CAMPYLOBACTER IN POULTRY: PHYSIOLOGY, GENETICS, AND DETECTION METHODS

EDITED BY: Steven C. Ricke and Michael J. Rothrock

PUBLISHED IN: Frontiers in Microbiology and Frontiers in Nutrition

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-763-6
DOI 10.3389/978-2-83250-763-6

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: frontiersin.org/about/contact

CAMPYLOBACTER IN POULTRY: PHYSIOLOGY, GENETICS, AND DETECTION METHODS

Topic Editors:
Steven C. Ricke, University of Wisconsin-Madison, United States
Michael J. Rothrock, Agricultural Research Service, United States Department of Agriculture (USDA), United States

Citation: Ricke, S. C., Rothrock, M. J., eds. (2022). *Campylobacter* in Poultry: Physiology, Genetics, and Detection Methods. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-763-6

Table of Contents

frontiers in Microbiology

ORIGINAL RESEARCH
published: 02 August 2018
doi: 10.3389/fmicb.2018.01676

Population Structure, Antimicrobial Resistance, and Virulence-Associated Genes in *Campylobacter jejuni* Isolated From Three Ecological Niches: Gastroenteritis Patients, Broilers, and Wild Birds

Yaidelys Iglesias-Torrens[1,2], Elisenda Miró[1], Pedro Guirado[3], Teresa Llovet[1,2], Carmen Muñoz[1,2], Marta Cerdà-Cuéllar[4], Cristina Madrid[3], Carlos Balsalobre[3]* and Ferran Navarro[1,2]*

[1] Hospital de la Santa Creu i Sant Pau, Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), Barcelona, Spain, [2] Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Departament de Genètica, Microbiologia i Estadística, Universitat de Barcelona, Barcelona, Spain, [4] Centre de Recerca en Sanitat Animal (CReSA)-IRTA, Campus de la Universitat Autònoma de Barcelona, Barcelona, Spain

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Ben Pascoe,
University of Bath, United Kingdom
Heriberto Fernandez,
Universidad Austral de Chile, Chile

***Correspondence:**
Elisenda Miró
emiro@santpau.cat
Carlos Balsalobre
cbalsalobre@ub.edu

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

Received: 02 May 2018
Accepted: 04 July 2018
Published: 02 August 2018

Citation:
Iglesias-Torrens Y, Miró E, Guirado P, Llovet T, Muñoz C, Cerdà-Cuéllar M, Madrid C, Balsalobre C and Navarro F (2018) Population Structure, Antimicrobial Resistance, and Virulence-Associated Genes in Campylobacter jejuni Isolated From Three Ecological Niches: Gastroenteritis Patients, Broilers, and Wild Birds. Front. Microbiol. 9:1676. doi: 10.3389/fmicb.2018.01676

Campylobacter jejuni is the causal agent of the food-borne infection with the highest incidence in Europe. Both poultry and wild birds are a major reservoir. To gain insight into the population structure, virulence potential, and antimicrobial resistance (AMR), a collection of 150 isolates from three different ecological niches (broilers, wild birds, and human patients) was studied. Despite the high genetic diversity found, the population structure defined two distinct clusters, one formed mostly by broiler and human isolates and another one by most wild bird isolates. The ST-21 complex exhibits highest prevalence (in humans and broilers), followed by ST-1275 complex (only in wild birds). The ST-48, -45, and -354 complexes were found in all three niches, but represent only 22 out of 150 studied strains. A higher occurrence of AMR and multidrug resistance was detected among broiler and human isolates. Moreover, significant differences were found in the distribution of certain putative virulence genes. Remarkably, many wild bird strains were negative for either *cdtA*, *cdtB*, or *cdtC* from the canonical strain 81-176, whereas all broiler and human strains were positive. These data suggest that the different variants of the *cdt* genes might be relevant for the efficient colonization of certain hosts by *C. jejuni*. Our study contributes to the understanding of the role of the diverse *Campylobacter* reservoirs in the transmission of campylobacteriosis to humans.

Keywords: *Campylobacter jejuni*, PFGE, MLST, antimicrobial resistance, pathogenicity genes

INTRODUCTION

In the last decade, there has been an increase in the incidence of campylobacteriosis worldwide, which is especially significant in North America, Europe, and Australia (Cody et al., 2012; Harvala et al., 2016). This zoonosis is therefore of economic and public health concern (Havelaar et al., 2015). Particularly in the European Union, *Campylobacter* has outnumbered *Salmonella* as the

most commonly reported cause of bacterial diarrheal disease in humans since 2005 (EFSA-ECDC, 2016). *Campylobacter jejuni* followed by *C. coli* are responsible for the vast majority of infections which lead to diarrhea and a range of other serious conditions affecting the gastrointestinal tract, especially in children under 5 years old (Eberle and Kiess, 2012; Kaakoush et al., 2015). Furthermore, two autoimmune neurological disorders, Guillain-Barre and Miller-Fisher syndromes have been associated in some patients with previous *C. jejuni* infections (Ang et al., 2001; van Doorn et al., 2008).

The natural reservoir of *Campylobacter* are a variety of domesticated and wild animals, including cats, dogs, cows, pigs, poultry, and wild birds, with the avian reservoir being the most important (Kaakoush et al., 2015). *Campylobacter* can be easily isolated from poultry retail meat, untreated water, unpasteurized milk, and soil (Workman et al., 2005; Engberg, 2006).

Since *C. jejuni* infections are usually self-limited and sporadic, antimicrobial therapy is only indicated in severe or long-lasting infections. The antimicrobials of choice are macrolides, sometimes combined with azithromycin and aminoglycosides in more severe cases of the disease (Moore et al., 2005; Bolinger and Kathariou, 2017). The use of fluoroquinolones and tetracyclines, often prescribed as empirical therapy for traveler's diarrhea (Guerrant et al., 2001), has been reduced due to the dramatic increase of the frequency of resistant strains to these antimicrobial agents. Unfortunately macrolide resistance is increasingly being reported too (Lim et al., 2016).

Although the infection route has been described, the pathogenicity of *Campylobacter* remains mostly elusive. Very little is known on the bacterial factors exploited by *Campylobacter* during the infectious process. Besides the flagella and lipoligosaccharide, some putative virulence factors have been defined such as the cytolethal distending toxin (CdtABC), the fibronectin binding protein (CadF), and the invasion antigen (CiaB) (Young et al., 2007; Dasti et al., 2010). Moreover, factors that can be relevant for *Campylobacter* host adaptation and transmission have recently been identified by whole-genome sequencing studies (Pascoe et al., 2015; Thépault et al., 2017; Yahara et al., 2017).

Characterization of the population structure of *Campylobacter* strains recovered from different sources within a region and its antimicrobial resistance is critical to identify the major source of infection and to implement efficient control measures to reduce human exposure to the pathogen. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been used to determine clonal diversity and population structure, respectively (Duarte et al., 2016). Moreover, gaining insight into the distribution of virulence-associated genes among strains might shed some light on the mechanisms exploited by *Campylobacter* to trigger infection.

In this study, we have characterized three populations of *Campylobacter* strains from different ecological niches: human patients suffering gastroenteritis, broilers, and wild birds. The relationship among the different strains in terms of population structure, antimicrobial resistance profile, and prevalence of virulence-associated genes has been established.

MATERIALS AND METHODS

Bacterial Strains

A total of 150 isolates were obtained from feces of three different sources: human patients, broilers, and wild birds. Human isolates were obtained from the Santa Creu i Sant Pau Hospital (Barcelona) strain collection. Isolates were originally recovered from routine stool samples of 50 patients with diarrhea attended through the year 2014. All samples were anonymized. The patients were 54% children ($\leq$ 16 years old), 30% adults, and 16% elderly people ($\geq$ 65 years old). From these, 58% were men and 42% women. Domestic and wild avian isolates belong to a *Campylobacter* strain collection from previous studies conducted at IRTA-CReSA. Broiler (*Gallus gallus*) isolates were obtained from caecal samples collected in seven different slaughterhouses (A–G), from 2009 and 2011 to 2013. Broilers were originally from 24 farms located in Barcelona, Lleida, and Tarragona (Catalonia) (**Table 1**). Finally, the wild bird fecal samples from cloacal swabs were obtained during 2008–2013. Wild bird species sampled were: 1 northern shoveler (*Spatula clypeata*), 2 white storks (*Ciconia ciconia*), 5 common ravens (*Corvus corax*), 14 feral pigeons (*Columba livia*), 7 yellow-legged gulls (*Larus michahellis*), and 21 Audouin's gulls (*Larus audouinii*) (**Table 2**). All wild bird samples were collected in Catalonia, except eight from Audouin's gulls which were obtained from Alboran Island. Sampling methods were in compliance with the Ethical Principles in Animal Research of the Universitat Autònoma de Barcelona and the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya. Field permits were authorized by Generalitat de Catalunya and Junta de Andalucia. All sampling procedures and/or experimental manipulations in the field were reviewed and approved as part of obtaining the field permit.

Isolates were recovered from stock cultures stored at −80°C in cryovials containing Brain Heart Infusion broth (BHI; Merck KGaA, Darmstadt, Germany) supplemented with 20% glycerol. Fresh cultures were obtained by streaking a loop of the frozen stock cultures onto blood agar plates (BioMérieux, Marcy l'Etoile, France); plates were incubated at 37°C for 48 h under a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2; Anaerocult©, Merck, Darmstadt, Germany).

