid from lactic acid consumption under anaerobic conditions (Johanningsmeier and McFeeters, 2013), whilst *Propionibacterium* and *Pectinatus* species are able to convert lactic acid to propionic acid (Breidt et al., 2013; Lucena-Padrós et al., 2014). Oxidative yeasts can also consume the lactic and acetic acids produced during olive fermentation under aerobic conditions (Ruiz Cruz and González Cancho, 1969). However, they are not able to use these acids in the absence of oxygen. Lactic acid consumption in table olives reduces the preservative power of fermented olives and increases the pH values, which can allow for the growth of others undesirable microorganisms with the consequent loss of product quality and food safety.

Table Olives: Hurdles against Biological Hazards

The olive fermentation process led by LAB involves the consumption of sugars to produce a wide range of final products with preservative effects; among the most important is lactic acid. These preservative compounds, together with low pH, protein and vitamin content, as well as reduced water activity (chloride salt is added to brine in a range of 5–11%), provide an acidic and salty environment which is adverse for the growth of undesirable microorganisms.

Other compounds excreted by microorganisms can also act as biopreservative agents. Bacteriocins are bacterial proteins or peptides that show a bactericide effect against closely related species. Jimenez-Díaz et al. (1993) isolated a bacteriocin producer *Lactobacillus plantarum* strain from green olive fermentation. The inhibitory compounds produced by this microorganism

TABLE 1 | Summary of the main types of biohazards reported in table olives.

Type of biological hazard	Microorganism or compound detected	Type of table olives	Reference
Biogenic amines	Putrescine	"zapatera" green olives	Hornero-Mendez and Garrido-Fernández (1994)
		Greek-style olives	García-García et al. (2001)
	Cadaverine		Tofalo et al. (2012)
	Tyramine		
Mycotoxins	Ochratoxin	"Greek-style" black olives	Gourama and Bullerman (1988)
			El Adlouni et al. (2006)
	Citrinin		Ghitakou et al. (2006)
			Franzetti et al. (2011)
	Aflatoxin B		
Presence of foodborne pathogenic bacteria	*Listeria monocytogenes*	Green olives	Caggia et al. (2004)
		Sliced black olives	RASFF Portal (2012a)
	Staphylococcus sp.	Black olives	Asehraou et al. (1992)
		Brined olives	Pereira et al. (2008)
	Coliforms	Black olives	Asehraou et al. (1992)
		Green olives	Franzetti et al. (2011)
	Yersinia and *Escherichia coli*	Spanish-style olives	Lucena-Padrós et al. (2014)
	Clostridium	Black olives	Fenicia et al. (1992)
			Debord et al. (1920)
		Olives stuffed with almonds	Jalava et al. (2011)
		Green olives from Italy	Cawthorne et al. (2005)
			RASFF Portal (2012b)

TABLE 2 | Major illness outbreaks associated with botulism in table olives.

Type of table olive	Reason	Relevance	Region/Country	Reference
Black olives	Improper sterilization	12 deaths	OH, USA	Debord et al. (1920)
		5 deaths	Detroit, MI, USA	
		4 deaths	NY, USA	
		7 deaths	TN, USA	
		1 death	CA, USA	
	Incorrect storage after opening	5 cases with 0 death	Italy	Fenicia et al. (1992)
Green olives	Incorrect homemade preparation (pH 6,2)	16 cases with 0 death	Molise, Italy	Cawthorne et al. (2005)
			Campania, Italy	
			Puglia, Italy	
Olives stuffed with almonds	Incorrect manufacturing by producer	2 cases with 1 death	Helsinki, Finland	Jalava et al. (2011)
Green olives paste	Incorrect thermal treatment by homemade producer	9 cases with 0 death	South–east and northern of France	Pingeon et al. (2011)

(plantaricins S and T) were active against bacteria that can cause spoilage in olive fermentations (*Propionibacteriaceae* and *Clostridium*) as well as natural competitors of *Lactobacillus plantarum* in olive fermentation brines (Ruíz-Barba et al., 1994). Likewise, yeasts produce toxic proteins or glycoproteins, also known as killer factors, are able to inhibit the growth of fungi and other non-desirable yeast species acting as biocontrol agents. *Debaryomyces*, *Pichia*, and *Candida* are genera with a considerable number of killer strains isolated from table olives (Hernández et al., 2008).

Table olive fermentations also contain antimicrobial compounds that limit the growth of LAB and others microorganisms, mainly in non-alkali treated olives (Medina et al., 2010). It has been recently demonstrated that some phenolic and oleosidic substances such as the dialdehydic form of decarboxymethyl elenolic acid (EDA), as well as EDA linked to hydroxytyrosol (Hy-EDA) present in olive brines, possess significant bacteriocide activity against foodborne pathogens, even greater than other phenolic compounds isolated from foods or synthetic biocides (Medina et al., 2009; Brenes et al., 2011). Thus, survival studies carried out with *E. coli* O157:H7 in Spanish-style table olive fermentation, show inhibition of the pathogen in all assayed conditions (Spyropoulou et al., 2001). Similar behavior was observed in the survival of

Bacillus cereus in green olive fermentation, where the population declined steadily during the fermentation process (Panagou et al., 2008). Recently, Grounta et al. (2013) investigated the survival of diverse foodborne pathogens artificially inoculated on natural black table olives. They demonstrated that natural black olives are not a favorable environment to support the growth of the assayed pathogens, and the population of all them showed a rapid decline throughout the first 2 days of storage. Medina et al. (2013) studied the survival of diverse food-borne pathogens (*E. coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *S. aureus*) in industrial olive brines from different cultivars (Manzanilla, Gordal, Hojiblanca, etc.). They found a correlation with the presence of polyphenols, considered inhibitory compounds from olives fruit. 5-log reduction of population inoculated was achieved between 5 min to 17 days in the least deleterious brine. Hence, according to the available data, table olive industrial brines of different olive cultivars and elaboration processes, do not constitute a favorable environment for any of the pathogenic bacteria tested.

How to Reduce Biological Hazards in Table Olives

The objective of table olive producers should be to achieve zero risk in the case of illness and injury caused by toxic microbial metabolites. This can only be achieved by following practices that ensure that the fruits selected for processing are: produced under Good Agricultural Practices (GAP); processed under the principles of Good Manufacturing Practices (GMP) and produced at premises with equipment and personnel strictly following Good Hygienic Practices (GHP). All these requisites must be considered in the framework of food safety management systems, which include not only the HACCP System, but also other food defense tools to prevent intentional adulterations, i.e., CARVER (Criticality Accesibility Recuperability Vulnerability Effect Recognizability), TACCP (Threat Assessment and Critical Control Points), VACCP (Vulnerability Analysis and Critical Control Points).

In many cases, the fermentation of olive fruit still occurs spontaneously, which can sometimes lead to spoilage of the final product or to sanitary risks. In order to prevent these problems, the processing can be controlled through physicochemical (addition of acids, salt, temperature control, preservatives, or application of modified atmospheres) or microbiological approaches. To improve fermentation and consistently produce high quality, safe, final products; many authors have recommended strict process control of the above parameters, in addition to the use of starter cultures (see Corsetti et al., 2012 for a complete review on this aspect). The search for starters with application in olive fermentation and vegetables in general, has for many years, been focused on the activity of LAB and their technological applications. However, in the last decade, several publications have emphasized the importance of the role that selected yeasts can play when used as starter cultures during table olive processing (Arroyo-López et al., 2012; Bevilacqua et al., 2012, 2013). Moreover, the selection of microorganisms as starters in olive fermentation and vegetables in general, has been exclusively based on diverse technological criteria (homo-fermentative metabolism; high acidification rate and fast consumption of fermentable substrates; organic acids, polyphenols, high pH and salt tolerance; flavor development or production of bacteriocins) (Duran-Quintana et al., 1999; Sánchez et al., 2001; Corsetti et al., 2012; Hurtado et al., 2012; Di Cagno et al., 2013; Heperkan, 2013). However, in addition to technological characteristics, recent studies on the development of starter cultures for table olives have focused on the study of the probiotic potential of native microorganisms. These studies must include both LAB and yeasts for the development of a mixed-multifunctional starter, in order to improve and expand the form of action of the culture by the use of two complementary microorganisms with different properties.

Conclusion

The harsh environmental conditions found in the fermentation process (low pH, high salt content, presence of inhibitory compounds, sugar consumption, etc.), and the presence of other additional hurdles (production of bacteriocins, killer factors, addition of preservatives, etc.), make table olives an adverse habitat for the development of foodborne pathogens. If such growth ultimately occurs, the presence of undesirable microorganisms or their metabolites is often linked to the storage or selling conditions, not to the fermentation/production process. For all the above mentioned reasons, this widespread Mediterranean fermented vegetable can be considered quite a safe product, if good hygiene and manufacturing practices are followed and appropriated levels of salt ($>5\%$) and pH (<4.3) are obtained in the final products.

Acknowledgments

This paper has received funding from Junta de Andalucía regional Government through the PrediAlo project (AGR-7755: www.predialo.science.com.es) and EU's Seventh Framework Program (FP7/2007-2013) under grant agreement n°243471 (PROBIOLIVES: www.probiolives.eu). FA-L wishes to express thanks to the Spanish government for his Ramón y Cajal postdoctoral research contract, while EM-P would like to thank The Junta de Andalucía for his postdoctoral research contract. Both authors also express their gratitude to Antonio de Castro and Antonio Garrido-Fernández from Instituto de la Grasa (CSIC, Seville, Spain) for their invaluable comments and help during the writing of this manuscript.

References

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García García, P., et al. (2012). Potential benefits of the application of yeast starters in table olive processing. *Front. Microbiol.* 3:161. doi: 10.3389/fmicb.2012.00161

Asehraou, A., Faid, M., and Jana, M. (1992). Physico-chemical properties and the microflora of Moroccan black table olives. *Grasas Aceites* 43, 130–133. doi: 10.3989/gya.1992.v43.i3.1164

Bevilacqua, A., Beneduce, L., Sinigaglia, M., and Corbo, M. R. (2013). Selection of yeasts as starters cultures for table olives. *J. Food Sci.* 78, M742–M751. doi: 10.1111/1750-3841.12117

Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2012). Selection of yeasts as starters cultures for table olives: a step-by-step procedure. *Front. Microbiol.* 3:194. doi: 10.3389/fmicb.2012.00194

Breidt, F., Medina, E., Wafa, D., Pérez-Díaz, I., Franco, W., Huang, H., et al. (2013). Characterization of cucumber fermentation spoilage bacteria by enrichment culture and 16S rDNA cloning. *J. Food Sci.* 78, 470–476. doi: 10.1111/1750-3841.12057

Brenes, M., García, A., De los Santos, B., Medina, E., Romero, C., Castro, A., et al. (2011). Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chem.* 125, 1262–1266. doi: 10.1016/j.foodchem.2010.10.055

Caggia, C., Randazzo, C. L., Salvo, M., Romeo, F., and Giudici, P. (2004). Occurrence of *Listeria monocytogenes* in green table olives. *J. Food Prot.* 10, 2189–2194.

Cawthorne, A., Celentano, L. P., D'Ancona, F., Bella, A., Masari, M., Aniballi, F., et al. (2005). Botulism and preserved green olives. *Emerg. Infect. Dis.* 11, 781–782. doi: 10.3201/eid1105.041088

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R., and Suzzi, G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Front. Microbiol.* 3:248. doi: 10.3389/fmicb.2012.00248

Debord, G. G., Edmondson, R. B., and Thom, C. (1920). Summary of bureau of chemistry investigations of poisoning due to ripe olives. *J. Ame. Med. Assoc.* 74, 1220–1221. doi: 10.1001/jama.1920.02620180018004

Di Cagno, R., Coda, R., De Angelis, M., and Gobbetti, M. (2013). Exploitation of vegetables and fruits through lactic acid fermentation. *Food Microbiol.* 33, 1–10. doi: 10.1016/j.fm.2012.09.003

Duran-Quintana, M. C., García-García, P., and Garrido-Fernández, A. (1999). Establishment of conditions for green table olive fermentation at low temperature. *Int. J. Food Microbiol.* 51, 133–143. doi: 10.1016/S0168-1605(99)00123-3

El Adlouni, C., Tozlovanu, M., Naman, F., Faid, M., and Pfohl-Leszkowicz, A. (2006). Preliminary data on the presence of mycotoxins (ochratoxin A, citrinin and aflatoxin B1) in black table olives "Greek style" of Moroccan origin. *Mol. Nutr. Food Res.* 50, 507–512. doi: 10.1002/mnfr.200600055

Fenicia, L., Ferrini, A. M., Aureli, P., and Padovan, M. T. (1992). Epidemia di botulismo da olive nere. *Industrie Alimentari* 31, 307–308.

Franzetti, L., Scarpellini, M., Vecchio, A., and Planeta, D. (2011). Microbiological and safety evaluation of green table olives marketed in Italy. *Ann. Microbiol.* 61, 843–851. doi: 10.1007/s13213-011-0205-x

García-García, P., Brenes-Balbuena, M., Romero-Barranco, C., and Garrido-Fernández, A. (2001). Biogenic amines in packed table olives and pickles. *J. Food Prot.* 64, 374–378.

Garrido-Fernández, A., Fernández-Díez, M. J., and Adams, R. M. (1997). *Table Olives Production and Processing*. London: Chapman & Hall. doi: 10.1007/978-1-4899-4683-6

Ghitakou, S., Koutras, K., Kanellou, E., and Markaki, P. (2006). Study of aflatoxin B1 and ochratoxin A production by natural microflora and *Aspergillus parasiticus* in black and green olives of Greek origin. *Food Microbiol.* 23, 612–621. doi: 10.1016/j.fm.2005.12.008

Gourama, H., and Bullerman, L. B. (1988). Mycotoxin production by molds isolated from "Greek-style" black olives. *Int. J. Food Microbiol.* 6, 81–90. doi: 10.1016/0168-1605(88)90087-6

Grounta, A., Nychas, G. J. E., and Panagou, E. Z. (2013). Survival of food-borne pathogens on natural black table olives after post-processing contamination. *Int. J. Food Microbiol.* 161, 197–202. doi: 10.1016/j.ijfoodmicro.2012.12.017

Heperkan, D. (2013). Microbiota of table olive fermentation and criteria of selection for their use as starters. *Front. Microbiol.* 4:143. doi: 10.3389/fmicb.2013.00143

Hernández, A., Martín, A., Córdoba, M. G., Benito, M. J., Aranda, E., and Pérez-Nevado, F. (2008). Determination of killer activity in yeasts isolated from the elaboration of seasoned green table olives. *Int. J. Food Microbiol.* 121, 178–188. doi: 10.1016/j.ijfoodmicro.2007.11.044

Hornero-Mendez, D., and Garrido-Fernández, A. (1994). Biogenic amines in table olives. Analysis by high-performance liquid chromatography. *Analyst* 119, 2037–2041. doi: 10.1039/an9941902037

Hurtado, A., Requant, C., Bordons, A., and Rozès, N. (2012). Lactic acid bacteria from fermented olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

International Olive Council [IOC]. (2004). *Trade standard applying to table olives*. Madrid: International Olive Council.

International Olive Council [IOC]. (2015). *World Table Olive Figures*. Available at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures [accessed 15 July, 2015].

Jalava, K., Selby, K., Pihlajasaari, A., Kolho, E., Dahlsten, E., Forss, N., et al. (2011). *Two Cases of Food-Borne Botulism in Finland Caused by Conserved Olives*. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20034

Jimenez-Díaz, R., Ríos-Sánchez, R. M., Desmazeaud, M., Ruíz-Barba, J. L., and Piard, J. C. (1993). Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Appl. Envirom. Microbiol.* 59, 1416–1424.

Johanningsmeier, S. D., and McFeeters, R. F. (2013). Metabolism of lactic acid in fermented cucumbers by *Lactobacillus buchneri* and related species, potential spoilage organisms in reduced salt fermentations. *Food Microbiol.* 35, 129–135. doi: 10.1016/j.fm.2013.03.004

Lanza, B. (2013). Abnormal fermentation in table-olive processing: microbial origin and sensory evaluation. *Front. Microbiol.* 4:91. doi: 10.3389/fmicb.2013.00091

Lucena-Padrós, H., González, J. M., Caballero-Guerrero, B., Ruiz-Barba, J. L., and Maldonado-Barragán, A. (2014). *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style green olives. *Int. J. Syst. Evol. Microbiol.* 64, 2980–2985. doi: 10.1099/ijs.0.063032-0

Medina, E., Brenes, M., García, A., Romero, C., and de Castro, A. (2009). Bactericidal activity of glutaraldehyde-like compounds from olives products. *J. Food Prot.* 72, 2611–2614.

Medina, E., Brenes, M., Romero, C., Ramirez, E., and de Castro, A. (2013). Survival of foodborne pathogenic bacteria in table olive brines. *Food Control* 34, 719–724. doi: 10.1016/j.foodcont.2013.06.026

Medina, E., Gori, C., Servili, M., de Castro, A., Romero, C., and Brenes, M. (2010). Main variables affecting the lactic acid fermentation of table olives. *Int. J. Food Sci. Technol.* 45, 1291–1296. doi: 10.1111/j.1365-2621.2010.02274.x

Panagou, E. Z., Tassou, C. C., Vamvakoula, P., Saravanos, E. K. A., and Nychas, G.-J. E. (2008). Survival of *Bacillus cereus* vegetative cells during Spanish-style fermentation of Conservolea green olives. *J. Food Prot.* 71, 1393–1400.

Pereira, A. P., Pereira, J. A., Bento, A., and Estevinho, M. L. (2008). Microbiological characterization of table olives commercialized in Portugal in respect to safety aspects. *Food Chem. Toxicol.* 4, 2895–2902. doi: 10.1016/j.fct.2008.05.033

Pingeon, J. M., Vanbockstael, C., Popoff, M. R., King, L. A., Deschamps, B., Pradel, G., et al. (2011). *Two Outbreaks of Botulism Associated with Consumption of Green Olive Paste*. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20035

RASFF Portal. (2012a). *Reference Number 2012.0703*. Available at: https://webgate.ec.europa.eu/rasff-window/portal

RASFF Portal. (2012b). *Reference Number 2012.1059*. Available at: https://webgate.ec.europa.eu/rasff-window/portal

Ruíz-Barba, J. L., Cathcart, D. P., Warner, P. J., and Jimenez-Díaz, R. (1994). Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. *Appl. Envirom. Microbiol.* 60, 2059–2064.

Ruiz Cruz, J., and González Cancho, F. (1969). Methabolism of yeasts isolated from brines of Spanish-style table olives. I. the assimilation of lactic, acetic and citric acid. *Grasas Aceites* 20, 6–11.

Sánchez, A. H., Rejano, L., Montaño, A., and De Castro, A. (2001). Utilization at high pH of starter cultures of lactobacilli for Spahish-style green olive fermentation. *Int. J. Food Microbiol.* 67, 115–122. doi: 10.1016/S0168-1605(01)00434-2

Spyropoulou, K. E., Chorianopoulos, N. G., Skandamis, P. N., and Nychas, G.-J. E. (2001). Survival of *Escherichia coli* O157:H7 during the fermentation of Spanish-style green table olives (conservolea variety) supplemented with different carbon sources. *Int. J. Food Microbiol.* 66, 3–11. doi: 10.1016/S0168-1605(00)00510-9

Tofalo, R., Schirone, M., Perpetuini, G., Angelozzi, G., Suzzi, G., and Corsetti, A. (2012). Microbiological and chemical profiles of naturally fermented table olives and brines from different Italian cultivars. *Int. J. General Mol. Microbiol.* 102, 121–131. doi: 10.1007/s10482-012-9719-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.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 June 2015
doi: 10.3389/fmicb.2015.00560

From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in *Bacillus cereus*

Nadja Jeßberger[1]†, Viktoria M. Krey[2†], Corinna Rademacher[3], Maria-Elisabeth Böhm[2], Ann-Katrin Mohr[1], Monika Ehling-Schulz[3], Siegfried Scherer[2] and Erwin Märtlbauer[1]*

[1] Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität München, Oberschleißheim, Germany, [2] Lehrstuhl für Mikrobielle Ökologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung, Wissenschaftszentrum Weihenstephan, Technische Universität München, Freising, Germany, [3] Functional Microbiology, Department of Pathobiology, Institute of Microbiology, University of Veterinary Medicine Vienna, Vienna, Austria

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Mehrdad Mark Tajkarimi,
University of North Carolina at Greensboro, USA
Maria Schirone,
Università degli Studi di Teramo, Italy

***Correspondence:**
Nadja Jeßberger,
Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität München, Schönleutnerstr. 8, 85764 Oberschleißheim, Germany
n.jessberger@mh.vetmed.uni-muenchen.de

†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: 07 May 2015
Accepted: 21 May 2015
Published: 10 June 2015

Citation:
Jeßberger N, Krey VM, Rademacher C, Böhm M-E, Mohr A-K, Ehling-Schulz M, Scherer S and Märtlbauer E (2015) From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in Bacillus cereus. Front. Microbiol. 6:560. doi: 10.3389/fmicb.2015.00560

In recent years *Bacillus cereus* has gained increasing importance as a food poisoning pathogen. It is the eponymous member of the *B. cereus sensu lato* group that consists of eight closely related species showing impressive diversity of their pathogenicity. The high variability of cytotoxicity and the complex regulatory network of enterotoxin expression have complicated efforts to predict the toxic potential of new *B. cereus* isolates. In this study, comprehensive analyses of enterotoxin gene sequences, transcription, toxin secretion and cytotoxicity were performed. For the first time, these parameters were compared in a whole set of *B. cereus* strains representing isolates of different origin (food or food poisoning outbreaks) and of different toxic potential (enteropathogenic and apathogenic) to elucidate potential starting points of strain-specific differential toxicity. While toxin gene sequences were highly conserved and did not allow for differentiation between high and low toxicity strains, comparison of *nheB* and *hblD* enterotoxin gene transcription and Nhe and Hbl protein titers revealed not only strain-specific differences but also incongruence between toxin gene transcripts and toxin protein levels. With one exception all strains showed comparable capability of protein secretion and so far, no secretion patterns specific for high and low toxicity strains were identified. These results indicate that enterotoxin expression is more complex than expected, possibly involving the orchestrated interplay of different transcriptional regulator proteins, as well as posttranscriptional and posttranslational regulatory mechanisms plus additional influences of environmental conditions.

Keywords: ***Bacillus cereus*, enterotoxins, Nhe, Hbl, host cell cytotoxicity, food poisoning**

Introduction

Bacillus cereus has become a hygienic and technological problem of increasing importance in the food industry. It is ubiquitous, produces heat resistant endospores and is able to form biofilms (Wijman et al., 2007; Stenfors Arnesen et al., 2008; Nam et al., 2014). Due to its lipo- and proteolytic properties it plays an important role in food spoilage (Andersson et al., 1995), but the main problem is the production of toxins, which are responsible for food poisoning. In 2011, the number of

B. cereus-associated food poisoning outbreaks within the European Union increased by 122% (Anonymous, 2013). Various efforts have been made to predict the toxic potential of newly isolated strains. The *B. cereus sensu lato* group consists of eight closely related species, i.e., *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus cytotoxicus,* and *Bacillus toyonensis*. Highly toxic as well as non-toxic strains exist among most of these species. Guinebretière et al. (2008) further divided the *B. cereus* group into seven phylogenetic groups and subgroups (Guinebretière et al., 2008). For *B. cereus sensu lato*, the most commonly used method in routine diagnostics is the detection and quantification of colonies on selective culture media such as PEMBA or MYP according to international standards (Ehling-Schulz and Messelhäusser, 2013), which does not allow to distinguish the species of the *B. cereus* group. Therefore, only *presumptive B. cereus* can be detected (ISO 7932). While molecular methods for quantification of *B. cereus* have been established, no differentiation between living and dead cell or between spores and vegetative cells could be achieved (Martinez-Blanch et al., 2009; Ceuppens et al., 2010; Dzieciol et al., 2013). Currently, the molecular detection of toxin genes rather than species differentiation is applied (Ehling-Schulz and Messelhäusser, 2013). Toward this end, multiplex PCR systems for the detection of *nhe*, *hbl*, *cytK,* and *ces* have been established (Guinebretière et al., 2002; Fricker et al., 2007; Wehrle et al., 2009). However, the presence or absence of toxin genes does not allow to reliably infer the toxic potential, as highly variable amounts of toxins are produced in strains sharing the same toxin genes (Dietrich et al., 2005; Jeßberger et al., 2014).

A peptide synthetase, encoded by *ces*, produces the emetic toxin cereulide, the causative agent of the emetic syndrome leading to vomiting (Ehling-Schulz et al., 2005b; Stenfors Arnesen et al., 2008). Due to its resistance against heat, acids and proteolysis, it can't be inactivated by heat treatment of contaminated food samples or during stomach passage (Ehling-Schulz et al., 2004). The amount of preformed cereulide present in foods can consequently be used as an indicator for the toxic potential and thus for the consumer's risk (Ehling-Schulz and Messelhäusser, 2013). Currently, an ISO method for quantitative detection of cereulide in food samples (EU-CEN action: TC 257/WG6) is under development.

Enteropathogenic *B. cereus* cause diarrhea due to the production of enterotoxins in the human intestine. This occurs after viable bacteria or most likely spores are ingested together with contaminated foods (Clavel et al., 2004; Ceuppens et al., 2012). So far, the two three component enterotoxin complexes Nhe (non haemolytic enterotoxin, Lund and Granum, 1996) and Hbl (haemolysin BL, Beecher et al., 1995) have been described, as well as the single protein CytK (cytotoxin K, Lund et al., 2000). Only very few strains bear the highly toxic variant CytK1 and these are classified as a separate species, *B. cytotoxicus* (Guinebretière et al., 2013). The *nhe* genes are present in all enteropathogenic *B. cereus* strains analyzed so far. The *hbl* operon is present in approximately 50% of the strains, whereas its prevalence seems to be higher in clinical and food isolates (Guinebretière et al., 2002; Ehling-Schulz et al., 2005a; Moravek et al., 2006). Prediction of toxicity is based on the quantification of the enterotoxin components in *B. cereus* culture supernatants. Currently, three test systems are commercially available, detecting the enterotoxin components Hbl L2, NheA, as well as NheB and Hbl L2, respectively. However, results may often be inappropriate for evaluating the risk of contaminated food samples, as the enterotoxins, unlike the emetic toxin cereulide, are largely produced in the intestine.