Species Identification and Antimicrobial Susceptibility Testing

Confirmation of *C. jejuni* strains was performed by conventional species-specific PCR using primers targeting the lipid A gene *lpxA* (Klena et al., 2004) and Matrix-Assisted Laser Desorption/Ionization-Time of flight (MALDI-TOF) Mass Spectrometry (Bruker Daltonics). Susceptibility to 12 antimicrobial agents was assessed by the disk diffusion method according to the Clinical Laboratory and Standard Institute [M100-S26; (CLSI, 2016)] using Mueller-Hinton medium supplemented with 5% defibrinated sheep blood (BioMérieux). The antimicrobials tested were: ampicillin (10 μg) (AMP), amoxicillin-clavulanic acid (30 μg) (AMC), imipenem (10 μg)

(IMP), tetracycline (30 μg) (TET), erythromycin (15 μg) (ERY), ciprofloxacin (5 μg) (CIP), nalidixic acid (30 μg) (NAL), gentamicin (10 μg) (G), streptomycin (10 μg) (S), kanamycin (30 μg) (K), chloramphenicol (30 μg) (CHL), and fosfomycin (200 μg) (FOS). The breakpoints were performed following the CLSI criteria except for TET, ERY and CIP where the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints criteria was used (Supplementary Table S1). All the clinical intermediate values were considered as resistant. The strains that showed resistance to three or more classes of antimicrobial agents were considered as multidrug-resistant (MDR) (Schwarz et al., 2010).

Pulsed Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST)

The PFGE was performed following the Standard Operating Procedure of PulseNet[1] for *C. jejuni*. Genomic DNA was digested with SmaI and KpnI restriction enzymes (Sigma-Aldrich, United States). Electrophoresis was performed in a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, United States). We performed a comparison analysis of PFGE profiles using the BioNumerics v7.6.3 software (AppliedMaths, Sint-Martens-Latem, Belgium). Similarity matrices were calculated by the Dice coefficient (2% optimization and 1% position tolerance) and dendrograms were constructed using the UPGMA method using the cited BioNumerics v7.6.3 software. Strains with a similarity $\geq$ 95% were considered as the same pulsotype, that means there are not more than two bands of difference.

Campylobacter jejuni strains were typed by MLST according to the procedures of PubMLST[2]. DNA was obtained using GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich, United States). Sanger sequence data were analyzed using BioNumerics v7.6.3 software. Alleles and sequence types (STs) were assigned based on the MLST scheme provided on the *Campylobacter* PubMLST[2] database. Novel alleles and STs were submitted to the database.

To represent the relationship among *Campylobacter* strains, we generated a complete minimum spanning tree (MST) using the BioNumerics v7.6.3 software.

Virulence-Associated Genes Detection

The 150 *C. jejuni* strains were tested by PCR for the presence of 8 genes encoding putative virulence factors. These included genes related to adhesion and colonization (*cadF*), invasion (*ciaB*, *virB11*, *htrA*, and *hcp*), and cytotoxin production (*cdtA*, *cdtB*, and *cdtC*). Genomic DNA was extracted by standard procedures using the InstaGene matrix Kit (Bio-Rad Laboratories). PCR reactions (PCR Master Mix x2, Thermo Scientific) were performed using 35 ng of DNA as a template and the specific primers indicated in Supplementary Table S2. As internal control of the PCR reaction, primers for the amplification of the housekeeping gene *gltA* were included in the PCR mixtures.

[1]http://www.pulsenetinternational.org/protocols/pfge

[2]http://pubmlst.org/campylobacter

Statistical Analysis

The virulence-associated genes data was analyzed using Pearson's chi-squared test (R Studio software). $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Genetic Diversity of *C. jejuni* Strains From the Three Different Ecological Niches

A collection of 150 *C. jejuni* isolates recovered from fecal samples from different ecological niches [50 isolates/each: human patients suffering gastroenteritis (H), broilers (B), and wild birds (W)] have been the focus of the study. The clonal relationship of the whole collection was determined by PFGE profiling. Four strains were non-typeable because of DNA smearing: one human (H49), one wild bird (W10), and two broiler (B24, B50) strains. As expected, a high clonal diversity was found among the 146 typeable strains. Genotyping using the SmaI restriction enzyme resulting 120 pulsotypes and 12 clones. To increase the clonal discrimination, the secondary KpnI enzyme was used (On et al., 1998). The combined analysis of SmaI and KpnI-PFGE banding patterns resulted in a wider clonal diversity with 137 pulsotypes and 4 clones (Supplementary Figure S1). The highest clonal diversity was found in the human population where no strains with the same pulsotype were found, consistent with the fact that human samples were not chosen in the context of an outbreak. By contrast, 13 strains, 6 from broilers and 7 from wild birds, were grouped in four clones (similarity $\geq$ 95%). Clone 1 included 6 broiler strains (B52–B57) from 2 different farms (5 from EU and 1 from CT) belonging to the same broiler company (**Table 1**). The five strains (B52–B56) from farm EU were recovered from broilers included in the same flock. This possible flock colonization is not a rare case and has also previously been reported by other authors (Ridley et al., 2011). Also, the recovery of the same clone in broilers from two different farms may be due to cross contamination between farms belonging to the same broiler company. Clone 2, included three strains (W06–W08) from Audouin's gulls (*Larus audouinii*) sampled at Alboran Island at the same breeding season, and Clones 3 and 4, both with two strains, W43 and W44 from pigeons (*Columba livia*, Barcelona) and W50 and W52 from common ravens (*Corvus corax*) (Sabadell, Barcelona province), respectively. It is not surprising the finding of different strains from the same host species belonging to the same clone, since samples were collected from different birds belonging to the same colony during the same time period.

The remaining PFGE patterns from the three different niches were scattered along the dendrogram, although different clusters could be observed (Supplementary Figure S1). Overall, human and broiler strains frequently grouped together at different similarity levels. On the contrary, a marked host specificity was found within some specific genus of wild birds (*Columba*, *Corvus*, and *Ciconia*) as previously described

(Griekspoor et al., 2013). Thus, pigeons' strains were grouped in different clusters with a similarity ranging from 65 to 80%; the three ravens' strains (two of them constitute the Clone 4) clustered with a similarity of 82% and the two storks clustered with a broiler strain with a 65% similarity. Finally, the northern shoveler strain showed low similarity ($\leq$ 50%) with all other wild birds strains (Supplementary Figure S1). Gulls strains were scattered along the dendrogram but some clusters were also defined. Interestingly, a cluster containing strains from two different gull species had a similarity of 65%. These gulls were from different geographical locations (Medes Islands, Ebro Delta, and Alboran Island). This fact has already been described by Griekspoor et al. (2013) who found a high similarity between strains from the same or closely related bird species from different geographical areas (Sweden, Australia, and United Kingdom). Notably, the gull isolate W30 cluster together with several pigeon isolates (W32, W36, W37). Since all those isolates were recovered from birds at the same geographical area (Barcelona), these data

TABLE 1 | Geographical relation between ST-complex and sequence types (STs) among *C. jejuni* from broiler caeca.

Slaughterhouse (*n*)	ST-complex**	ST	Year	Farm	Farm Location	Strain
A (8)	S	441	2011	ES44	Lleida	B44
	ST-21	21	2013	ES28	Lleida	B36
	ST-45	45	2013	ES3	Lleida	B31
		137	2012	ES41	Lleida	B42
	ST-353	5	2013	ES52	Lleida	B29
	ST-354	354	2013	ES52	Lleida	B45
	ST-607	904	2011	ES28	Lleida	B47
			2013	ES3	Lleida	B38
B (6)	S	531	2013	ES138	Barcelona	B16
	ST-45	652	2013	ES138	Barcelona	B22
	ST-464	464	2013	ES129	Barcelona	B15
	ST-574	305	2015	ES129	Barcelona	B21
			2013	ES138	Barcelona	B25
	ST-607	1707	2013	ES129	Barcelona	B24
C (6)	S	7114	2013	ES191	Barcelona	B39
	ST-283	267	2013	ES191	Barcelona	B33
	ST-353	5	2013	ES193	Barcelona	B28
		400	2013	ES193	Barcelona	B18
	ST-48	48	2011	ES193	Barcelona	B09
	ST-464	464	2011	ES191	Barcelona	B08
D (9)	S	1710	2013	ES125	Tarragona	B26
	S	1710	2013	ES128	Tarragona	B35
	ST-21	50	2012	ES116/CAT5	Tarragona	B14/B50
		883	2013	ES116	Tarragona	B17
	ST-353	400	2013	ES116/ES115	Tarragona	B30/B40
	ST-354	8498	2013	ES125	Tarragona	B37
	ST-607	607	2013	ES128	Tarragona	B41
E (9)	S	1710	2011	ES84	Lleida	B10
	S	2331	2011	ES84	Lleida	B02
	ST-21	21	2013	ES84	Lleida	B20/B46
	ST-45	45	2013	ES85	Lleida	B27
	ST-257	2254	2012	ES202	Lleida	B13
	ST-353	356	2013	ES202	Lleida	B48
	ST-354	354	2013	ES84	Lleida	B23
	ST-607	7110	2012	CAT1	Lleida	B51
F (6)	ST-21	883	2011	ES156	Barcelona	B06
		50	2011	ES184	Tarragona	B07
	ST-206	46	2012	ES156	Barcelona	B49
	ST-353	400	2012	ES154	Lleida	B05
	ST-607	607	2011	ES147	Lleida	B04
		7110	2013	ES154	Lleida	B19
G* (6)	ST-257	367	2009	CT1/EU	Lleida	B57/B52, B53, B54, B55, B56

*n means number of strains. *Includes the six strains belonging to Clone 1 of PFGE pattern. **S means singleton.*