According to recent studies, further virulence factors such as sphingomyelinase, haemolysin II or exoproteases contribute to pathogenicity. A role of sphingomyelinase as a virulence factor against insects and murine intestinal epithelial cells as well as its interaction with Nhe have been reported (Doll et al., 2013). HlyII was shown to induce *in vitro* and *in vivo* apoptosis to macrophages (Tran et al., 2011). In another study, *hlyII* was preferably found in pathogenic *B. cereus*, and *inhA1* and *nprA* expression (both genes encoding metalloproteases) was higher in pathogenic than in non-pathogenic strains (Cadot et al., 2010).

So far, it is still not clear why toxin production and thus toxicity is so highly variable among different *B. cereus* strains. Thus, we aimed to explore whether distinct bacterial (genetic) factors, mechanisms or regulatory levels can be identified upon which high and low toxicity of enteropathogenic *B. cereus* strains can be distinguished unequivocally. Therefore, we chose a representative set of *B. cereus* isolates and characterized it with respect to enterotoxin gene presence, sequence and transcription as well as protein secretion, enterotoxin production and cytotoxicity.

Materials and Methods

Bacterial Strains, Growth Conditions, and Sample Preparation

All *B. cereus* strains used in this study are listed in **Table 1** and Supplemental Table S1. Strains were grown at 30°C on CGY plates or in CGY broth. Unless stated otherwise, 30 ml cultures were inoculated from 17 h pre-cultures, and were grown in 300 ml Erlenmeyer flasks with 125 rpm at 30°C. For determination of enterotoxin production and toxicity, all strains listed in Table S1 were routinely grown for 6 h in CGY medium, inoculated with an optical density at 600 nm (OD_{600}) of 0.2. For growth tests in this study, CGY medium was inoculated with a 17 h pre-culture to an OD_{600} of 0.05 and OD_{600} was recorded every 30 min. 5 milliliter (at 2 h) and 3 ml (at 6 h) samples were taken and centrifuged for 15 min at RT and 3500 rpm. Cell pellets were immediately frozen at −80°C and used for preparation of RNA and measurement of intracellular protein concentrations. Supernatants were filtered through 0.2 μm filters and separated for measurement of extracellular protein concentrations and enterotoxin production. For the latter, 1 mM EDTA was added to the culture supernatant. Supernatants were frozen and stored at −20°C. All growth tests and sample preparations were carried out in triplicates.

Cell Lines and Culture Conditions

CaCo-2 cells were from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)

TABLE 1 | Set of the 19 *B. cereus* strains used in this study.

B. cereus strain	Origin		Genotype clade (group)	Toxin gene profiling								Toxin titer NheB	Toxicity Vero
				ces	*hbl*	*nhe*	*cytK2*	*hlyII*	*inhA1*	*nprA*	profile		
14294-3 (M6)	Ice cream	Food	I (III)	–	+	+	+	–	+	+	A	m	m
SDA KA96	Raw milk	Food	I (III)	–	+	+	+	–	+	+	A	hi	hi
INRA A3	Starch	Food	II (IV)	–	+	+	+	–	+	+	A	lo	lo
INRA C3	Past. Carrot	Food	II (IV)	–	+	+	+	–	+	+	A	hi	hi
6/27/S	Human feces	Diarrheal	II (IV)	–	+	+	+	–	+	+	A	m	m
F3175/03 (D7)	Human feces	Diarrheal	II (IV)	–	+	+	+	–	+	+	A	hi	m
RIVM BC 934	Lettuce	Food	II (IV)	–	+	+	+	–	+	+	A	lo	lo
F528/94	Beef and chow mein and rice, food poisoning outbreak	Diarrheal	I (II)	–	+	+	–	+	+	+	C	lo	lo
F837/76	Human, postoperative infection	*hbl* reference	I (III)	–	+	+	–	+	+	+	C	hi	hi
RIVM BC 126	Human feces	Diarrheal	I (II)	–	+	+	–	–	+	+	C	hi	hi
MHI86	Infant food	Food	I (III)	–	–	+	+	–	+	+	D	lo	lo
F4429/71	Vanilla pudding	Diarrheal	I (III)	–	–	+	+	–	+	+	D	hi	hi
RIVM BC 964	Kebab	Food	II (IV)	–	–	+	+	+	+	+	D	hi	hi
F3162/03 (D8) *	Human feces	Clinical	I (III)	–	–	+	+	–	+	+	D	lo	hi
MHI226 **	Milk and milk products	Food	I (III)	–	–	+	–	+	+	+	F	lo	lo
NVH 0075–95	Stew with vegetables, foodpoisoning	*nhe* reference	I (III)	–	–	+	–	–	+	+	F	hi	hi
WSBC10035	Past. Milk	Food	I (III)	–	–	+	–	–	+	+	F	hi	hi
RIVM BC 90	Human feces	Diarrheal	I (III)	–	–	+	–	–	+	+	F	lo	lo
7/27/S	Human feces	Diarrheal	I (III)	–	–	+	–	–	+	+	F	hi	hi

Genotyping was performed by sequence analyses of the genetic markers spoIIIAB and panC, toxin profiling by PCR analyses using specific primers for ces, enterotoxin genes and virulence factors, toxin titers were determined by sandwich EIAs against NheB and toxicity was analyzed by WST-1-bioassay on Vero cells. Isolates were classified highly (hi), medium (m) or low (lo) toxic according to their cytotoxicity (hi: >500; m: 250-500; lo: <250) and NheB titers (hi: >4000; m: 2000-4000; lo: <2000).

**, strain showed high toxicity but particularly low NheB titers due to binding failure of mab 2B11 in sandwich EIA (A. Didier, in preparation).*

***, sequence analysis revealed a truncated hbl operon; as strain is not able to produce Hbl L2 and Hbl B protein (negative in EIAs), it was allocated to profile F.*

and Vero cells from ECACC (European Collection of Cell Cultures). Both cell lines were cultured in media recommended by the supplier and as described elsewhere (Jeßberger et al., 2014).

Toxin Gene Profiling and Sequence Typing

Total DNA was isolated using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre). Detection of *nhe*, *hbl*, *cytK* and *ces* was performed by multiplex PCR (Ehling-Schulz et al., 2006). The sporulation stage IIIAB gene (*spoIIIAB*) was used as a genetic locus for sequence typing and classification of *B. cereus* isolates to clades (Ehling-Schulz et al., 2005a; Ehling-Schulz and Messelhäusser, 2013). Therefore, sequences were aligned using CLUSTAL X software (Thompson et al., 2002) and cluster analysis of aligned sequences was performed by TREECON using UPGMA method for inferring tree topology (Van De Peer and De Wachter, 1997). Alternatively sequence typing and clade classification was carried out by using the pantothenate synthetase gene *panC* (Guinebretière et al., 2008).

Comparison of Toxin Operon Nucleotide and Amino Acid Sequences

Nucleotide sequences of *nheABC* and *hblCDA* were obtained from a *de novo* genome sequencing approach (MiSeq® Reagent Kit v2, 500 cycles, Illumina) (Böhm et al., in preparation), identified via BLASTN algorithm and compared using CloneManager 7. Multiple amino acid sequence alignments of single Nhe and Hbl enterotoxin components were constructed with Clustal Omega v1.2.0 (Sievers et al., 2011). Signal peptide sequences were predicted using the SignalP 4.1 server (Petersen et al., 2011).

RNA Isolation, cDNA Synthesis, and Quantitative Real-time PCR (qRT-PCR)

Isolation of total RNA and DNAse I digestion was performed as previously described (Dommel et al., 2010). Using the random primers of the qScript cDNA Supermix (Quanta Biosciences), first strand synthesis of 1 mg of total RNA was performed. qRT-PCR was done as reported repeatedly and the $2^{-\Delta\Delta C_T}$ method was used for calculation of relative gene expression

(Livak and Schmittgen, 2001; Lücking et al., 2009; Dommel et al., 2010). Primer amplification efficiencies (E) were in the desirable range (E = 1.7 − 2.1) for all primer pairs listed in Table S2 as evaluated according to the equation $E = 10^{(-1/slope)}$ (Pfaffl et al., 2002). 16S *rrn* transcription levels determined by qRT-PCR (Martineau et al., 1996) served as the reference control for normalization applying the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). This method is based on the following formula: amount of target transcript $= 2^{-\Delta\Delta C_T}$ with $-\Delta\Delta C_T = -(\Delta C_{T(sample)} - \Delta C_{T(calibrator)}) = -((C_{T(reference\ gene)} - C_{T(target\ gene)})_{sample} - (C_{T(reference\ gene)} - C_{T(target\ gene)})_{calibrator})$. C_T denotes the cycle number of the amplification reaction that exceeds the quantification threshold of the instrument. To determine the relative transcription of a target gene, *rrn*-normalized transcription level of a sample ($\Delta C_{T(sample)}$) was set relative to the transcript level of an external calibrator ($\Delta C_{T(calibrator)}$) and multiplied with 100 to obtain relative transcription in percent [%]. Expression level of the *hblD* gene of the toxin reference strain *B. cereus* F837/76 at 6 h served as calibrator that was set to 100% (log-2 = 0), all other transcript levels were compared to this condition using the $2^{-\Delta\Delta C_T}$ method.

Enzyme Immunoassays (EIAs)

The enterotoxin components NheB and Hbl L2, L1 and B were detected in indirect and sandwich enzyme immunoassays as described elsewhere (Dietrich et al., 1999, 2005; Moravek et al., 2006). For NheB detection, the sandwich EIA using mabs 2B11 and 1E11-HRP or the indirect EIA using only mab 1E11 were performed. Titers were defined as the reciprocal of the highest dilutions resulting in an absorbance value of ≥1.0.

WST-1 Bioassays

WST-1 bioassays were performed as previously described (Dietrich et al., 1999, 2005; Didier et al., 2012; Jeßberger et al., 2014). Characterization of the toxic activity of the 136 *B. cereus* strains was carried out on Vero cells, as this assay is used in our routine *B. cereus* diagnostic. For the 19 strains in this study, the human colon carcinoma cell line CaCo-2 was further used. For detection, the optical density at 450 nm was measured with a Tecan photometer. With Ridawin software dose-response curves and thus 50% inhibitory concentrations were calculated, shown as reciprocal titers.

Propidium Iodide (PI) Influx Studies

Enterotoxin-induced pore formation in the membranes of CaCo-2 cells was measured by propidium iodide influx tests as described before (Jeßberger et al., 2014). For each *B. cereus* strain, a specific curve (increase of fluorescence per time) was obtained, of which the value of the highest linear slope was calculated. Strains were compared according to those values.

Quantification of Total Protein Amount

Extracellular proteins were obtained from culture supernatants. For isolation of intracellular proteins cell pellets were resuspended in 50 mM Tris, pH 7,5 and disrupted by two passages through a French Pressure cell press (2 kbar). The soluble proteins were collected by centrifugation (15.000 rpm, 45 min, 4°C). Intra- and extracellular protein concentrations were quantified by Roti-Nanoquant Kit (Roth) in microtiterplates according to the manufacturer's instructions. Colorimetric reactions were measured with Infinite F200 reader (Tecan) at wavelengths of 610/450 nm. Protein concentrations were determined by quotient of optical densities $OD_{610}/_{450}$ referred to internal standard generated by Quick Start Bovine Serum Albumin Standard (Biorad).

Isolation of Extracellular Protein Extracts for Gelelectrophoretic Separation

Protein extracts were precipitated by addition of ice-cold trichloroacetic acid solution to a final concentration of 10% (v/v) overnight at 4°C. Precipitated proteins were centrifuged (1 h, 10.000 rpm, 4°C) and protein pellet was washed 3 times with acetone. Pellets were dried overnight at 4°C and finally resuspended in DIGE lysis buffer (7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5).

DIGE Labeling and SDS PAGE

For differential comparison of secretion patterns 5 μg of protein extracts per sample were labeled with CyDye Fluor minimal dyes (GE Healthcare) according to the manufacturer's instructions. Protein extracts of low toxin producing strains were labeled with Cy5, extracts of high toxin producing strains were labeled with Cy3. Afterwards samples of corresponding low and high toxic strains were pooled, reduced with Laemmli sample buffer containing 0,5 M DTT for 10 min on ice, and alkylated with 14% (w/*v*) iodoacetamide for 10 min at room temperature. Finally labeled protein extracts were separated by SDS PAGE using SE600 vertical electrophoresis system (Hoefer). Fluorescence images were scanned with a Typhoon 9400 imager (GE Healthcare).

Statistical Analyses

Mean values and standard deviations (SD) were calculated from at least three independent experiments each. For statistical analysis, 2-tailed Student's *t* test was used, where applicable, to determine statistically significant differences by Microsoft Excel. Graphs of raw data were plotted using Microsoft Excel or GraphPad Prism (version 6.0). Spearman correlation was calculated using SigmaStat 3.5.

Results

Selection of the *B. cereus* Strain Set

For selection of the *B. cereus* strain set to be used in this study, 136 isolates from the strain collections of our institutes were characterized according to their genetic toxin profile. Additionally, they were classified to be of high, medium or low toxicity according to their cytotoxicity in routinely performed WST-1 bioassays on Vero cells and their production of enterotoxin component NheB (Table S1). 19 of these strains were selected for genome sequencing (Böhm et al., in preparation) and growth, enterotoxin gene transcription, enterotoxin production, protein secretion, and cytotoxicity were studied. The genetic relationship of the strains is depicted in Figure S1. The strains

were of different origins (food or food poisoning outbreaks), of different genetic toxin profiles and of either high or low toxic activity (**Table 1**). Strains involved in food poisoning outbreaks were isolated either from the contaminated food or from patient feces. Also, the presence of *hlyII*, *inhA1,* and *nprA* was considered. Originally, equal numbers of strains per toxin profile were chosen, later two isolates from recent food poisoning outbreaks in Austria and England were added to profile A.

Strong Genetic Conservation of Enterotoxin Components

Whole genome sequences of all 19 *B. cereus* strains were obtained using a *de novo next generation sequencing* approach (MiSeq®, Illumina®) (Böhm et al., in preparation). Enterotoxin genes *nheABC* and *hblCDA* were identified via BLASTN analysis using published *nhe* and *hbl* operon sequences from the reference strains NVH 0075-95 and F837/76 as template. Alignments of the concatenated operon genes using CloneManager 7 showed that nucleotide sequences of both enterotoxin operons are highly conserved over all *B. cereus* strains investigated. Similarity of *nheABC* genes ranges from 93 to 99%, while *hblCDA* genes are even more conserved (95–98%). In contrast to all other *hbl* encoding strains of the set that harbor the major *hblCDAB* operon, *B. cereus* MHI226 solely possesses the hbl_a gene variant (Beecher and Wong, 2000) with a sequence similarity of 80–81% to the other *hblCDA* genes. *B. cereus* MHI226 does not form a functional Hbl toxin (data not shown) and was therefore reclassified in toxin profile F (only encoding *nheABC*). Hbl_a ($hblCDA_a$) is present in addition to the major toxin operon *hblCDAB* in about one third of all *hbl*-encoding strains (Böhm et al., in preparation), as it appears in strains 14294-3 (M6), 6/27/S and RIVM BC 126. Similarity between *hbl* and hbl_a variants ranges from 75 to 82%.

As a consequence of the pronounced enterotoxin gene conservation, very high sequence identity can be observed in multiple amino acid (aa) sequence alignments for all enterotoxin components (Figures S2, S3). NheB is the most conserved Nhe component with 15 variable out of 402 aa (3.73%) (Figure S2B), compared to 35 out of 386 aa (9.07%) in NheA (Figure S2A) and 47 out of 359 aa (13.09%) in NheC (Figure S2C). Hbl L2 exhibits 37 variable out of 439 aa (8.43%) (Figure S3A), Hbl B 24 out of 375 (6.40%) (Figure S3C) and the highly conserved Hbl L1 6 out of 406 (1.48%) (Figure S3B).

Additionally, amino acid sequences of the putative virulence factors HlyII, InhA1, NprA, and sphingomyelinase (Cadot et al., 2010; Tran et al., 2011; Doll et al., 2013) were compared. As for Nhe, all 19 strains possess the genes for InhA1, NprA and SMase, while only 21.1% (4 out of 19, among them two high and two low toxic strains) bear *hlyII* (**Table 1**). Thus, *hlyII* was excluded from further investigations. The low toxic strain MHI226 even possesses two *hlyII* genes. In comparison to the enterotoxins, InhA1, NprA and SMase are less conserved harboring 11.3%, 9.0% and 16.2% variable aa positions, respectively (data not shown). Neither comparison of toxin or virulence factor genes, nor amino acid sequences displayed discriminatory differences between high and low toxic *B. cereus* strains or showed any clustering according to their origin (food, food poisoning, or feces). These results show that neither presence or absence nor gene and protein sequences of enterotoxin and virulence factors provide a basis to assess virulence of a *B. cereus* strain. Therefore, it may be suspected that differential toxicity may be due to regulatory processes at the transcriptional or translational level of toxin expression.

Highly Similar Growth Kinetics within the *B. cereus* Strain Set

For comparative analyses of toxin transcription, production and secretion, all 19 *B. cereus* strains were grown under standard laboratory conditions. In **Figure 1**, the growth kinetics of strains grouped according to their toxin profile are shown. In general, growth kinetics of all *B. cereus* strains were very similar except for the low toxicity strain MHI226, which had a more shallow slope and reached the lowest maximal optical density of OD_{600} 11.9, while $OD_{600max.}$ of all other stains in toxin profile F ranged between OD_{600} 15.8–23.2 (**Figure 1D**). Maximal OD_{600} in toxin profile A (**Figure 1A**) was between OD_{600} 15.2–27.4, between OD_{600} 20.0–23.7 in toxin profile C (**Figure 1B**) and between OD_{600} 14.6–20.8 in toxin profile D (**Figure 1C**). Strains isolated from human feces (6/27/S, F3175/03 (D7), RIVM BC 126, F3162/04 (D8), RIVM BC 90, 7/27/S) tended to grow to higher maximal optical densities ($OD_{600} > 20.0$) than non-feces strains of the same toxin profile. However, significant differences in growth kinetics were only seen for MHI226. To account for any growth differences, further data were normalized to the optical density of the respective *B. cereus* strain at the time point analyzed.

Strain-dependent Toxin Gene Transcription

Transcription of enterotoxin genes was analyzed using qRT-PCR. As previously described, *B. cereus* cytotoxicity strongly correlates with NheB and Hbl L1 concentrations in the supernatant (Jeßberger et al., 2014). Therefore, transcript levels (Livak and Schmittgen, 2001) of the corresponding genes *nheB* and *hblD* were determined. Relative *nheB* and *hblD* transcription [%] of all 19 *B. cereus* strains is presented in **Figures 2A,B**, respectively. In general, relative toxin gene transcription increased from 2 to 6 h in CGY medium, except for *B. cereus* 14294-3 (M6). Relative *hblD* transcription was higher than *nheB* transcription, except for strains 14294-3 (M6), RIVM BC 934 and RIVM BC 126 which showed 2.4 and 3.9-fold increase of *nheB* over *hblD* transcription in 6 h cultures. Unexpectedly, maximum *hblD* transcription occurred in F528/94 (**Figure 2B**), a strain classified as low toxic in the cytotoxicity assay. Relative transcription was further normalized to the OD_{600} and resulting transcription efficiencies (% transcription/OD_{600}) are depicted in **Figures 2C,D**. In contrast to relative toxin transcript levels, toxin transcription efficiency did not uniformly increase from 2 to 6 h for all *B. cereus* strains, but showed strain-dependent kinetics. Eight strains of toxin profile A, C, D and F and of various origins showed decreased *nheB* transcription efficiency in 6 h cultures compared to 2 h, six strains had increased efficiency and five were similar at both growth stages (**Figure 2C**). In contrast, 80% of strains expressing Hbl showed higher *hblD* transcription efficiency at 2 h compared to 6 h except for SDA KA96 and

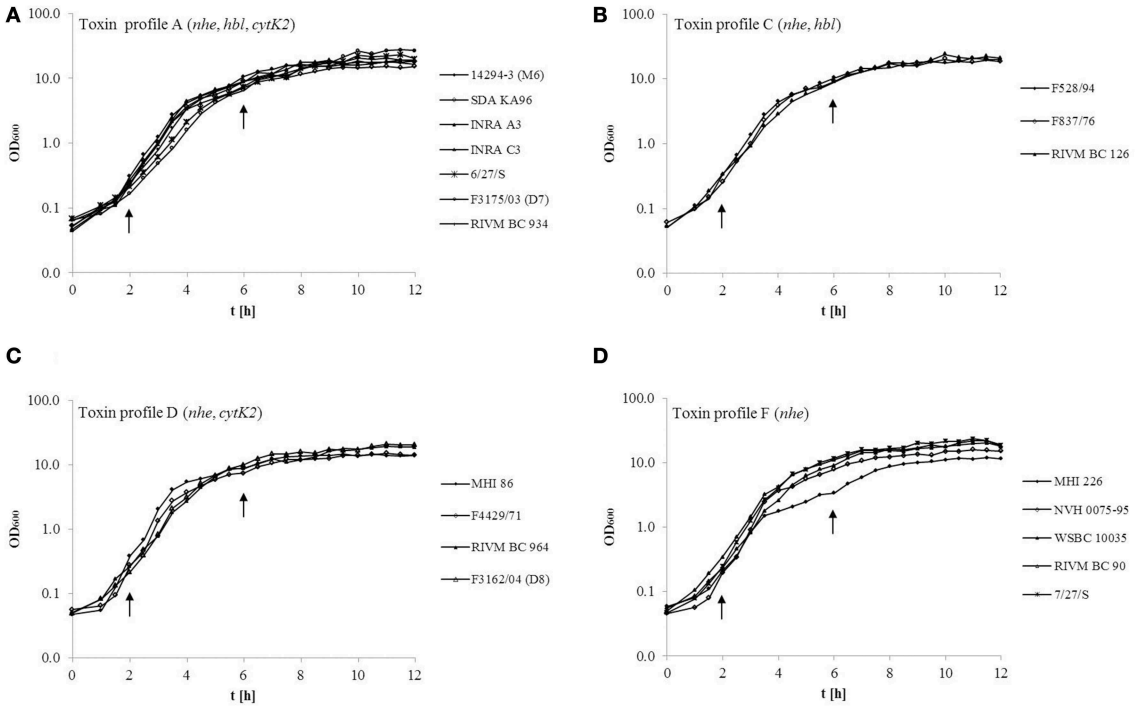


FIGURE 1 | Growth curves of 19 *B. cereus* strains grouped according to their toxin profile. (A) toxin profile A (*nhe, hbl, cytK*), (**B)** toxin profile C (*nhe, hbl*), (**C)** toxin profile D (*nhe, cytK*), (**D)** toxin profile F (*nhe*). Strains were grown in CGY medium at 30°C, 125 rpm. Arrows indicate the time were samples were taken.

F528/94 (**Figure 2D**). Interestingly, maximal *nheB* transcription efficiency was observed in the low toxic strain 14294-3 (M6) after 2 h in CGY medium, followed by the high toxic strains F837/76, RIVM BC 126 and F4429/71 (**Figure 2C**). Highest *hblD* transcription efficiency was achieved in 2 h cultures by F837/76, followed by INRA C3 and 14294-3 (M6) (**Figure 2D**).

In principle, toxin transcript levels of both *nheB* and *hblD* reflect the classification into high and low toxin producing strains according to their cytotoxicity (**Table 1**) to a certain extent. However, this is not generally the case. Setting a random threshold of 20% relative *nheB* and 40% *hblD* transcription for strains showing high toxin transcript levels, high cytotoxic strains F3175/03 (D7), F4429/71, F3162/04 (D8), WSBC10035, and 7/27/S cannot be considered to transcribe high levels of *nheB*, neither RIVM BC 126 for *hblD*. Moreover, some strains, e.g., 14294-3 (M6) are low toxic even though toxin gene transcription efficiency is amongst the highest, indicating that posttranscriptional and/or posttranslational regulation plays an important role for the manifestation of *B. cereus* toxicity. Furthermore, transcription of *inhA1*, *sph* and *nprA*, discussed as additional virulence contributors, proved to be highly strain-dependent (data not shown). In summary, there was no correlation of a strain's origin or genetic toxin profile and its toxin or virulence factor transcript level.

Strain-specific Enterotoxin Production

Reciprocal titers of the enterotoxin components NheB, Hbl L2, Hbl L1, and Hbl B in the culture supernatants were determined performing specific EIAs. As a strong correlation of NheB with cytotoxicity has already been demonstrated (Moravek et al., 2006; Jeßberger et al., 2014), titers of NheA or NheC were not investigated. The productivity for all toxin components was calculated as titer/OD_{600} to exclude growth effects (**Figure 3**). After 2 h, no Hbl and only trace amounts of NheB protein were detectable. After 6 h, NheB titers generally confirmed prior classification into high, medium and low toxin producing strains (compare **Table 1**). Only 14294-3 (M6) showed higher NheB levels than in previous experiments (**Figure 3A**). Strains producing high, medium and low amounts of NheB were found among all toxin profiles as well as among food- and food poisoning-associated strains. NheB titers of strain F3162/04 (D8) were determined using only NheB-specific mab 1E11, as this strain is negative in sandwich EIA (A. Didier, in preparation). As the indirect EIA is not as sensitive as the sandwich, NheB production of this strain most likely is higher than detected with our tools.