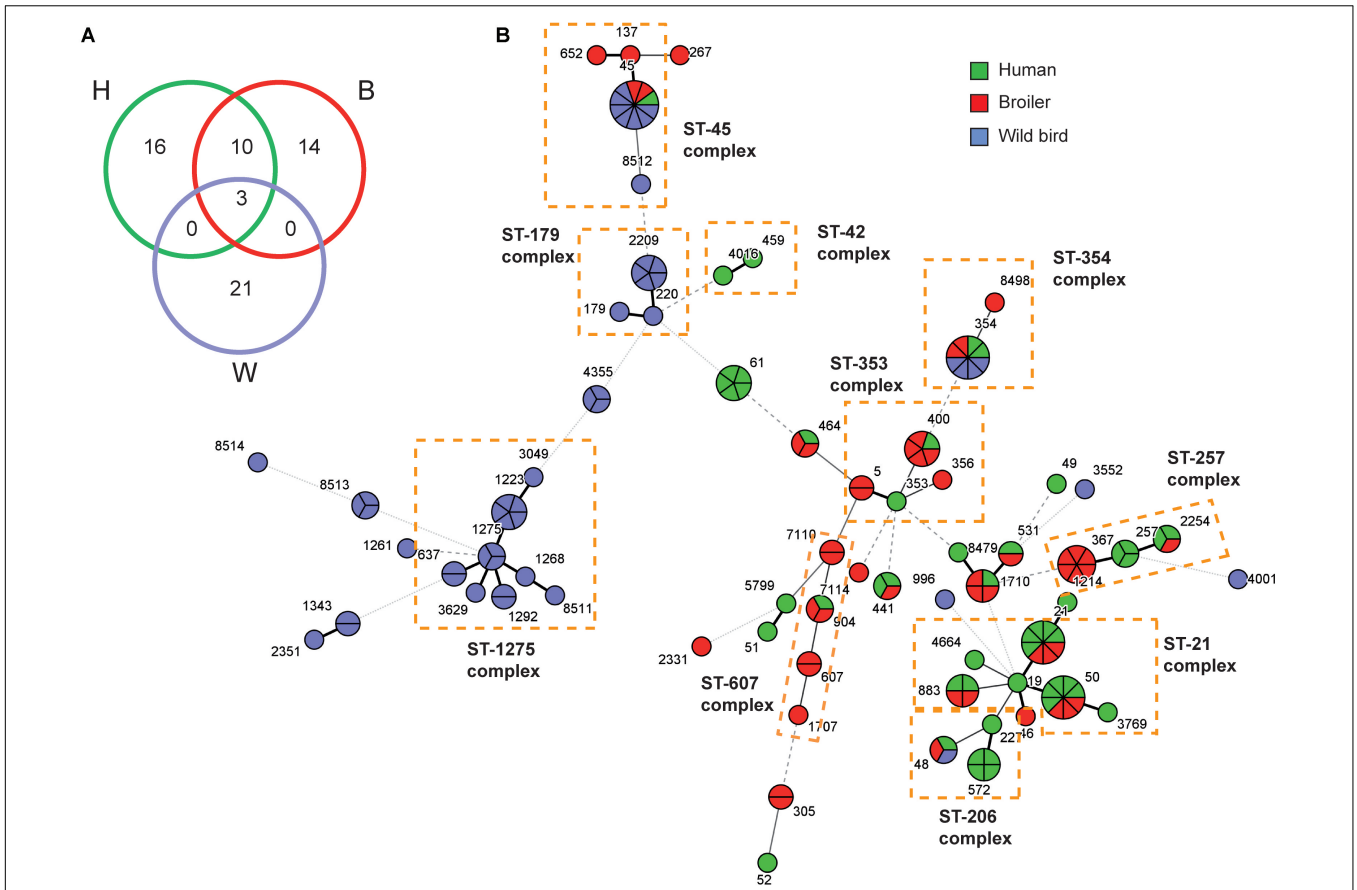

FIGURE 1 | Minimum spanning tree showing the distribution of the *C. jejuni* strains. **(A)** The number of STs shared among the three niches was represented by a Venn-diagram. **(B)** Minimum spanning tree (MST) of *C. jejuni* sequence types (STs) based on allele numbers. Each circle corresponds to a ST, being its size proportional to the number of strains within it. The ST of each strain are indicated in different color attending to the origin of the strain: green for humans (H), red for broilers (B), and blue for wild birds (W). The thickness and the dotting of the lines indicate the distance between STs. A thicker line denotes closer distance than a thin line and a thin line denotes closer distance than a dotted line. The ST-complexes including several STs are indicated in dotted color lines. The MST analysis was carried out by BioNumerics v7.6.

suggest that both the gull and the pigeons have a common source of infection or it is a consequence of gulls being pigeons predators.

MLST Analysis and Population Structure

The MLST analysis corroborates the genetic diversity observed by PFGE typing among the *C. jejuni* strains and confirms a closer relationship between human and broiler strains.

Among the 150 strains studied, 64 different STs grouped in 21 clonal complexes (ST-complexes) and 12 singletons (S) were recognized (**Table 3** and Supplementary Figure S2). Six novel STs were identified: ST-8479 (human), ST-8498 (broiler), ST-8511, ST-8512, ST-8513, and ST-8514 (wild birds). In this study, the ST-21 (24 strains), ST-1275 (16 strains), and ST-45 (13 strains) clonal complexes were the most frequent. Strains from the ST-1275 complex were only isolated from two different gull species. The ST-21 complex was found in humans (16 strains) and broilers (8 strains), whereas the ST-45 was found in the three environments studied. Thus, the ST-45 complex was predominant in wild birds (8 strains), followed by broilers (4 strains) and by humans (1 strain). Both, the ST-21 and ST-45 complexes are described as multihost genotypes and have been isolated from a wide variety of agricultural and environmental sources (Sopwith et al., 2006; French et al., 2009; Sheppard et al., 2011; Colles and Maiden, 2012).

The relationship among the three studied populations is reflected in a minimum spanning tree diagram (**Figure 1**). Three STs (ST-45, ST-48, and ST-354) were found in all three niches representing 14% (21/150) of the 150 studied strains. The spanning tree diagram distributes the strains in two main clusters, characterized – with some exceptions – by its ecology. The first cluster, in the left side of the **Figure 1B**, grouped STs from wild birds, whereas the second cluster (right side) agglutinate most human and broiler STs. This distribution clearly establishes a closer relationship between broiler and human strains, consistent with broiler meat being the most frequent source of *Campylobacter* human infection (EFSA-ECDC, 2016; Ramonaite et al., 2017). This can also be observed when looking at the number of STs shared among the different niches (**Figure 1**): 10 STs are shared between human and broiler strains,

only 3 in the 3 niches, and no ST is shared among wild birds and either humans or broilers.

From the first cluster (STs from wild birds), the ST-179 and ST-1275 complexes were mainly found in pigeons and seagulls, respectively. These data support that *Campylobacter* genotypes isolated from non-agricultural sources, such as wild birds, are found only rarely among broilers (Griekspoor et al., 2010). However, these clonal complexes have also been recovered from stool samples from humans with sporadic cases of gastroenteritis (Hughes et al., 2009). STs from the ST-179 complex have been isolated from environmental sources, specifically from the sand of bathing beaches in the United Kingdom, presumably ultimately coming from wild birds (Dingle et al., 2001). The ST-1275 complex has also been found in several wild bird species such as pigeons (Sheppard et al., 2009). Certain host specificity was found among the ST-complexes present in different wild birds. Hence, the ST-1275 complex was predominant in gulls, the ST-179 complex in pigeons and the ST-952 complex in ravens (**Table 2**).

With regards to the second cluster (STs from human and broiler strains), five ST- complexes were found as predominant (ST-21, ST-206, ST-257, ST-353, and ST-607). These clonal complexes have been previously associated with human infections, poultry, other farm animals, and environmental samples (Colles and Maiden, 2012; Cody et al., 2015; Ramonaite et al., 2017). Overall, a great diversity of ST-complexes was found among both human and broiler strains. A total of 16 STs were found in humans and 14 in broilers (**Figure 1A**). Strains from Clones 1, 2, 3, and 4 by PFGE belong to ST-367, ST-4355, ST-45, and ST-8513, respectively, while the remaining strains showed unrelated PFGE pattern.

Antimicrobial Susceptibility

Antimicrobial susceptibility was tested for all the isolates and antimicrobial resistance (AMR) profiles were defined (**Figure 2**). In agreement with the increase in the occurrence of AMR and MDR detected in *Campylobacter* strains in many countries (Luangtongkum et al., 2009), a high frequency of AMR was detected among the strains of our collection. A total of 127 strains (84.6%) were resistant to one or more antimicrobial agents. The highest percentage of antimicrobial resistance was found for tetracycline (71.3%), quinolones [ciprofloxacin (67.3%), nalidixic acid (64.6%)], and ampicillin (63.3%) (**Figure 2A**). In agreement with the genotyping data, the antimicrobial susceptibility describes important differences between wild bird strains and the clustered broiler and human strains, with the occurrence of antibiotic resistance much lower among wild bird strains. In our strain collection, 23 strains were susceptible to all antimicrobial agents tested. From those, 21 strains were

TABLE 2 | Distribution of ST-complexes and sequence types (STs) of *C. jejuni* strains from wild birds.