The toxin pattern of the three Hbl components was similar to Nhe, i.e., strains earlier classified as *high* showed high amounts of all Hbl components, *low* strains showed lower Hbl production (**Figures 3B–D**). Interestingly, the two strains 6/27/S and F3175/03 (D7) showed similar toxic activity (**Table 1** and **Figure 6**), but a different pattern. While 6/27/S produced comparably high amounts of Hbl L1 but low amounts of L2 and B, F3175/03 (D7) showed a comparably high titer and productivity for Hbl L2 but low L1 and B. Strain F528/94 showed similar Hbl titers compared to strain F837/76, though classified as a pair of low and high toxicity strains of the same genetic

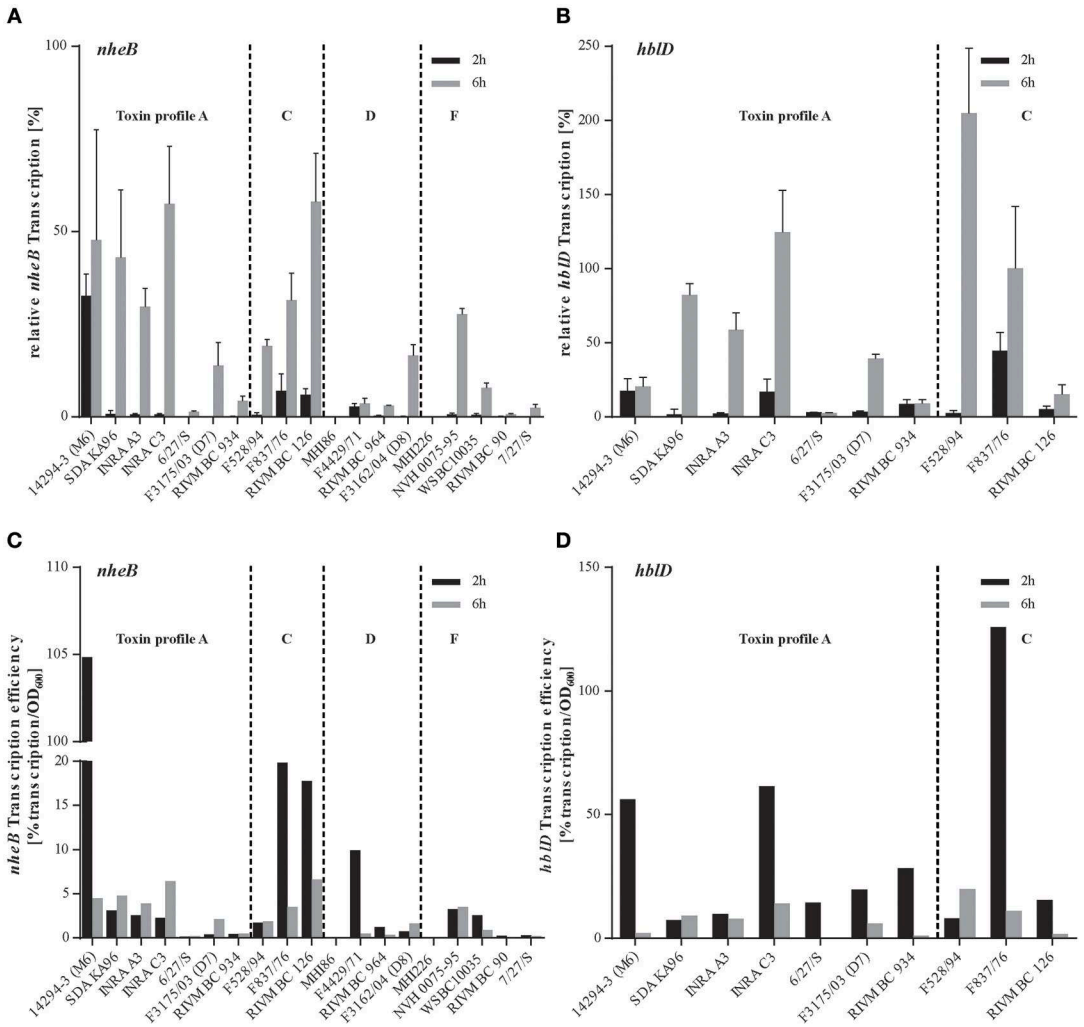


FIGURE 2 | Analyses of relative toxin gene transcription and transcription efficiency of the *B. cereus* strain set using qRT-PCR and the $2^{-\Delta\Delta C_T}$ method. Total RNA was purified from 19 *B. cereus* strains harvested 2 (black) and 6 (gray) h after inoculation in CGY medium. Levels of *nheB* **(A)** and *hblD* **(B)** transcript were determined by qRT-PCR, normalized to 16S *rrn* levels of the same sample and relative to the transcript level of an external calibrator. The expression level of *hblD* of the toxin reference strain *B. cereus* F837/76 at 6 h served as calibrator that was set to 100% (log-2 = 0), all other transcript levels (A and B) were compared to this condition using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Transcription efficiency was calculated as the ratio of mean relative transcript level of *nheB* **(C)** and *hblD* **(D)** in % per mean optical density of each strain (compare **Figure 1**).

toxin profile (**Table 1**), suggesting that the total toxic activity as determined by WST-1-bioassays on Vero cells depends on Nhe rather than Hbl production. As for NheB, no specific pattern of Hbl production was found among the toxin profiles or food and food poisoning strains. NheB and Hbl L1 titers were compared to the transcription levels of their corresponding genes *nheB* and *hblD* using the Spearman correlation test. Significant dependence (P values < 0.05) of secreted NheB on relative *nheB* transcript level could be demonstrated for all time points investigated. Spearman correlation coefficient R was 0.817 (2 vs. 2 h), 0.605 (6 vs. 6 h) and 0.600 for comparing 2 h transcription with 6 h toxin titers. Poor influence ($P > 0.05$) of *hblD* transcription on Hbl L1 titer was detected.

Comparable Protein Secretion Ability

To analyze whether enterotoxin production generally correlates with the amount of secreted proteins, total extra- and intracellular protein concentrations were quantified. Intracellular protein concentrations were similar among all isolates tested (data not shown). Extracellular protein contributed to up to 10% of total protein content. 2 h after inoculation, only few extracellular protein concentrations were detectable (**Figure 4A**), while extracellular proteins of about 20–60 ng/μl were isolated after 6 h of growth under the standard laboratory conditions. For MHI226 total extracellular protein concentrations of 90 ng/μl were found. Extracellular protein content was normalized to the optical density of the strains to calculate the efficiency of protein secretion. As depicted in **Figure 4B**, all strains,

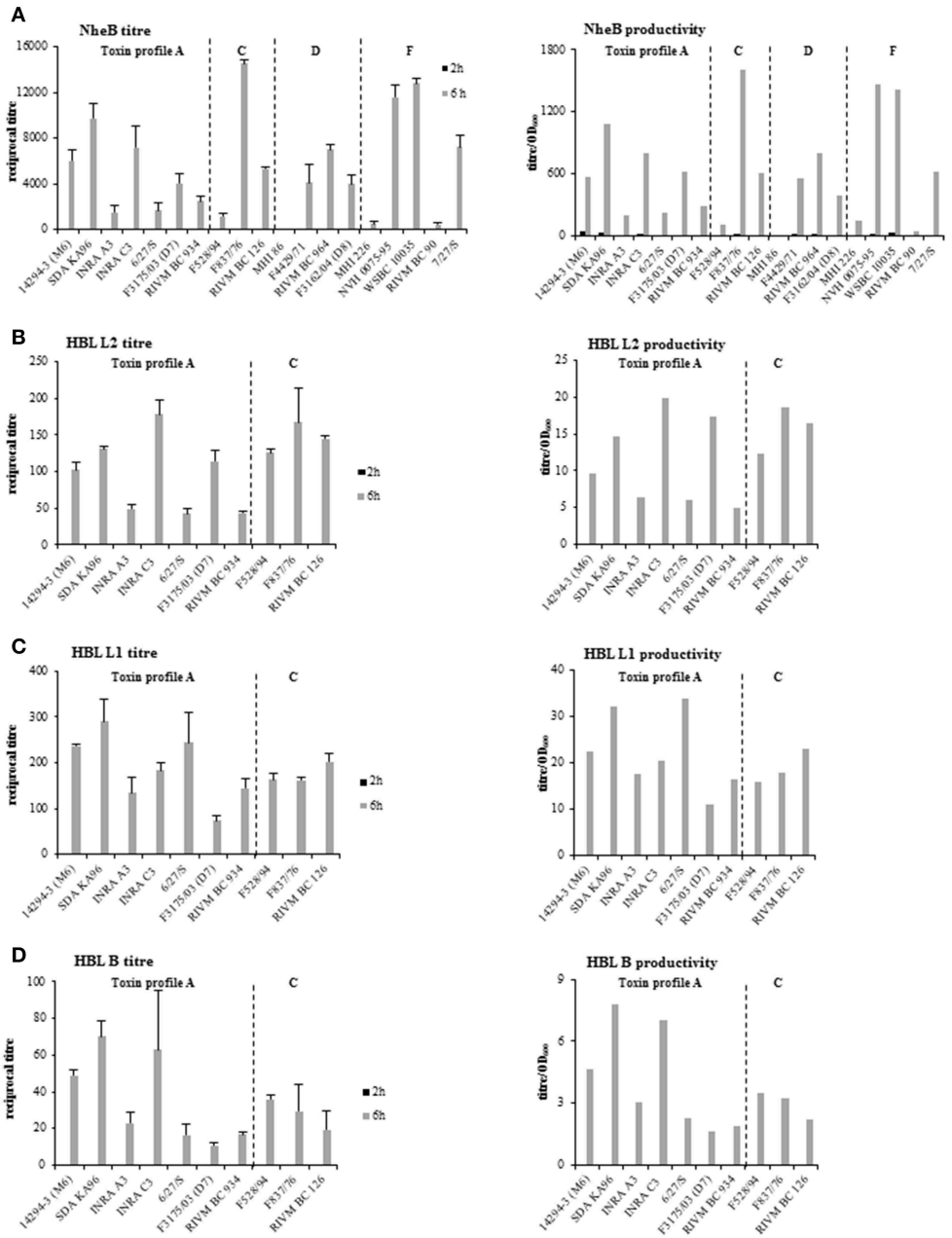


FIGURE 3 | Enterotoxin production of the *B. cereus* strain set as determined by sandwich and indirect EIAs. Reciprocal titres as well as the productivity (titre/OD_{600}) are shown. **(A)** NheB. **(B)** Hbl L2. **(C)** Hbl L1. **(D)** Hbl B.

except MHI226, showed comparable values. Generally, secretion efficiency was higher after 6 h of growth than at early growth phase. Only isolates 14294-3 (M6), F528/94 and NVH0075-95 revealed comparable efficiency values at 2 and 6 h. However, no significant differences in extracellular protein concentrations or efficiency of protein secretion were found between low and high toxic strains.

Exoprotein Profiling by DIGE Analysis

To study secretion patterns of selected *B. cereus* isolates, secreted proteins were isolated 6 h after inoculation and analyzed by SDS-PAGE. Extracellular protein extracts of high and low toxin producing strains of the same genetic toxin profile were labeled with fluorescent Cyanine Dyes and separated in one single lane. Analysis of exoprotein profiles revealed distinct differences between the *B. cereus* isolates (**Figure 5**). Various proteins were differentially regulated in high and low toxic strains. However, secretion patterns of *B. cereus* isolates show high variability between the strains and no marker bands specific for high or low toxic strains could be detected by DIGE analysis. Silver staining of SDS-PAGE analyzed exoprotein extracts of the remaining 9 strains

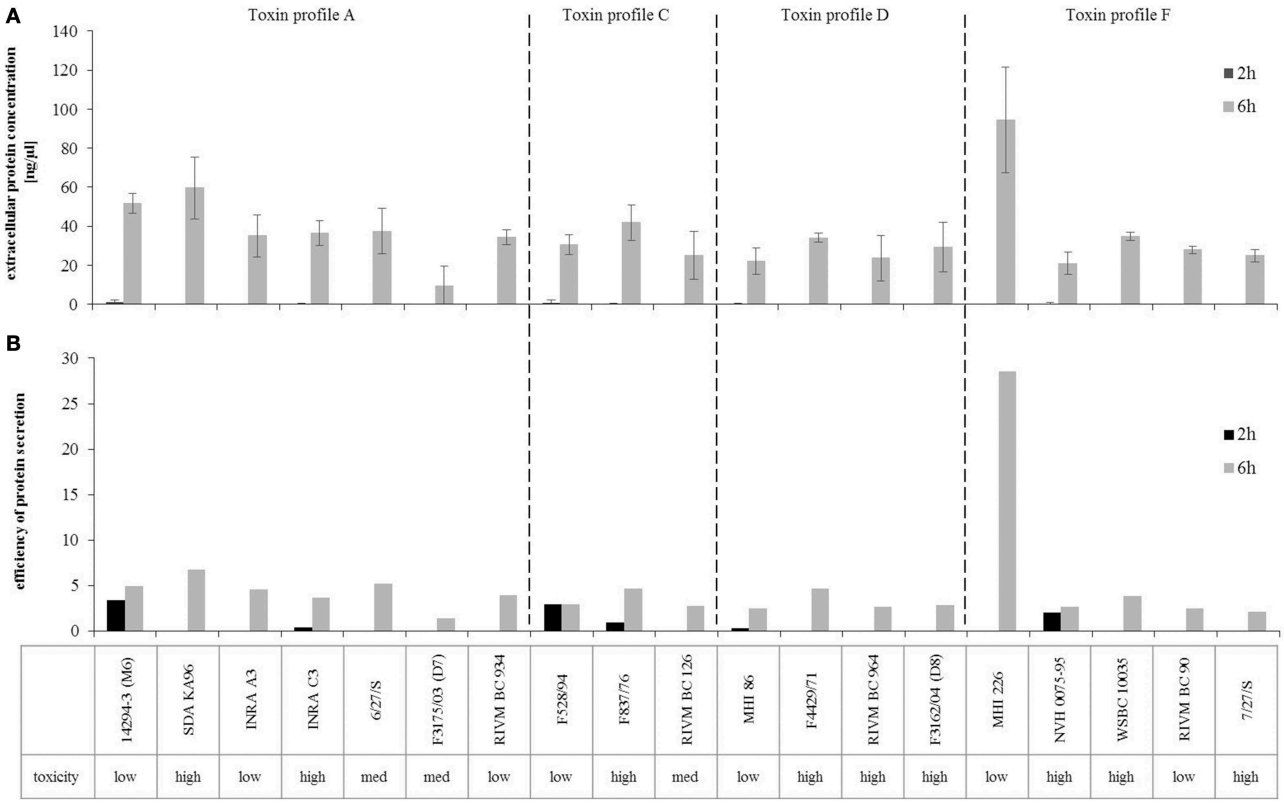


FIGURE 4 | Extracellular protein concentrations and secretion efficiency of the *B. cereus* strain set. (A) Extracellular protein was quantified 2 and 6 h after inoculation. **(B)** To determine the efficiency of protein secretion, extracellular protein concentrations were normalized to the optical density (OD_{600}) of the strains.

confirmed the high variability of secretion patterns (data not shown).

Strain-specific Cytotoxic Activity

Cytotoxicity assays were performed using the human colon carcinoma cell line CaCo-2 instead of the routinely used Vero cells. As a consequence, the classification as high, medium or low toxic (**Table 1**) was altered. 14294-3 (M6), RIVM BC 934 and MHI226, former low toxic, now showed relatively high cytotoxicity in comparison with all strains. On the other hand, for INRA C3 and F4429/71, former highly toxic strains, comparably low cytotoxicity titers were obtained (**Figure 6A**). Of special interest were the strains F837/76 and F528/94 (high and low toxic, sharing the same genetic enterotoxin profile). Although F837/76 showed 12 x higher NheB production than F528/94 (**Figure 3A**), host cell toxicity was only 1.8 x increased (**Figure 6A**). Hbl titers were similar in both strains (**Figures 3B–D**), underlining that CaCo-2 cells are more sensitive to Hbl than Nhe. Our data indicate that results of the WST-1 bioassay depend very much on the target cell line and that for studying the toxic potential of enteropathogenic *B. cereus* in the consumer human colon cell lines such as CaCo-2 must strictly be used to avoid underestimation of certain strains.

Additionally, propidium iodide influx tests were performed to determine the speed of pore formation by the enterotoxins. Strains were compared according to the speed of PI influx shown as the maximum linear slope of the fluorescence curves (**Figure 6B**). Compared to the WST-1 bioassay, almost no differences between high and low toxic strains appeared, whereas strains producing Nhe and Hbl caused a significantly faster PI influx than solely Nhe producing strains. This phenomenon, as observed before (Jeßberger et al., 2014), suggests that pore formation in CaCo-2 cell membranes is triggered by the presence of Hbl and additionally that even low amounts of Hbl cause rapid PI influx.

Discussion

So far, the reasons why *B. cereus* strains with identical genetic toxin profile produce such variable amounts of enterotoxins are not understood, i.e., it is yet to be investigated at which levels the varying pathogenic potential originates and is regulated. Moreover, it is unclear, whether further markers (proteins or nucleotides) can be identified for the differentiation between high and low toxic strains. To our knowledge, the presented study is the first comprehensive analysis of a representative set of enteropathogenic *B. cereus* strains that integrates many levels of toxin formation, from gene sequence to host cell cytotoxicity.

Strain Set

Currently, the most reliable method for predicting the toxic potential of enteropathogenic *B. cereus* is the quantification of the

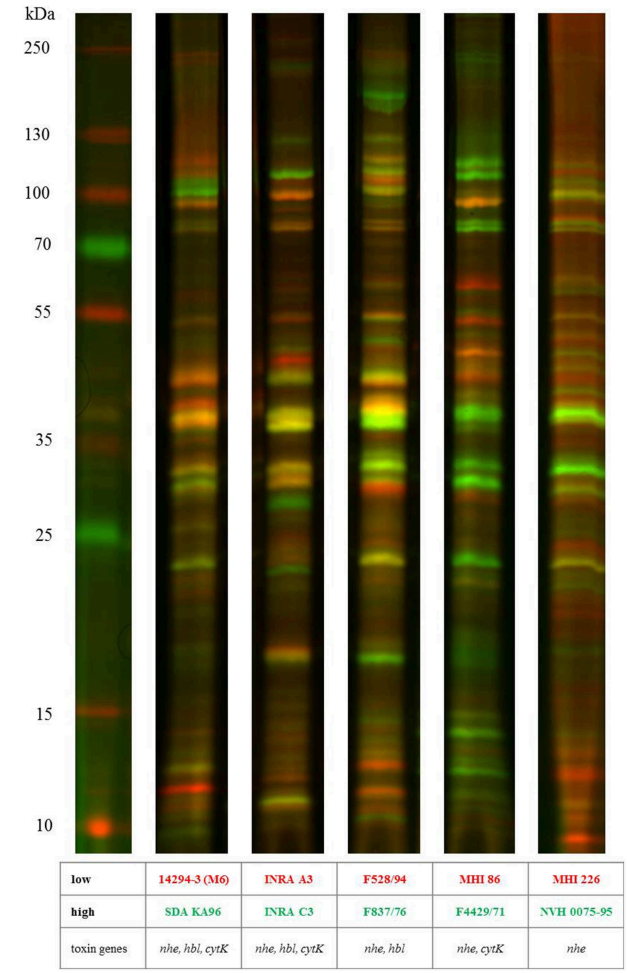


FIGURE 5 | 1D DIGE exoprotein profiling of selected *B. cereus* isolates. Exoproteins were isolated 6 h after inoculation and precipitated by trichloroacetic acid overnight. Five microgram of protein extracts were labeled with CyDye Fluor minimal dyes and separated by SDS PAGE. Proteins present in low toxin producing strains are shown in red (Cy5), proteins present in high toxin producing strains are displayed in green (Cy3). Similar protein levels are indicated by yellow bands.

secreted enterotoxins (Guinebretière et al., 2002; Moravek et al., 2006). This can also be seen in our strain set, which can be divided into "pairs" of high and low toxic strains with identical genetic background. For example, INRA A3 and INRA C3, both clade II, toxin profile A, *hlyII*−, *inhA1*+, *nprA*+, share over 99% and 98% sequence similarity for Nhe and Hbl, but low vs. high amounts of the enterotoxin components are found in the respective culture supernatant. This was also observed for F528/94 (low) and F837/76 (high), both clade I, toxin profile C, *hlyII*+, *inhA1*+, *nprA*+, with 96% and 97% sequence similarity for Nhe and Hbl, respectively. The Nhe toxin components of MHI86 (low) and F4429/71 (high) (clade I, profile D, *hlyII*−, *inhA1*+, *nprA*+) even were 100% identical. So far, no connection between a certain enterotoxin gene profile, i.e., the presence of certain enterotoxin genes, and the toxic potential was found (Ceuppens et al., 2013). Even the presence of the PlcR/PapR regulatory system is no reliable indication for toxic activity (Fagerlund et al., 2007). Our data suggest that cytotoxicity of *B. cereus* is rather independent of toxin gene presence and sequence polymorphisms.

Enterotoxin Gene Transcription

In a first systematic approach we compared enterotoxin gene expression of a representative *B. cereus* strain set. Previous studies compared enterotoxin expression in single *B. cereus* strains under varying growth temperature (Van Netten et al., 1990; Fermanian et al., 1997; Rejasse et al., 2012), oxygen availability (Duport et al., 2004; Van Der Voort and Abee, 2009), lowered oxidation-reduction potential (ORP) (Duport et al., 2006; Zigha et al., 2007; Esbelin et al., 2009) and differing availability of nutrients, for instance sugars (Ouhib et al., 2006; Ouhib-Jacobs et al., 2009). In our study, quantification of *nheB* and *hblD* mRNA levels at early exponential and late exponential growth phase/transition phase by qRT-PCR revealed highly strain-dependent variations in toxin gene transcript levels and transcription efficiency. It was further shown that toxin production is only to a certain extent determined by the level of enterotoxin transcription, which suggests that further posttranscriptional and posttranslational processes are involved.

The global virulence regulator PlcR strongly induces transcription of all enterotoxin genes during entry into the stationary growth phase (Gohar et al., 2008). PlcR-driven toxin gene expression might continue until *B. cereus* faces nutrient limitation at late stationary growth and undergoes sporulation, while at the same time *plcR* transcription is repressed by the transition state regulator Spo0A (Lereclus et al., 2000). However, in the present study transcription of *nheB* and *hblD* could be detected as early as 2 h after inoculation, and increased markedly during exponential growth until 6 h. These results coincide with the findings of Frenzel et al. (2012) on high *plcR* transcript levels during early *B. cereus* growth phase and reemphasize the direct positive transcriptional regulation of the enterotoxin operons by PlcR (Frenzel et al., 2012).

Comparison of the PlcR regulator proteins of our strain set revealed minor differences, especially in the less conserved C-terminal part of the protein (data not shown), which is also truncated in *B. anthracis* (Agaisse et al., 1999; Gohar et al., 2005). With the exception of one strain, PlcR of all *B. cereus* strains investigated shared between 87 and 100% similarity. Transcription analysis of the *plcR* regulator gene for a subset of *B. cereus* strains resulted in less pronounced strain-specificity compared to toxin gene transcription, but demonstrated similar *plcR* transcription kinetics for toxin profile A/C and D/F. Generally, no enhanced *plcR* transcript levels could be measured for *B. cereus* isolates classified as high in contrast to low toxic strains, except for the pair MHI86 (low) and F4429/71 (high) (Figure S4). Furthermore, no correlation between *plcR* transcription and *nheB* or *hblD* transcript levels could be determined. However, another pattern for differentiation of isolates was found: all four strains of toxin profile A and C showed increased *plcR* transcription after 6 h compared to 2 h, while the remaining six strains of toxin profile D and F transcribed lower amounts of *plcR* and tend to decrease *plcR* transcription at the later growth stage (Figure S4).

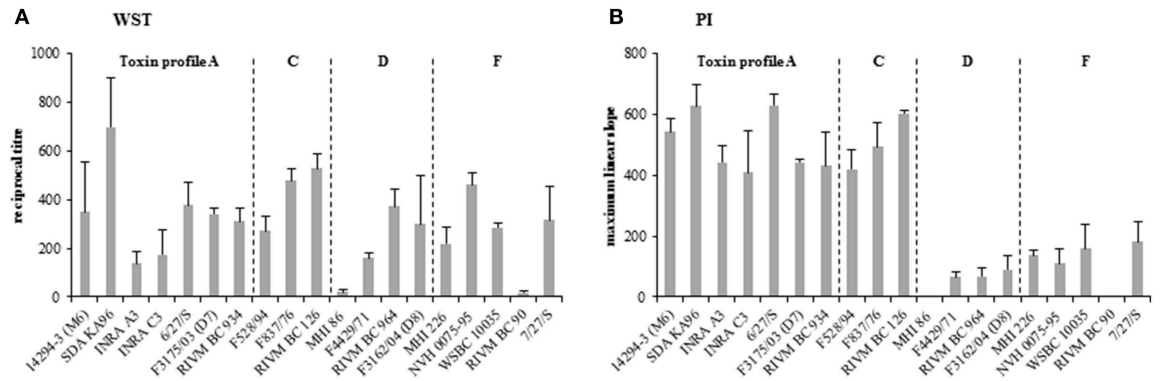


FIGURE 6 | Analysis of the toxic potential of the *B. cereus* strain set. (A) Results of a WST-1 bioassay are shown as reciprocal titres. **(B)** Results of a propidium iodide influx test. The maximum linear slope of the fluorescence curve of each strain is shown. As after 2 h of bacterial growth no cytotoxicity was detected, only the toxicity after 6 h of growth is shown.

The present study focused on the comparison of a *B. cereus* strain set under identical aerobic culture conditions. Hence, induction of environment-sensing regulator proteins Fnr, ResDE, CcpA or CodY (Duport et al., 2006; Zigha et al., 2007; Van Der Voort et al., 2008; Esbelin et al., 2009, 2012; Messaoudi et al., 2010) was assumed to be comparable between the strains, their impact on differential toxin gene expression being negligible. Furthermore, since Fnr, ResDE, CcpA, and CodY protein sequences are highly conserved (sequence similarity > 98% and identity > 96%, data nor shown), variations in functionality are unlikely to account for strain-specific differences in toxin gene activation. Also, comparison of the *nheABC* and *hblCDAB* operon promoter regions of the strain set revealed only minor DNA sequence differences between essential promoter elements and regulator binding sites, precluding mutations that could explain single strain *nheB* or *hblD* transcript levels (data not shown).

Enterotoxin gene transcription only partially reflects our isolates' actual toxicity, indicating posttranscriptional and/or translational regulation. Alternative regulation of gene expression by non-coding RNA molecules is well-known in bacteria. RNAs might act as antisense RNA or form alternative structures, so called riboswitches, that promote or inhibit target gene translation in response to small molecule binding or temperature changes (for review see Johansson and Cossart, 2003; Batey, 2006). It has been demonstrated that several virulence determinants of the Gram-positive pathogen *Listeria monocytogenes* are controlled posttranscriptionally by long 5′ untranslated RNAs (Wong et al., 2004; Shen and Higgins, 2005; Loh et al., 2006). Involvement of stabilized mRNA transcripts in the regulation of *cryIIIA* toxin gene expression has been shown in *B. thuringiensis* and more recently gene control by riboswitches has been characterized in *B. anthracis* and *B. subtilis* (Welz and Breaker, 2007; Irnov et al., 2010; Wilson-Mitchell et al., 2012). The observation of unusually long 5′ untranslated regions (UTR) upstream of the start codons of both enterotoxin operons that span over 600 bp for P_{hbl} and 300 bp for P_{nhe} (data not shown; see also Agaisse et al., 1999) may point to a role in posttranscriptional regulation via formation of regulatory mRNA structures. Hence, deciphering a possible function of the long 5′ UTRs in enterotoxin expression of *B. cereus* is subject to ongoing investigations.