Wild bird	ST-complex	ST	Location	Strains (Year of recollection)
Audouin's gull (21)	ST-446 (1)	3552	Alboran Island	W12 (2010)
	ST-1034 (1)	4001	Ebro Delta	W05 (2009)
	ST-1275 (12)	1223	Ebro Delta	W03 (2009), W10 (2010), W15 (2010), W18 (2011), W21 (2011)
		1268	Ebro Delta	W22 (2011)
		1275	Ebro Delta	W04 (2009), W09 (2010), W20 (2010)
		1292	Alboran Island	W14 (2010), W16 (2010)
		3629	Ebro Delta	W19 (2010)
	Singletons (7)	1261	Ebro Delta	W11 (2010)
		1343	Alboran Island	W13 (2010), W23 (2010)
		2351	Ebro Delta	W17 (2011)
		4355	Alboran Island	W06 (2009), W07 (2009), W08 (2009)
Yellow-legged gull (7)	ST-45 (1)	45	Barcelona	W30 (2014)
	ST-48 (1)	48	Barcelona	W29 (2014)
	ST-354 (1)	354	Medes Islands	W28 (2013)
	ST-1275 (4)	637	Medes Islands	W24 (2012), W27 (2013)
		3049	Medes Islands	W26 (2013)
		8511	Medes Islands	W25 (2012)
Common Ravens (5)	ST-354 (1)	354	Barcelona province	W54 (2008)
	ST-952 (3)	8513	Barcelona province	W50 (2008), W51 (2008), W52 (2008)
	Singleton (1)	8514	Barcelona province	W53 (2008)
Fereal Pigeons (14)	ST-45 (7)	45	Barcelona	W32 (2015), W36 (2015), W37 (2015), W40 (2015), W43 (2015), W44 (2015)
		8512	Barcelona	W47 (2015)
	ST-179 (7)	179	Barcelona	W33 (2015)
		220	Barcelona	W41 (2015)
		2209	Barcelona	W34 (2015), W39 (2015),W46 (2015), W48 (2015), W49 (2015)
Northern shoveler (1)	Singleton (1)	996	Ebro Delta	W02 (2008)
White storks (2)	ST-354 (2)	354	Lleida	W55 (2011), W56 (2011)

In brackets, number of strains.

A

	Antimicrobial agent	H (%)	B (%)	W (%)	Total (%)
Q	Tetracycline (TET)	44 (88)	48 (96)	15 (30)	107 (71.3)
Q	Ciprofloxacin (CIP)	44 (88)	49 (98)	8 (16)	101 (67.3)
Q	Nalidixic Acid (NAL)	39 (78)	49 (98)	9 (16)	97 (64.6)
	Ampicillin (AMP)	40 (80)	37 (74)	18 (36)	95 (63.3)
A	Streptomycin (S)	2 (4)	7 (14)	2 (4)	11 (7.3)
A	Kanamycin (K)	3 (6)	2 (4)	2 (4)	7 (4.6)
	Gentamicin (G)	1 (2)	0	0	1 (0.6)

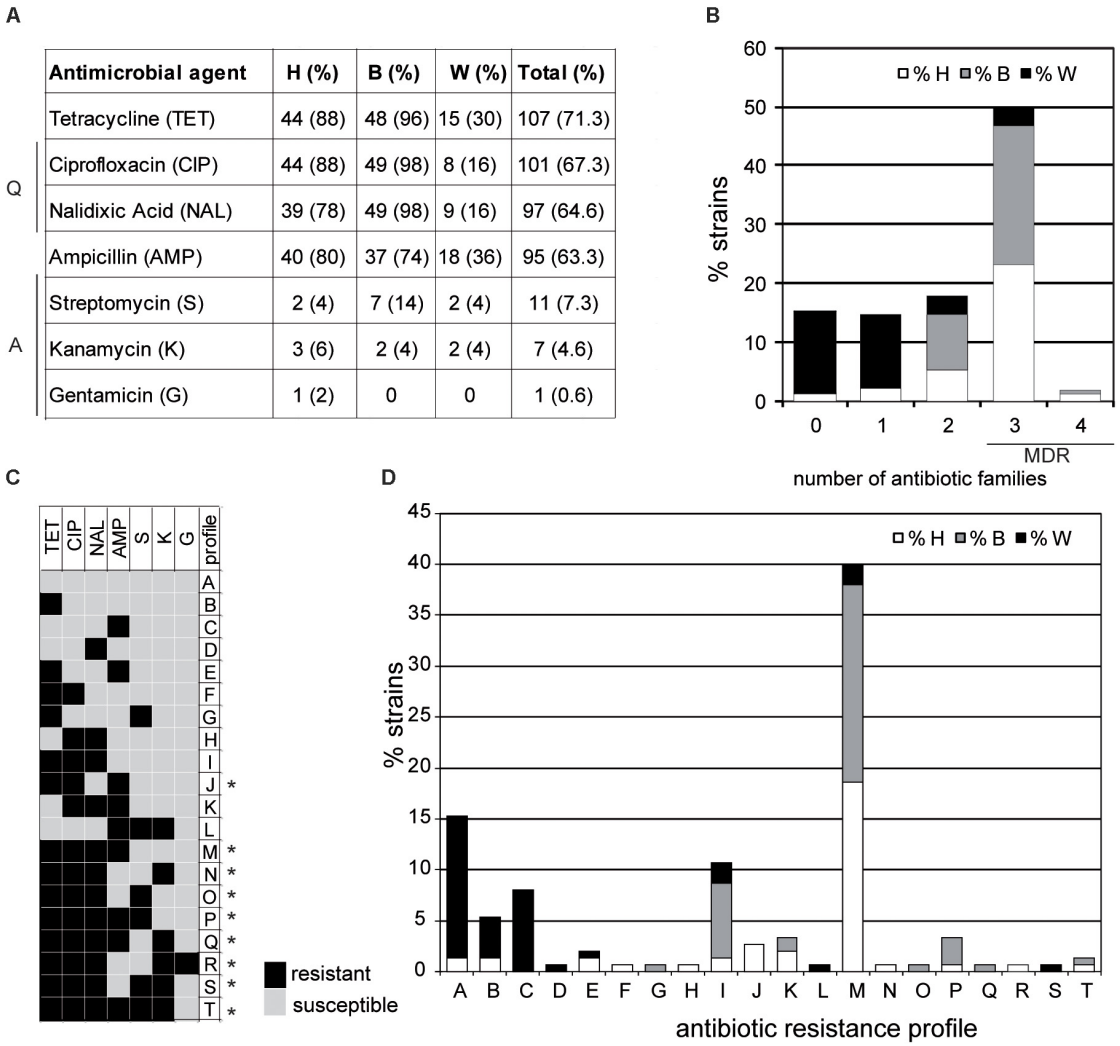

FIGURE 2 | Antimicrobial resistance in *C. jejuni* studied strains. **(A)** Absolute and relative values of antimicrobial resistance in human (H), broiler (B), and wild bird (W) strains. Antibiotic families (Q: quinolones and A: aminoglycosides) are indicated. All strains were susceptible to amoxicillin-clavulanic acid, imipenem, erythromycin, chloramphenicol, and fosfomycin. **(B)** Percentage of resistant strains to 0 to 4 families of antibiotics. MDR indicates multidrug-resistant strains. **(C)** Antimicrobial resistance profiles obtained. Asterisks indicate the MDR profiles. **(D)** Percentage of the different antimicrobial resistance profiles in each subgroup, as defined in **(C)**.

recovered from wild birds and the other two from human samples. Moreover, the occurrence of tetracycline, quinolones, and ampicillin resistance was higher in human and broiler strains compared to wild birds. In contrast, the low occurrence resistance to aminoglycosides is shared among strains from the three niches. In our study, 7.3 and 4.6% of the strains were resistant to streptomycin and kanamycin, respectively, whereas only one strain from a human sample was resistant to gentamicin.

The high occurrence of resistance to some antibiotics cannot be accounted to the non-therapeutic use of antimicrobial drugs in poultry production since this practice was banned in the EU in 2006. The therapeutic antibiotics used in poultry are mainly quinolones (enrofloxacin), tetracyclines (doxycycline), penicillins (amoxicillin), and macrolides (erythromycin, tylosin), which is reflected – in general – with the occurrence of antibiotic resistance detected. Also in agreement with the detected occurrence, the aminoglycosides tested (streptomycin, kanamycin, and gentamicin) are not of common use for treatment of enteric diseases in poultry (Cantero et al., 2018). All strains were susceptible to amoxicillin-clavulanic acid, imipenem, erythromycin, chloramphenicol, and fosfomycin. The rare occurrence of imipenem, erythromycin, and amoxicillin-clavulanic acid resistance among *Campylobacter* strains has been previously described (Gallay et al., 2007; Deckert et al., 2010; Deng et al., 2015). In contrast, resistance to fosfomycin has been detected in *C. jejuni* strains (Novoa-Farías et al., 2016) as well as to chloramphenicol in *C. coli* strains isolated from humans and broilers (Yang et al., 2017).

The MDR, defined as resistance to three or more families of antimicrobial agents (Schwarz et al., 2010), has increased

worldwide among *C. jejuni* strains (Giacomelli et al., 2014). In this study, 49.3% of the strains were MDR (**Figure 2B**). The most frequent MDR profile (M profile, TET-NAL-CIP-AMP) was detected in 40% of the strains, being mostly identified in humans (23.3%) and broilers (23.3%) (**Figures 2C,D**). Only five strains from wild birds were MDR, four of them with the above mentioned M profile and one with the S profile (TET-NAL-CIP-S-K). Those MDR strains were recovered from birds with insalubrious feeding habits (feeding on refuse dumps and carrion) such as gulls, storks, and ravens (Ramos et al., 2010).

These MDR strains were included in five out of the six predominant ST-complexes found in humans and broilers (ST-21, ST-206, ST-257, ST-354, and ST-607) (**Table 3**). Interestingly, the percentage of MDR among strains of ST-353 complex, also defined as predominant in humans and broilers, was much lower (33.3 %). No MDR strains have been found among the two ST- complexes exclusive for wild birds (ST-179 and ST-1275). It should be highlighted that there is a high variability in the AMR profile among genetically closely related strains. For instance, within the ST-45 and ST-61, strains susceptible to all antimicrobial agents tested and MDR were found. These suggest a rapid acquisition of antibiotic resistance determinants among *C. jejuni* strains. Accordingly, transfer of antibiotic resistance genes by mobile genetic elements, such as plasmids and transposons that can help acquisition and diffusion of drug resistance, has been described (Bennett, 2008).

Prevalence and Distribution of Virulence-Associated Genes Among the *C. jejuni* Strains

The presence of genes coding for putative virulence factors in the 150 *C. jejuni* strains have been tested by PCR. The genes detected were: (1) *cdtA*, *cdtB*, and *cdtC* genes, conforming a polycistronic operon, coding for synthesis and deliver of the cytolethal distending toxin that causes host cell cycle arrest; (2) three genes involved in adherence and invasion of host cells: *cadF* coding for a putative OmpA-like protein that mediates bacterial adhesion by binding to host fibronectin, *ciaB* coding for an invasive antigen that translocate into the cytoplasm of host cells facilitating the *C. jejuni* invasion, and *htrA* coding for a serine-protease that contributes to stress tolerance and with ability to cleave E-cadherin; (3) the *hcp* gene coding for a host surface adhesion protein that is a component of a type 6 secretion system (T6SS) that has been associated with virulence; and (4) the *virB11*, a gene located in the virulence related plasmid pVir (Bacon et al., 2002; Tracz et al., 2005; Cróinín and Backert, 2012; Bleumink-Pluym et al., 2013). The results showed a high prevalence of six out of eight genes tested (**Figure 3**). Remarkably, significant differences exist in the prevalence of some of the genes when comparing among the strains from different origin. A different distribution of the *cdtA*, *cdtB*, and *cdtC* genes was detected among the three subgroups of strains. The three genes were present in all the human and broiler strains whereas 46% of the wild bird strains were negative for at least one of the *cdt* genes (Supplementary Figure S2). The fact that many strains (23) lacked one or two of the three open reading frames (ORF) present in the polycistronic *cdtABC* operon is an interesting phenomenon that has been reported earlier (Bang et al., 2003; Koolman et al., 2015). Remarkably, the *cdtA* gene had the more uneven distribution since it was detected in all human and broilers strains, while it was only present in 58% of the wild bird strains. Among the 23 strains lacking at least one *cdt* gene, 14 lacked *cdtA*, 1 *cdtB*, 1 *cdtC*, 3 *cdtAB*, and 4 *cdtAC*. The negative PCR-amplification of one or two of the three ORF present in the *cdtABC* operon, when using primers designed from the 81-176 genome sequence (Supplementary Table S2), may indicate significant differences among alleles from different strains. Our results suggest that: (1) a high diversity of *cdt* alleles exists among *C. jejuni* strains found in natural environments (wild bird) and (2) the *cdt* alleles promoting efficient broiler colonization are much less diverse. *cdtA* and *cdtC* are involved in the binding to the target host cells allowing internalization of the *cdtB* toxin. Therefore, we hypothesize that different *cdtA* might have distinct target molecules during tissue recognition and consequently might play a relevant role defining the host susceptibility of *C. jejuni* strains. Interesting observations can be made when having a closer look to the phylogenetic and ecological relationships of the strains that were negative for any of the *cdt* genes (**Table 3** and Supplementary Figure S2): (1) all 16 strains belonging to the ST-1275 complex and different PFGE pattern were *cdtA* negative and were isolated from the two different seagull species located in three geographically distinct colonies along the western Mediterranean coast; three of them were also *cdtC*-negative. From these seagulls' colonies, $cdtABC^{+}$ strains, belonging to diverse ST-complexes distinct to ST-1275, were also isolated; (2) all three strains belonging to ST-952 complex are *cdtAB* negative but two share the same PFGE pattern (W50 and W52), these strains were isolated from commons ravens. The other two ravens' strains are *cdtA* negative (W53, singleton ST-8514) and $cdtABC^{+}$ (W54, ST-354 complex), respectively; (3) the ST-48 complex includes three strains, belonging each one to a different niche, the broiler (B09) and human (H38) strains are $cdtABC^{+}$ and showed different PFGE pattern, whereas the wild bird strain (W29) from a Yellow-legged gull is *cdtB* negative; (4) from the wild bird subgroup, all the strains from pigeons and storks, birds that are more in contact with the human population, are positive for all three *cdt* genes; and (5) the unique $cdtC^{-}$ strain belongs to the ST-1034 complex. The relevant diversity among *cdtABC* found among wild bird strains and the apparently highly conserved distribution among human and broiler strains might indicate that the specific sequence of certain *cdt* alleles can be related to the ability to colonize different hosts. Moreover, the fact that there are differences in the presence of *cdt* alleles among strains from the same ST-complex and/or ST indicates the high plasticity of this genetic locus (the *cdtABC* operon).

Regarding the genes coding for putative factors involved in host cell invasion, only *cadF* showed an uneven prevalence distribution among strains from the different niches. The tested *cadF* allele was present in all broiler and wild bird strains and in 45 out of 50 human strains. This result suggests that either some strains lack the *cadF* gene or carry a *cadF*

TABLE 3 | Clonal complex (ST-complex), sequence type (ST), virulence factors profile (VF), and antimicrobial resistance profile (AMR) found in 150 *C. jejuni* strains from humans, broilers, and wild birds niches.

			Humans		Broilers		Wild Birds	
ST complexes	**ST**		**VF[a]**	**AMR[b]**	**VF**	**AMR**	**VF**	**AMR**
ST-21 complex (24)	19	(1)	#2	M (1)				
	21	(8)	#2	M (1) I (2) J (1) T (1)	#2	M (2) O (1)		
	50	(8)	#2	M(4) K (1)	#2	M (1) K (2)		
	883	(4)	#2	M (1) J (1)	#1	I (1)		
					#2	I (1)		
	1214	(1)	#2	J (1)				
	3769	(1)	#2	M (1)				
	4664	(1)	#2	B (1)				
ST-42 complex (2)	459	(1)	#2	M (1)				
	4016	(1)	#2	E (1)				
ST-45 complex (13)	45	(10)	#2	K (1)	#2	M (1) G (1)	#2	A (3) C (3) I (1)
	137	(1)			#2	P (1)		
	652	(1)			#2	P (1)		
	8512	(1)					#2	A (1)
ST-48 complex (3)	48	(3)	#5	M (1)	#9	M (1)	#14	M (1)
ST-49 complex (1)	49	(1)	#2	M (1)				
ST-52 complex (1)	52	(1)	#2	H (1)				
ST-61 complex (5)	61	(5)	#6	M (2) A (1) E (1)				
			#12	B (1)				
ST-179 complex (7)	179	(1)					#5	A (1)
	220	(1)					#3	D (1)
	2209	(5)					#2	A (1)
							#5	A (2) C (2)
ST-206 complex (6)	46	(1)			#2	M (1)		
	227	(1)	#2	A (1)				
	572	(4)	#5	M (4)				
ST-257 complex (12)	257	(3)	#2	M (1) N (1) P(1)				
	367	(6)			#2	M (6)		
	2254	(3)	#2	M (2)	#2	I (1)		
ST-283 complex (1)	267	(1)			#2	M (1)		
ST-353 complex (9)	5	(2)			#1	I (1)		
					#3*	I (1)		
	353	(1)	#1*	K (1)				
	356	(1)			#2	M (1)		
	400	(5)	#3	M (1)	#3	I (3) M (1)		
ST-354 complex (9)	354	(8)	#2	M (2)	#2	M (2)	#2	M (2) I (2)
	8498	(1)			#2	M (1)		
ST-443 complex (2)	51	(1)	#2	M (1)				
	5799	(1)	#2	M (1)				
ST-446 complex (1)	3552	(1)					#1	M (1)
ST-464 complex (3)	464	(3)	#1	F (1)	#2	M (1)		
					#1	M (1)		
ST-574 complex (2)	305	(2)			#2	M (1) I (1)		
ST-607 complex (8)	607	(2)			#1	M (1) I (1)		
	904	(3)	#1	M (1)	#1	M (2)		
	1707	(1)			#1	M (1)		
	7110	(2)			#2	M (1)		
					#1	I (1)		
ST-952 complex (3)	8513	(3)					#10	A (2) C (1)
ST-1034 complex (1)	4001	(1)					#13	L (1)
ST-1275 complex (16)	637	(2)					#4	A (1) B (1)

(Continued)

TABLE 3 | Continued

			Humans		Broilers		Wild Birds	
ST complexes	**ST**		**VF[a]**	**AMR[b]**	**VF**	**AMR**	**VF**	**AMR**
	1223	(5)					#4	C (1)
							#7	A (2) C (1)
							#15	C (1)
	1268	(1)					#4	B (1)
	1275	(3)					#4	B (2)
							#8	B (1)
	1292	(2)					#4	C (1)
							#8	C (1)
	3049	(1)					#4	A (1)
	3629	(1)					#4	E (1)
	8511	(1)					#4	B (1)
Singletons	441	(3)	#2	R (1)	#5	P (1)		
			#11	J (1)				
	531	(2)	#3	M (1)	#1	M (1)		
	996	(1)					#16	A (1)
	1261	(1)					#2	A (1)
	1343	(2)					#2	A (2)
	1710	(4)	#1	M (1)	#1	M (1) Q (1)		
					#3	P (1)		
	2331	(1)			#1	M (1)		
	2351	(1)					#2	C (1)
	4355	(3)					#2	A (3)
	7114	(1)			#2	T (1)		
	8479	(1)	#1	M (1)				
	8514	(1)					#7	S (1)

[a] *The VF profile is described in* ***Figure 3****.* [b] *The AMR profile is described in* ***Figure 2****. *virB11 positive. In brackets, number of strains.*