Protein Secretion

All 19 *B. cereus* strains included in this study were capable of significant protein secretion. Under laboratory growth conditions all strains showed comparable extracellular protein concentrations and no differentiation between high and low toxicity strains were obvious. Thus, a defect in general protein secretion can be excluded as a potential cause for the low toxicity of certain strains.

B. cereus enterotoxins are most likely secreted via the *sec* translocation pathway (Tjalsma et al., 2004; Fagerlund et al., 2010; Vörös et al., 2014). Complete amino acid sequence comparisons performed in our study showed that Sec-type secretion signal peptides of all Nhe and Hbl enterotoxin components, as predicted using the SignalP method, are highly conserved, for NheB and Hbl L1 even up to 100% (Figures S2, S3). Especially, signal peptidase cleavage sites are identical with the exception of NheA, where 14294-3 (M6) showed an exchange of the basic amino acid lysine against the negatively charged, hydrophilic amino acid residue glutamate K27E (Figure S2A). It has already been shown that a modification of the signal peptide sequence of Hbl B within the hydrophobic region leads to loss of secretion and intracellular accumulation of the protein (Fagerlund et al., 2010). In our study, most amino acid exchanges could be found in the signal peptide sequence of NheC and Hbl B. While most changes are neutral for the charge of the signal peptide sequences of Nhe and Hbl, some substitutions of the neutral alanine residue to threonine render the hydrophobic region more hydrophilic: A19T (NheB), A12T (NheC), A21T (Hbl B) (Figures S2B, S2C, S3C). However, no correlation of signal peptide mutations with high and low toxicity was found. Only the signal peptide sequence of Hbl B in the highly toxic isolate RIVM BC 126 showed several amino acid residue substitutions that might explain why high levels of Hbl L1 and Hbl L2 were detected in the secretome of RIVM BC 126, but only relatively low amounts of Hbl B (**Figure 3**).

Additionally, we compared extra- and intracellular amounts of NheB and Hbl L1 to exclude potential defects in the secretory mechanisms of our strains. Though taken in an independent approach, NheB and Hbl L1 titers in the culture supernatants were comparable to those in **Figure 3**. No or only trace amounts of NheB and Hbl L1 were detectable in the cytoplasm (data not shown), suggesting that the enterotoxins of all strains are secreted immediately and effectively after translation. Furthermore, intracellular titers rose in comparison to extracellular titers after treatment of the strains with sodium azide (data not shown), which inhibits the ATPase function of SecA (according to Fagerlund et al., 2010). This indicates that the enterotoxins of all 19 strains are indeed secreted via a fully functional Sec translocation pathway.

Besides enterotoxins, *B. cereus* secretes a variety of other proteins such as proteases, phospholipases and other members of the PlcR regulon (Gohar et al., 2008). For *B. anthracis* it has been shown that own bacterial proteases contribute to virulence by directly degrading anthrax toxins or modulating concentrations and activity of other extracellular proteases (Pflughoeft et al., 2014). The proteases InhA1 and NprX can represent up to 90% of the bacterium's secretome (Chitlaru et al., 2006). The concentrations of the respective proteins in the secretome of the *B. cereus* strain set cannot be detected so far, as suitable detection methods (e.g., EIAs based on specific antibodies) still have to be developed. We suppose it may rather be possible that the low toxic strains generally secrete higher levels of proteases, which subsequently degrade the secreted enterotoxins, than secreting low amounts of enterotoxins and proteases. Enterotoxin gene expression of strain MHI226 was hardly detectable, but cytotoxic activity on CaCo-2 cells was comparably high. Perhaps, the lack of enterotoxin production was compensated by enhanced protein secretion. Generally, MHI226 showed quite extraordinary properties concerning growth, enterotoxin gene expression, toxicity and especially protein secretion, thus it might be a suitable candidate to further study the relationship of protein secretion and toxicity in *B. cereus*.

Enterotoxin Production and Toxicity

Enterotoxin titers generally confirmed the previous classification of high, medium and low toxin producing strains. Comparison of F3162/04 (D8) with the other strains must be done with caution, as NheB titers were obtained in indirect EIA using only mab 1E11. In preliminary experiments, this strain showed low NheB production but in comparison to that unusually high cytotoxicity (**Table 1**, Table S1). Sequence analyses revealed a point mutation in the *nheB* gene resulting in the exchange of amino acid residue 151 from glutamic to aspartic acid. This residue is situated in direct proximity to the predicted epitope of mab 2B11, which is used in sandwich EIAs for NheB detection (Didier et al., 2012). This mutation might contribute to a loss of 2B11 binding capacity and thus, detection of the NheB protein, but without affecting its functionality (A. Didier, in preparation). During selection of our strain set, three strains (F3162/04 (D8), MHI 124 and HWW 274-1) out of 136 showed this phenomenon, implying that the toxic potential of 2.2% of all enteropathogenic *B. cereus* isolates might be underestimated when NheB production is investigated with sandwich EIAs based on mab 2B11.

Pearson correlation tests showed significant correlation of cytotoxicity, obtained with WST-1 bioassays on CaCo-2 cells, and NheB titers after 6 h of growth (correlation coefficient 0.61). The same WST test was performed on Vero cells (data not shown), with which NheB titers correlated even better (correlation coefficient 0.84). This confirmed prior observations that cytotoxicity depends significantly on NheB production and that Vero cells are even more sensitive, particularly toward Nhe and generally to *B. cereus* culture supernatants than CaCo-2 cells (Moravek et al., 2006; Jeßberger et al., 2014). Nevertheless, CaCo-2 cells should be used rather than Vero cells to investigate the cytotoxic activity, as *B. cereus* enterotoxins operate rather in the human intestine than in kidney. Furthermore, discrepancies between high and low toxic strains are bigger on Vero cells, which might lead to underestimation of putative non-pathogenic strains. Contrary to earlier studies (Jeßberger et al., 2014) no significant correlation between Hbl production and cytotoxicity was found, possibly due to the small number of strains investigated (10 *hbl* positive strains). We further observed that for uncovering the varying toxic potential of different *B. cereus* isolates the choice of cytotoxicity assay is very important. While differences between high and low toxic strains of our strain set became obvious in WST-1 bioassays, these differences were no longer detectable when PI influx tests were performed (**Figure 6**). The 10 strains expressing Nhe and Hbl showed comparably quick PI influx and thus pore formation while the 9 strains expressing only Nhe showed only poor PI influx. This supports the assumption that, although high sequence homologies between the Nhe and the Hbl components exist (Granum et al., 1999), the mode of action and the way of pore assembly and formation of these two enterotoxin complexes differs significantly.

Conclusion

In summary, our data suggest that the highly variable toxic potential of *B. cereus* is determined by complex and dynamic regulatory processes involving toxin gene transcription strictly regulated by known regulator proteins as well as strain-specific posttranscriptional or posttranslational toxin modifications affecting mRNA stability, translation initiation or protein durability and, perhaps, resistance to extracellular proteolytic degradation. Furthermore, high and low toxic strains could not be correlated with certain groups such as food or food poisoning isolates or enterotoxin gene profiles. Also, the presence of genes encoding further putative virulence factors such as hemolysin II, sphingomyelinase or the metalloproteases InhA1 and NprA provided no further information to predict high toxicity in *B. cereus* strains.

Besides these intrinsic bacterial factors, the condition of the host (age, immunity, etc.) as well as the composition of the contaminated food may contribute to the high variability of enterotoxin production (Ceuppens et al., 2013), which makes the toxic potential even more difficult to assess and to predict. In this study, using standard laboratory growth conditions which are routinely applied for the assessment of *B. cereus* toxicity,

no distinct bacterial factors or mechanisms responsible for the highly variable enterotoxin production and thus cytotoxic potential were found. Therefore, the question arises whether enterotoxin production in the intestine is triggered or activated by environmental factors and whether potentially high and low toxic strains respond differentially to such factors. We showed that the pattern of high and low toxic strains was already altered when an alternative target cell line was used in cytotoxicity assays (CaCo-2 instead of Vero). It may be speculated that this pattern is further influenced by growth of bacteria under simulated intestinal instead of standard laboratory conditions or in the presence of colon epithelial cells.

Acknowledgments

This research project was supported by the German Ministry of Economics and Energy (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 17506 N. We thank Scharifa Bornschier, Christine Braig, Romy Wecko, Tatjana Svoboda and Cornelia Berger for excellent technical assistance.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00560/abstract

References

Agaisse, H., Gominet, M., Okstad, O. A., Kolsto, A. B., and Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol. Microbiol.* 32, 1043–1053. doi: 10.1046/j.1365-2958.1999.01419.x

Andersson, A., Ronner, U., and Granum, P. E. (1995). What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and Clostridium perfringens? *Int. J. Food Microbiol.* 28, 145–155. doi: 10.1016/0168-1605(95)00053-4

Anonymous. (2013). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks 2011. *EFSA J.* 11:3129. doi: 10.2903/j.efsa.2013.3129

Batey, R. T. (2006). Structures of regulatory elements in mRNAs. *Curr. Opin. Struct. Biol.* 16, 299–306. doi: 10.1016/j.sbi.2006.05.001

Beecher, D. J., Schoeni, J. L., and Wong, A. C. (1995). Enterotoxic activity of hemolysin BL from *Bacillus cereus*. *Infect. Immun.* 63, 4423–4428.

Beecher, D. J., and Wong, A. C. (2000). Tripartite haemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. *Microbiology* 146(Pt 6), 1371–1380.

Cadot, C., Tran, S. L., Vignaud, M. L., De Buyser, M. L., Kolsto, A. B., Brisabois, A., et al. (2010). InhA1, NprA, and HlyII as candidates for markers to differentiate pathogenic from nonpathogenic *Bacillus cereus* strains. *J. Clin. Microbiol.* 48, 1358–1365. doi: 10.1128/JCM.02123-09

Candelon, B., Guilloux, K., Ehrlich, S. D., and Sorokin, A. (2004). Two distinct types of rRNA operons in the Bacillus cereus group. *Microbiology* 150, 601–611. doi: 10.1099/mic.0.26870-0

Ceuppens, S., Boon, N., Rajkovic, A., Heyndrickx, M., Van De Wiele, T., and Uyttendaele, M. (2010). Quantification methods for Bacillus cereus vegetative cells and spores in the gastrointestinal environment. *J. Microbiol. Methods* 83, 202–210. doi: 10.1016/j.mimet.2010.09.009

Ceuppens, S., Boon, N., and Uyttendaele, M. (2013). Diversity of Bacillus cereus group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiol. Ecol.* 84, 433–450. doi: 10.1111/1574-6941.12110

Ceuppens, S., Uyttendaele, M., Drieskens, K., Rajkovic, A., Boon, N., and Wiele, T. V. (2012). Survival of *Bacillus cereus* vegetative cells and spores during *in vitro* simulation of gastric passage. *J. Food Prot.* 75, 690–694. doi: 10.4315/0362-028X.JFP-11-481

Chitlaru, T., Gat, O., Gozlan, Y., Ariel, N., and Shafferman, A. (2006). Differential proteomic analysis of the *Bacillus anthracis* secretome: distinct plasmid and chromosome CO2-dependent cross talk mechanisms modulate extracellular proteolytic activities. *J. Bacteriol.* 188, 3551–3571. doi: 10.1128/JB.188.10.3551-3571.2006

Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C., and Schmitt, P. (2004). Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. *J. Appl. Microbiol.* 97, 214–219. doi: 10.1111/j.1365-2672.2004.02292.x

Didier, A., Dietrich, R., Gruber, S., Bock, S., Moravek, M., Nakamura, T., et al. (2012). Monoclonal antibodies neutralize *Bacillus cereus* Nhe enterotoxin by inhibiting ordered binding of its three exoprotein components. *Infect. Immun.* 80, 832–838. doi: 10.1128/IAI.05681-11

Dietrich, R., Fella, C., Strich, S., and Märtlbauer, E. (1999). Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*. *Appl. Environ. Microbiol.* 65, 4470–4474.