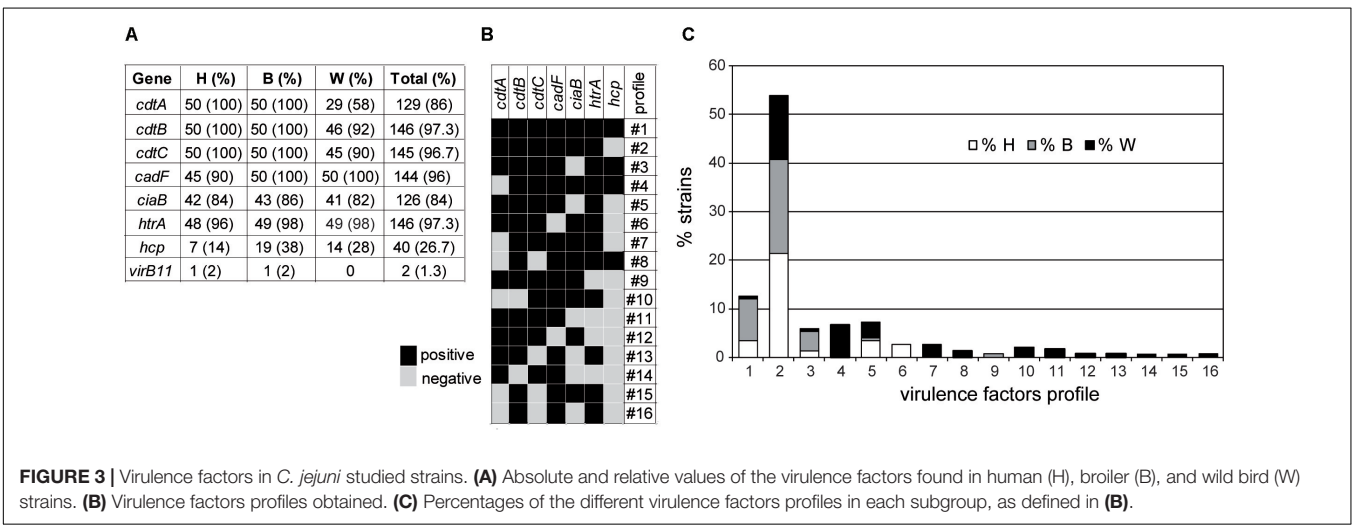

FIGURE 3 | Virulence factors in *C. jejuni* studied strains. **(A)** Absolute and relative values of the virulence factors found in human (H), broiler (B), and wild bird (W) strains. **(B)** Virulence factors profiles obtained. **(C)** Percentages of the different virulence factors profiles in each subgroup, as defined in **(B)**.

allele with significant differences from the $cadF_{81-176}$ allele chosen as template in this study (Supplementary Table S2). Interestingly, all the *cadF* negative strains belong to ST-61, which is often found in cattle/bovine reservoirs (Kwan et al., 2008). Our results suggest that the presence of the *cadF* tested may not be essential for the ability of *Campylobacter* to cause infection in humans. It cannot be disregarded that the five $cadF^-$ strains carry a different *cadF* allele not detected with the primers used (Supplementary Table S2). In a recent report studying the distribution of virulence-associated genes in a collection of 24 *Campylobacter* strains, *cadF* was detected in all *C. jejuni* strains whereas some *C. coli* from both humans

and broilers were negative for this gene (Koolman et al., 2015).

No significant differences exist when comparing the prevalence of the *ciaB* gene among the three populations. The *ciaB* negative strains are the majority for certain ST-complexes such as ST-48, ST-179, ST-353, ST-206, and two singletons (ST-441, ST-1710). However, we also found *ciaB* negative strains belonging to ST-complex where most strains are positive for this genetic locus. Only four strains were negative for *htrA*, two from humans (ST-61 and ST-441), one from a broiler (ST-48), and one from a wild bird (ST-48).

The presence of the *hcp* gene is a hallmark of the ability of *C. jejuni* to express a T6SS (Lindmark et al., 2009). A functional T6SS has been identified in some *C. jejuni* strains, those carrying the integrative element CJIE3. Its presence had been related to the ability to infect humans and cause disease by influencing cell adhesion and cytotoxicity toward erythrocytes (Lertpiriyapong et al., 2012; Bleumink-Pluym et al., 2013; Harrison et al., 2014). Notably, the *hcp* gene was detected in a low proportion in all three populations. The detection frequency was in a range similar to that described in previous reports (Harrison et al., 2014; Siddiqui et al., 2015). Surprisingly, our data indicate that *hcp* is significantly less represented among the human strains (14%) as compared to broiler (38%) and wild bird (28%) strains (**Figure 3A**). Interestingly, the *hcp*-positive clones are mostly distributed among certain ST-complexes: ST-1275 (12 out of 16 are positive), ST-607 (7/8), ST-353 (8/9), ST-464 (2/3), and ST-446 (1/1). The differential prevalence of *hcp*, with lower prevalence among human strains, suggests that T6SS does not promote human infection. The last putative virulence factor encoding gene tested was *virB11*. Previous reports have argued on the contribution of the pVir plasmid in the virulence potential of *C. jejuni* (Tracz et al., 2005; Louwen et al., 2006). Among our strains the prevalence of the *virB11* gene is very low since only two strains, one from human and one from broiler, were positive for the tested *virB11* allele, suggesting that the pVir plasmid is not required for *C. jejuni* to either colonize birds or infect humans.

Virulence profiles were defined attending to the presence/absence of virulence-related genes of chromosomal location (all genes except *virB11*) (**Figure 3**). The virulence profile #2 ($cadF^+$, cdt^+, $htrA^+$, $ciaB^+$) is overrepresented among the collection, being detected in a 54% of the strains and being the most prevalent in all three subgroups (human, broiler and wild birds). In addition to profile #2, only three more virulence profiles (#1, #3, #5) were detected in all the three subgroups. The highest variability was found among the wild bird strains since up to 14 different profiles were identified, in contrast to human and broiler strains where five profiles were found within each subgroup. From the 14 profiles described in wild birds, 10 were only found in this subgroup. One virulence profile (#6, cdt^+, $cadF^-$, $ciaB^+$, $htrA^+$ hcp^-) was only found among human strains, being detected in four clinical strains belonging to ST-61, but with different PFGE patterns. Moreover the profile #9 has only been detected among broiler strains.

CONCLUSION

In this report, a comparative study has been performed among *C. jejuni* strains from three different sources: humans, broilers, and wild birds from Catalonia. As expected, by PFGE a great genetic diversity was observed among all included strains, with the exception of a few clones. In our *Campylobacter* population, the ST-45, ST-48, and ST-354 were present in all three niches; the ST-21 complex was the predominant in human and the ST-1275 complex in wild birds, while in broilers different complexes: ST-21, ST-257, ST-353, and ST-607 were present equally. MLST analysis clearly distinguished the wild bird *Campylobacter* population from that of humans and chickens, suggesting that certain host specificity may exist among *C. jejuni* clonal complexes. The drug antimicrobial profiles show an overall high percentage of MDR strains (49%). Again, a closer relationship between human and broiler strains was detected. A similar high percentage of MDR strains (72%) was detected among human and broiler strains whereas the percentage among wild bird strains was much lower (8%). This discrepancy in MDR prevalence correlates with the differential antibiotic pressure. The broiler and human strains are under a high antibiotic pressure due to the use of antimicrobial drugs whereas the selective pressure is barely inexistent in wild bird population. The presence of several putative virulence genes have been detected by PCR. The genes *cadF*, *ciaB*, *htrA*, and the operon *cdtABC*, coding for proteins presumably involved in bacterial adherence, invasion of epithelial cells and toxin production, are highly prevalent among *Campylobacter* strains. Correlation between certain virulence factors profiles and specific STs has been detected, which may have ecological implications. For instance, our data indicates a great diversity in *cdtA* alleles among wild bird strains as compared with broiler and human strains, suggesting that colonization of specific hosts might be promoted by certain *cdtA* variants.

All the genotype- and phenotype-based analysis indicate that most strains isolated from wild birds form a cluster clearly differentiated from those strains isolated from broilers and humans. Nonetheless, some wild bird strains belong to clonal complexes also detected among broiler or human strains suggesting a reverse zoonosis transmission, most likely consequence of the scavenging feeding habitats of the studied birds. Overall, our report provides new insights into the distribution of circulating *C. jejuni* strains among different ecological niches.

AUTHOR CONTRIBUTIONS

YI-T performed the investigation, formal analysis, and statistical analysis, and wrote the manuscript. PG performed the investigation, formal analysis, and statistical analysis. TL and CMu performed the conception and design of the study, and revised the manuscript. MC-C and FN performed the formal analysis, statistical analysis, conception, and design of the study, and revised the manuscript. EM, CMa, and CB performed the

formal analysis, statistical analysis, conception, and design of the study, and wrote the manuscript.

FUNDING

This study was supported by the Spanish Ministry of Economy and Competitiveness (Grant No. AGL2013-45339R), the Spanish National Institute of Agrifood Research and Technology (INIA, Grant No. FAU2008-00012-C02-01 and RTA2009-00117-00-00), the Catalonian Government (Grant No. 2017SGR499), the Recercaixa Program (Grant No. 2012/ACUP/00048), and the European Community's Seventh Framework Program (FP7/2007-2013) under Grant agreement No. 244547 (CamCon project). CERCA Program from the Generalitat de Catalunya is also acknowledged.

ACKNOWLEDGMENTS

Pigeon samples were kindly provided by the Barcelona Public Health Agency (ASPB).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01676/full#supplementary-material

REFERENCES

Ang, C. W., De Klerk, M. A., Endtz, H. P., Jacobs, B. C., Laman, J. D., Van Der Meché, F. G. A., et al. (2001). Guillain-Barré syndrome- and Miller Fisher syndrome-associated *Campylobacter jejuni* lipopolysaccharides induce anti-GM_1 and anti-GQ_{1b} antibodies in rabbits. *Infect. Immun.* 69, 2462–2469. doi: 10.1128/IAI.69.4.2462-2469.2001

Bacon, D. J., Alm, R. A., Hu, L., Hickey, T. E., Ewing, C. P., Batchelor, R. A., et al. (2002). DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. *Infect. Immun.* 70, 6242–6250. doi: 10.1128/IAI.70.11.6242-6250.2002

Bang, D. D., Nielsen, E. M., Scheutz, F., Pedersen, K., Handberg, K., and Madsen, M. (2003). PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *J. Appl. Microbiol.* 94, 1003–1014. doi: 10.1046/j.1365-2672.2003.01926.x

Bennett, P. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153, 347–357. doi: 10.1038/sj.bjp.0707607

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

Bolinger, H., and Kathariou, S. (2017). The current state of macrolide resistance in *Campylobacter* spp: trends and impacts of resistance mechanisms. *Appl. Environ. Microbiol.* 83:e00416-17. doi: 10.1128/AEM.00416-17

Cantero, G., Correa-Fiz, F., Ronco, T., Strube, M., Cerdà-Cuellar, M., and Pedersen, K. (2018). Characterization of *Campylobacter jejuni* and *Campylobacter coli* broiler isolates by whole-genome sequencing. *Foodborne Pathog. Dis.* 15, 145–152. doi: 10.1089/fpd.2017.2325

CLSI (2016). *Performance Standards for Antimicrobial Susceptibility Testing CLSI Supplement M100S*. Wayne, PA: CLSI.

Cody, A. J., Mccarthy, N. D., Bray, J. E., Wimalarathna, H. M. L., Colles, F. M., Jansen Van Rensburg, M. J., et al. (2015). Wild bird-associated *Campylobacter jejuni* isolates are a consistent source of human disease, in Oxfordshire, United Kingdom. *Environ. Microbiol. Rep.* 7, 782–788. doi: 10.1111/1758-2229.12314

Cody, A. J., Mccarthy, N. M., Wimalarathna, H. L., Colles, F. M., Clark, L., Bowler, I. C. J. W., et al. (2012). A longitudinal 6-year study of the molecular epidemiology of clinical *Campylobacter* isolates in Oxfordshire, United Kingdom. *J. Clin. Microbiol.* 50, 3193–3201. doi: 10.1128/JCM.01086-12

Colles, F. M., and Maiden, M. C. J. (2012). *Campylobacter* sequence typing databases: applications and future prospects. *Microbiology* 158, 2695–2709. doi: 10.1099/mic.0.062000-0

Cróinín, O. T., and Backert, S. (2012). Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front. Cell. Infect. Microbiol.* 2:25. doi: 10.3389/fcimb.2012.00025

Dasti, J. I., Tareen, A. M., Lugert, R., Zautner, A. E., and Gross, U. (2010). *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int. J. Med. Microbiol.* 300, 205–211. doi: 10.1016/j.ijmm.2009.07.002

Deckert, A., Valdivieso-Garcia, A., Reid-Smith, R., Tamblyn, S., Seliske, P., Irwin, R., et al. (2010). Prevalence and antimicrobial resistance in *Campylobacter* spp. isolated from retail chicken in two health units in Ontario. *J. Food Prot.* 73, 1317–1324. doi: 10.4315/0362-028X-73.7.1317

Deng, F., Shen, J., Zhang, M., Wu, C., Zhang, Q., and Wang, Y. (2015). Constitutive and inducible expression of the rRNA methylase gene erm(B) in *Campylobacter*. *Antimicrob. Agents Chemother.* 59, 6661–6664. doi: 10.1128/AAC.01103-15

Dingle, K. E., Colles, F. M., Wareing, D. R. A., Ure, R., Fox, A. J., Bolton, F. E., 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

Duarte, A., Seliwiorstow, T., Miller, W. G., De Zutter, L., Uyttendaele, M., Dierick, K., et al. (2016). Discriminative power of *Campylobacter* phenotypic and genotypic typing methods. *J. Microbiol. Methods* 125, 33–39. doi: 10.1016/j.mimet.2016.03.004

Eberle, K. N., and Kiess, A. S. (2012). Phenotypic and genotypic methods for typing *Campylobacter jejuni* and *Campylobacter coli* in poultry. *Poult. Sci.* 91, 255–264. doi: 10.3382/ps.2011-01414

EFSA-ECDC (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA J.* 14:4634. doi: 10.2903/j.efsa.2016.4634

Engberg, J. (2006). Contributions to the epidemiology of *Campylobacter* infections. A review of clinical and microbiological studies. *Dan. Med. Bull.* 53, 361–389.

French, N. P., Midwinter, A., Holland, B., Collins-Emerson, J., Pattison, R., Colles, F., et al. (2009). Molecular epidemiology of *Campylobacter jejuni* isolates from wild-bird fecal material in children's playgrounds. *Appl. Environ. Microbiol.* 75, 779–783. doi: 10.1128/AEM.01979-08

Gallay, A., Prouzet-Mauléon, V., Kempf, I., Lehours, P., Labadi, L., Camou, C., et al. (2007). *Campylobacter* antimicrobial drug resistance among humans, broiler chickens, and pigs, France. *Emerg. Infect. Dis.* 13, 259–266. doi: 10.3201/eid1302.060587

Giacomelli, M., Salata, C., Martini, M., Montesissa, C., and Piccirillo, A. (2014). Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* from poultry in Italy. *Microb. Drug Resist.* 20, 181–188. doi: 10.1089/mdr.2013.0110

Griekspoor, P., Colles, F. M., McCarthy, N. D., Hansbro, P. M., Ashhurst-Smith, C., Olsen, B., et al. (2013). Marked host specificity and lack of phylogeographic population structure of *Campylobacter jejuni* in wild birds. *Mol. Ecol.* 22, 1463–1472. doi: 10.1111/mec.12144

Griekspoor, P., Engvall, E. O., Olsen, B., and Waldenström, J. (2010). Multilocus sequence typing of *Campylobacter jejuni* from broilers. *Vet. Microbiol.* 140, 180–185. doi: 10.1016/j.vetmic.2009.07.022

Guerrant, R. L., Gilder, T., Van Steiner, T. S., Thielman, N. M., Slutsker, L., Tauxe, R. V., et al. (2001). Practice guidelines for the management of infectious diarrhea. *Clin. Infect. Dis.* 32, 331–350. doi: 10.1086/318514

Harrison, J. W., Dung, T. T. N., Siddiqui, F., Korbrisate, S., Bukhari, H., Vu Tra, M. P., et al. (2014). Identification of possible virulence marker from *Campylobacter jejuni* isolates. *Emerg. Infect. Dis.* 20, 1026–1029. doi: 10.3201/eid2006.130635

Harvala, H., Rosendal, T., Lahti, E., Engvall, E. O., Brytting, M., Wallensten, A., et al. (2016). Epidemiology of *Campylobacter jejuni* infections in Sweden, November 2011-October 2012: is the severity of infection associated with *C. jejuni* sequence type? *Infect. Ecol. Epidemiol.* 6:31079. doi: 10.3402/iee.v6.31079

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Med.* 12:e1001923. doi: 10.1371/journal.pmed.1001923

Hughes, L. A., Bennett, M., Coffey, P., Elliott, J., Jones, T. R., Jones, R. C., et al. (2009). Molecular epidemiology and characterization of *Campylobacter* spp. isolated from wild bird populations in northern england. *Appl. Environ. Microbiol.* 75, 3007–3015. doi: 10.1128/AEM.02458-08

Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi: 10.1128/CMR.00006-15

Klena, J. D., Parker, C. T., Knibb, K., Claire Ibbitt, J., Devane, P. M. L., Horn, S. T., et al. (2004). Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. *J. Clin. Microbiol.* 42, 5549–5557. doi: 10.1128/JCM.42.12.5549-5557.2004

Koolman, L., Whyte, P., Burgess, C., and Bolton, D. (2015). Distribution of virulence-associated genes in a selection of *Campylobacter* isolates. *Foodborne Pathog. Dis.* 12, 424–432. doi: 10.1089/fpd.2014.1883

Kwan, P. S., Birtles, A., Bolton, F. J., French, N. P., Robinson, S. E., Newbold, L. S., et al. (2008). Longitudinal study of the molecular epidemiology of *Campylobacter jejuni* in cattle on dairy farms. *Appl. Environ. Microbiol.* 74, 3626–3633. doi: 10.1128/AEM.01669-07

Lertpiriyapong, K., Gamazon, E. R., Feng, Y., Park, D. S., Pang, J., Botka, G., et al. (2012). *Campylobacter jejuni* type VI secretion system: roles in adaptation to deoxycholic acid, host cell adherence, invasion, and *in vivo* colonization. *PLoS One* 7:e42842. doi: 10.1371/journal.pone.0042842

Lim, S.-K., Moon, D.-C., Chae, M. H., Kim, H. J., Nam, H.-M., Kim, S.-R., et al. (2016). Macrolide resistance mechanisms and virulence factors in erythromycin-resistant *Campylobacter* species isolated from chicken and swine feces and carcasses. *J. Vet. Med. Sci.* 78, 1791–1795. doi: 10.1292/jvms.16-0307

Lindmark, B., Rompikuntal, P. K., Vaitkevicius, K., Song, T., Mizunoe, Y., Uhlin, B. E., et al. (2009). Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. *BMC Microbiol.* 9:220. doi: 10.1186/1471-2180-9-220

Louwen, R. P. L., Van Belkum, A., Wagenaar, J. A., Doorduyn, Y., Achterberg, R., and Endtz, H. P. (2006). Lack of association between the presence of the pVir plasmid and bloody diarrhea in *Campylobacter jejuni* enteritis. *J. Clin. Microbiol.* 44, 1867–1868. doi: 10.1128/JCM.44.5.1867-1868.2006

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C. M., and Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol.* 4, 189–200. doi: 10.2217/17460913.4.2.189

Moore, J., Corcoran, D., Dooley, J., and Lucey, B. (2005). *Campylobacter*. *Vet. Res.* 36, 351–382. doi: 10.1051/vetres:2005012