Dietrich, R., Moravek, M., Burk, C., Granum, P. E., and Märtlbauer, E. (2005). Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. *Appl. Environ. Microbiol.* 71, 8214–8220. doi: 10.1128/AEM.71.12.8214-8220.2005

Doll, V. M., Ehling-Schulz, M., and Vogelmann, R. (2013). Concerted action of sphingomyelinase and non-hemolytic enterotoxin in pathogenic *Bacillus cereus*. *PLoS ONE* 8:e61404. doi: 10.1371/journal.pone.0061404

Dommel, M. K., Frenzel, E., Strasser, B., Blochinger, C., Scherer, S., and Ehling-Schulz, M. (2010). Identification of the main promoter directing cereulide biosynthesis in emetic *Bacillus cereus* and its application for real-time monitoring of ces gene expression in foods. *Appl. Environ. Microbiol.* 76, 1232–1240. doi: 10.1128/AEM.02317-09

Duport, C., Thomassin, S., Bourel, G., and Schmitt, P. (2004). Anaerobiosis and low specific growth rates enhance hemolysin BL production by *Bacillus cereus* F4430/73. *Arch. Microbiol.* 182, 90–95. doi: 10.1007/s00203-004-0688-y

Duport, C., Zigha, A., Rosenfeld, E., and Schmitt, P. (2006). Control of enterotoxin gene expression in *Bacillus cereus* F4430/73 involves the redox-sensitive ResDE signal transduction system. *J. Bacteriol.* 188, 6640–6651. doi: 10.1128/JB.00702-06

Dzieciol, M., Fricker, M., Wagner, M., Hein, I., and Ehling-Schulz, M. (2013). A novel diagnostic real-time PCR assay for quantification and differentiation of emetic and non-emetic *Bacillus cereus*. *Food Control* 32, 176–185. doi: 10.1016/j.foodcont.2012.11.010

Ehling-Schulz, M., Fricker, M., and Scherer, S. (2004). *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. *Mol. Nutr. Food Res.* 48, 479–487. doi: 10.1002/mnfr.200400055

Ehling-Schulz, M., Guinebretière, M. H., Monthan, A., Berge, O., Fricker, M., and Svensson, B. (2006). Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. *FEMS Microbiol. Lett.* 260, 232–240. doi: 10.1111/j.1574-6968.2006.00320.x

Ehling-Schulz, M., and Messelhäusser, U. (2013). Bacillus "next generation" diagnostics: moving from detection toward subtyping and risk-related strain profiling. *Front. Microbiol.* 4:32. doi: 10.3389/fmicb.2013.00032

Ehling-Schulz, M., Svensson, B., Guinebretière, M. H., Lindbäck, T., Andersson, M., Schulz, A., et al. (2005a). Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology* 151, 183–197. doi: 10.1099/mic.0.27607-0

Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Märtlbauer, E., et al. (2005b). Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide

production in emetic *Bacillus cereus*. *Appl. Environ. Microbiol.* 71, 105–113. doi: 10.1128/AEM.71.1.105-113.2005

Esbelin, J., Armengaud, J., Zigha, A., and Duport, C. (2009). ResDE-dependent regulation of enterotoxin gene expression in *Bacillus cereus*: evidence for multiple modes of binding for ResD and interaction with Fnr. *J. Bacteriol.* 191, 4419–4426. doi: 10.1128/JB.00321-09

Esbelin, J., Jouanneau, Y., and Duport, C. (2012). *Bacillus cereus* Fnr binds a [4Fe-4S] cluster and forms a ternary complex with ResD and PlcR. *BMC Microbiol.* 12:125. doi: 10.1186/1471-2180-12-125

Fagerlund, A., Brillard, J., Furst, R., Guinebretière, M. H., and Granum, P. E. (2007). Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group. *BMC Microbiol.* 7:43. doi: 10.1186/1471-2180-7-43

Fagerlund, A., Lindbäck, T., and Granum, P. E. (2010). *Bacillus cereus* cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. *BMC Microbiol.* 10:304. doi: 10.1186/1471-2180-10-304

Fermanian, C., Lapeyre, C., Fremy, J. M., and Claisse, M. (1997). Diarrhoeal toxin production at low temperature by selected strains of *Bacillus cereus*. *J. Dairy Res.* 64, 551–559. doi: 10.1017/S0022029997002379

Frenzel, E., Doll, V., Pauthner, M., Lücking, G., Scherer, S., and Ehling-Schulz, M. (2012). CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits. *Mol. Microbiol.* 85, 67–88. doi: 10.1111/j.1365-2958.2012.08090.x

Fricker, M., Messelhäusser, U., Busch, U., Scherer, S., and Ehling-Schulz, M. (2007). Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Appl. Environ. Microbiol.* 73, 1892–1898. doi: 10.1128/AEM.02219-06

Gohar, M., Faegri, K., Perchat, S., Ravnum, S., Okstad, O. A., Gominet, M., et al. (2008). The PlcR virulence regulon of *Bacillus cereus*. *PLoS ONE* 3:e2793. doi: 10.1371/journal.pone.0002793

Gohar, M., Gilois, N., Graveline, R., Garreau, C., Sanchis, V., and Lereclus, D. (2005). A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. *Proteomics* 5, 3696–3711. doi: 10.1002/pmic.200401225

Granum, P. E., O'sullivan, K., and Lund, T. (1999). The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiol. Lett.* 177, 225–229. doi: 10.1111/j.1574-6968.1999.tb13736.x

Guinebretière, M. H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M. L., et al. (2013). Bacillus cytotoxicus sp. nov. is a novel thermotolerant species of the *Bacillus cereus* Group occasionally associated with food poisoning. *Int. J. Syst. Evol. Microbiol.* 63, 31–40. doi: 10.1099/ijs.0.030627-0

Guinebretière, M. H., Broussolle, V., and Nguyen-The, C. (2002). Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *J. Clin. Microbiol.* 40, 3053–3056. doi: 10.1128/JCM.40.8.3053-3056.2002

Guinebretière, M. H., Thompson, F. L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., et al. (2008). Ecological diversification in the *Bacillus cereus* Group. *Environ. Microbiol.* 10, 851–865. doi: 10.1111/j.1462-2920.2007.01495.x

Irnov, I., Sharma, C. M., Vogel, J., and Winkler, W. C. (2010). Identification of regulatory RNAs in Bacillus subtilis. *Nucleic Acids Res.* 38, 6637–6651. doi: 10.1093/nar/gkq454

Jeßberger, N., Dietrich, R., Bock, S., Didier, A., and Märtlbauer, E. (2014). *Bacillus cereus* enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. *Toxicon* 77, 49–57. doi: 10.1016/j.toxicon.2013.10.028

Johansson, J., and Cossart, P. (2003). RNA-mediated control of virulence gene expression in bacterial pathogens. *Trends Microbiol.* 11, 280–285. doi: 10.1016/S0966-842X(03)00118-5

Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S., and Gominet, M. (2000). Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. *Int. J. Med. Microbiol.* 290, 295–299. doi: 10.1016/S1438-4221(00)80024-7

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

Loh, E., Gripenland, J., and Johansson, J. (2006). Control of Listeria monocytogenes virulence by 5'-untranslated RNA. *Trends Microbiol.* 14, 294–298. doi: 10.1016/j.tim.2006.05.001

Lücking, G., Dommel, M. K., Scherer, S., Fouet, A., and Ehling-Schulz, M. (2009). Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator AbrB, but not by the virulence regulator PlcR. *Microbiology* 155, 922–931. doi: 10.1099/mic.0.024125-0

Lund, T., De Buyser, M. L., and Granum, P. E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol.* 38, 254–261. doi: 10.1046/j.1365-2958.2000.02147.x

Lund, T., and Granum, P. E. (1996). Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiol. Lett.* 141, 151–156. doi: 10.1111/j.1574-6968.1996.tb08377.x

Martineau, F., Picard, F. J., Roy, P. H., Ouellette, M., and Bergeron, M. G. (1996). Species-specific and ubiquitous DNA-based assays for rapid identification of *Staphylococcus epidermidis*. *J. Clin. Microbiol.* 34, 2888–2893.

Martinez-Blanch, J. F., Sanchez, G., Garay, E., and Aznar, R. (2009). Development of a real-time PCR assay for detection and quantification of enterotoxigenic members of *Bacillus cereus* group in food samples. *Int. J. Food Microbiol.* 135, 15–21. doi: 10.1016/j.ijfoodmicro.2009.07.013

Messaoudi, K., Clavel, T., Schmitt, P., and Duport, C. (2010). Fnr mediates carbohydrate-dependent regulation of catabolic and enterotoxin genes in *Bacillus cereus* F4430/73. *Res. Microbiol.* 161, 30–39. doi: 10.1016/j.resmic.2009.11.003

Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretière, M. H., Granum, P. E., et al. (2006). Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. *FEMS Microbiol. Lett.* 257, 293–298. doi: 10.1111/j.1574-6968.2006.00185.x

Nam, H., Seo, H. S., Bang, J., Kim, H., Beuchat, L. R., and Ryu, J. H. (2014). Efficacy of gaseous chlorine dioxide in inactivating *Bacillus cereus* spores attached to and in a biofilm on stainless steel. *Int. J. Food Microbiol.* 188, 122–127. doi: 10.1016/j.ijfoodmicro.2014.07.009

Ouhib, O., Clavel, T., and Schmitt, P. (2006). The production of *Bacillus cereus* enterotoxins is influenced by carbohydrate and growth rate. *Curr. Microbiol.* 53, 222–226. doi: 10.1007/s00284-006-0094-6

Ouhib-Jacobs, O., Lindley, N. D., Schmitt, P., and Clavel, T. (2009). Fructose and glucose mediates enterotoxin production and anaerobic metabolism of *Bacillus cereus* ATCC14579(T). *J. Appl. Microbiol.* 107, 821–829. doi: 10.1111/j.1365-2672.2009.04254.x

Petersen, T. N., Brunak, S., Von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30:e36. doi: 10.1093/nar/30.9.e36

Pflughoeft, K. J., Swick, M. C., Engler, D. A., Yeo, H. J., and Koehler, T. M. (2014). Modulation of the *Bacillus anthracis* Secretome by the Immune Inhibitor A1 Protease. *J. Bacteriol.* 196, 424–435. doi: 10.1128/JB.00690-13

Rejasse, A., Gilois, N., Barbosa, I., Huillet, E., Bevilacqua, C., Tran, S., et al. (2012). Temperature-dependent production of various PlcR-controlled virulence factors in *Bacillus weihenstephanensis* strain KBAB4. *Appl. Environ. Microbiol.* 78, 2553–2561. doi: 10.1128/AEM.07446-11

Shen, A., and Higgins, D. E. (2005). The 5' untranslated region-mediated enhancement of intracellular listeriolysin O production is required for Listeria monocytogenes pathogenicity. *Mol. Microbiol.* 57, 1460–1473. doi: 10.1111/j.1365-2958.2005.04780.x

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539. doi: 10.1038/msb.2011.75

Stenfors Arnesen, L. P., Fagerlund, A., and Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32, 579–606. doi: 10.1111/j.1574-6976.2008.00112.x

Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2002). Multiple sequence alignment using ClustalW and ClustalX. *Curr. Protoc. Bioinformatics* Chapter 2, Unit 2 3. doi: 10.1002/0471250953.bi0203s00

Tjalsma, H., Antelmann, H., Jongbloed, J. D., Braun, P. G., Darmon, E., Dorenbos, R., et al. (2004). Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* 68, 207–233. doi: 10.1128/MMBR.68.2.207-233.2004

Tran, S. L., Guillemet, E., Ngo-Camus, M., Clybouw, C., Puhar, A., Moris, A., et al. (2011). Haemolysin II is a *Bacillus cereus* virulence factor that induces

apoptosis of macrophages. *Cell. Microbiol.* 13, 92–108. doi: 10.1111/j.1462-5822.2010.01522.x

Van De Peer, Y., and De Wachter, R. (1997). Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. *Comput. Appl. Biosci.* 13, 227–230. doi: 10.1093/bioinformatics/13.3.227

Van Der Voort, M., and Abee, T. (2009). Transcriptional regulation of metabolic pathways, alternative respiration and enterotoxin genes in anaerobic growth of *Bacillus cereus* ATCC 14579. *J. Appl. Microbiol.* 107, 795–804. doi: 10.1111/j.1365-2672.2009.04252.x

Van Der Voort, M., Kuipers, O. P., Buist, G., De Vos, W. M., and Abee, T. (2008). Assessment of CcpA-mediated catabolite control of gene expression in *Bacillus cereus* ATCC 14579. *BMC Microbiol.* 8:62. doi: 10.1186/1471-2180-8-62

Van Netten, P., Van De Moosdijk, A., Van Hoensel, P., Mossel, D. A., and Perales, I. (1990). Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *J. Appl. Bacteriol.* 69, 73–79. doi: 10.1111/j.1365-2672.1990.tb02913.x

Vörös, A., Simm, R., Slamti, L., Mckay, M. J., Hegna, I. K., Nielsen-Leroux, C., et al. (2014). SecDF as part of the Sec-translocase facilitates efficient secretion of *Bacillus cereus* toxins and cell wall-associated proteins. *PLoS ONE* 9:e103326. doi: 10.1371/journal.pone.0103326

Wehrle, E., Moravek, M., Dietrich, R., Burk, C., Didier, A., and Märtlbauer, E. (2009). Comparison of multiplex PCR, enzyme immunoassay and cell culture methods for the detection of enterotoxinogenic *Bacillus cereus*. *J. Microbiol. Methods* 78, 265–270. doi: 10.1016/j.mimet.2009.06.013

Welz, R., and Breaker, R. R. (2007). Ligand binding and gene control characteristics of tandem riboswitches in *Bacillus anthracis*. *RNA* 13, 573–582. doi: 10.1261/rna.407707

Wijman, J. G., De Leeuw, P. P., Moezelaar, R., Zwietering, M. H., and Abee, T. (2007). Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Appl. Environ. Microbiol.* 73, 1481–1488. doi: 10.1128/AEM.01781-06

Wilson-Mitchell, S. N., Grundy, F. J., and Henkin, T. M. (2012). Analysis of lysine recognition and specificity of the *Bacillus subtilis* L box riboswitch. *Nucleic Acids Res.* 40, 5706–5717. doi: 10.1093/nar/gks212

Wong, K. K., Bouwer, H. G., and Freitag, N. E. (2004). Evidence implicating the 5' untranslated region of Listeria monocytogenes actA in the regulation of bacterial actin-based motility. *Cell. Microbiol.* 6, 155–166. doi: 10.1046/j.1462-5822.2003.00348.x

Zigha, A., Rosenfeld, E., Schmitt, P., and Duport, C. (2007). The redox regulator Fnr is required for fermentative growth and enterotoxin synthesis in *Bacillus cereus* F4430/73. *J. Bacteriol.* 189, 2813–2824. doi: 10.1128/JB.01701-06

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on