Novoa-Farías, O., Frati-Munari, C., Peredo, M., Flores-Juárez, S., Novoa-García, O., Galicia-Tapia, J., et al. (2016). Susceptibilidad de las bacterias aisladas de infecciones gastrointestinales agudas a la rifaximina y otros agentes antimicrobianos en México. *Rev. Gastroenterol. Mex.* 81, 3–10. doi: 10.1016/j.rgmx.2015.07.003

On, S. L. W., Nielsen, E. M., Engberg, J., and Madsen, M. (1998). Validity of SmaI-defined genotypes of *Campylobacter jejuni* examined by SalI, KpnI, and BamHI polymorphisms: evidence of identical clones infecting humans, poultry, and cattle. *Epidemiol. Infect.* 120, 231–237. doi: 10.1017/S0950268898008668

Pascoe, B., Méric, G., Murray, S., Yahara, K., Mageiros, L., Bowen, R., et al. (2015). Enhanced biofilm formation and multi-host transmission evolve from divergent genetic backgrounds in *Campylobacter jejuni*. *Environ. Microbiol.* 17, 4779–4789. doi: 10.1111/1462-2920.13051

Ramonaite, S., Tamuleviciene, E., Alter, T., Kasnauskyte, N., and Malakauskas, M. (2017). MLST genotypes of *Campylobacter jejuni* isolated from broiler products, dairy cattle and human campylobacteriosis cases in Lithuania. *BMC Infect. Dis.* 17:430. doi: 10.1186/s12879-017-2535-1

Ramos, R., Cerdà-Cuéllar, M., Ramírez, F., Jover, L., and Ruiz, X. (2010). Influence of refuse sites on the prevalence of *Campylobacter* spp. and *Salmonella* serovars in seagulls. *Appl. Environ. Microbiol.* 76, 3052–3056. doi: 10.1128/AEM.02524-09

Ridley, A. M., Morris, V. K., Cawthraw, S. A., Ellis-Iversen, J., Harris, J. A., Kennedy, E. M., et al. (2011). Longitudinal molecular epidemiological study of thermophilic campylobacters on one conventional broiler chicken farm. *Appl. Environ. Microbiol.* 77, 98–107. doi: 10.1128/AEM.01388-10

Schwarz, S., Silley, P., Simjee, S., Woodford, N., Van Duijkeren, E., Johnson, A. P., et al. (2010). Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* 65, 601–604. doi: 10.1016/j.vetmic.2009.12.013

Sheppard, S. K., Colles, F. M., McCarthy, N. D., Strachan, N. J. C., Ogden, I. D., Forbes, K. J., et al. (2011). Niche segregation and genetic structure of *Campylobacter jejuni* populations from wild and agricultural host species. *Mol. Ecol.* 20, 3484–3490. doi: 10.1111/j.1365-294X.2011.05179.x

Sheppard, S. K., Dallas, J. F., Macrae, M., Mccarthy, N. D., Gormley, F. J., Strachan, N. J. C., et al. (2009). Europe PMC funders group *Campylobacter* genotypes from food animals, environmental sources and clinical disease in Scotland 2005 / 6. *Int. J. Food Microbiol.* 134, 96–103. doi: 10.1016/j.ijfoodmicro.2009.02.010

Siddiqui, F., Champion, O., Akram, M., Studholme, D., Eqani, S. A., Wren, B. W., et al. (2015). Molecular detection identified a type six secretion system in *Campylobacter jejuni* from various sources but not from human cases. *J. Appl. Microbiol.* 118, 1191–1198. doi: 10.1111/jam.12748

Sopwith, W., Birtles, A., Matthews, M., Fox, A., Gee, S., Painter, M., et al. (2006). *Campylobacter jejuni* multilocus sequence types in humans, Northwest England, 2003-2004. *Emerg. Infect. Dis.* 12, 1500–1507. doi: 10.3201/eid1210.060048

Thépault, A., Méric, G., Rivoal, K., Pascoe, B., Mageiros, L., Touzain, F., et al. (2017). Genome-wide identification of host-segregating epidemiological markers for source attribution in *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 83:e03085-16. doi: 10.1128/AEM.03085-16

Tracz, D. M., Keelan, M., Ahmed-Bentley, J., Gibreel, A., Kowalewska-Grochowska, K., and Taylor, D. E. (2005). pVir and bloody diarrhea in *Campylobacter jejuni* enteritis. *Emerg. Infect. Dis.* 11, 838–843. doi: 10.3201/eid1106.041052

van Doorn, P., Ruts, L., and Jacobs, B. C. (2008). Clinical features, pathogenesis, and treatment of Guillain-Barre syndrome. *Lancet Neurol.* 7, 939–950. doi: 10.1016/S1474-4422(08)70215-1

Workman, S. N., Mathison, G. E., Marc, C., and Lavoie, M. C. (2005). Pet dogs and chicken meat as reservoirs of *Campylobacter* spp. in Barbados. *J. Clin. Microbiol.* 43, 2642–2650. doi: 10.1128/JCM.43.6.2642

Yahara, K., Méric, G., Taylor, A. J., de Vries, S. P. W., Murray, S., Pascoe, B., et al. (2017). Genome-wide association of functional traits linked with *Campylobacter jejuni* survival from farm to fork. *Environ. Microbiol.* 19, 361–380. doi: 10.11/1462-2920.13628

Yang, W., Zhang, M., Zhou, J., Pang, L., Wang, G., and Hou, F. (2017). The molecular mechanisms of ciprofloxacin resistance in clinical *Campylobacter jejuni* and their genotyping characteristics in Beijing, China. *Foodborne Pathog. Dis.* 14, 386–392. doi: 10.1089/fpd.2016.2223

Young, K. T., Davis, L. M., and Dirita, V. J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* 5, 665–679. doi: 10.1038/nrmicro1718

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: 24 August 2018
doi: 10.3389/fmicb.2018.02002

A Review of the Effect of Management Practices on *Campylobacter* Prevalence in Poultry Farms

Nompilo Sibanda[1,2], Aaron McKenna[1,2], Anne Richmond[2], Steven C. Ricke[3], Todd Callaway[4], Alexandros Ch. Stratakos[5], Ozan Gundogdu[6] and Nicolae Corcionivoschi[5]**

[1] School of Biological Sciences, Queen's University Belfast, Belfast, United Kingdom, [2] Moy Park, Ltd., Craigavon, United Kingdom, [3] Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [4] Department of Animal and Dairy Science, University of Georgia, Athens, GA, United States, [5] Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, United Kingdom, [6] London School of Hygiene and Tropical Medicine, London, United Kingdom

OPEN ACCESS

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

Reviewed by:
Odile Tresse, INRA, Centre Angers-Nantes Pays de la Loire, France
Robin Anderson, Agricultural Research Service (USDA), United States

***Correspondence:**
Nompilo Sibanda
Nompilo.Sibanda@moypark.com
Nicolae Corcionivoschi
nicolae.corcionivoschi@afbini.gov.uk

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

Received: *04 June 2018*
Accepted: *08 August 2018*
Published: *24 August 2018*

Citation:
Sibanda N, McKenna A, Richmond A, Ricke SC, Callaway T, Stratakos AC, Gundogdu O and Corcionivoschi N (2018) A Review of the Effect of Management Practices on Campylobacter Prevalence in Poultry Farms. Front. Microbiol. 9:2002. doi: 10.3389/fmicb.2018.02002

Poultry is frequently associated with campylobacteriosis in humans, with *Campylobacter jejuni* being the most usual *Campylobacter* associated with disease in humans. Far-reaching research on *Campylobacter* was undertaken over the past two decades. This has resulted in interventions being put in place on farms and in processing plants. Despite these interventions, coupled with increased media coverage to educate the consumer on *Campylobacter* prevalence and campylobacteriosis, human health incidents are still high. Recent research is now shifting toward further understanding of the microorganisms to challenge interventions in place and to look at further and more relevant interventions for the reduction in human incidents. Farm practices play a key role in the control of colonization within poultry houses and among flocks. Prevalence at the farm level can be up to 100% and time of colonization may vary widely between flocks. Considerable research has been performed to understand how farm management and animal health practices can affect colonization on farms. This review will focus on farm practices to date as a baseline for future interventions as the microorganism becomes better understood. Further research is required to understand the chicken microbiome and factors influencing vertical transmission. The persistence of *Campylobacter* in animal and environmental reservoirs within and around farms requires further investigation to tailor farm practices toward preventing such reservoirs.

IMPLICATIONS

This review gives an overview of farm practices and their effect on *Campylobacter* prevalence in poultry. Various elements of farm practices have been captured in this review.

Keywords: *Campylobacter*, poultry, farm practices, biosecurity, management

INTRODUCTION

Campylobacter, as a genus are corkscrew shaped and motile Gram negative microorganisms with an optimal growth at 42°C. They are generally isolated from the intestines of cattle, sheep, swine, and the poultry caecum. Because of a higher body temperature poultry and other avian species are the most common food animal that hosts for *Campylobacter* spp., representing the main source of infection for humans (Silva et al., 2011). While three *Campylobacter* species (*C. jejuni*, *C. coli,* and *C. lari*) are related to the poultry digestive system and to foodborne infections, *Campylobacter jejuni* is considered the dominant specie in relation to its impact on human health (Shane, 1992; Corcionivoschi et al., 2012; Cean et al., 2015; Ugarte-Ruiz et al., 2018). A limited number of studies have reported possible negative health implications in chickens caused by *C. jejuni* colonization of the gut, therefore this bacterium is considered to have a near-commensal relationship with the chicken (Thibodeau et al., 2015). Only a few *Campylobacter* cells from undercooked or raw chicken are able to cause human illness (Bhaduri and Cottrell, 2004) and over 100 deaths in the United Kingdom annually, with an estimated cost of £1 billion to the United Kingdom National Health Service (Tam and O'Brien, 2016). Most human cases of campylobacteriosis in the United Kingdom are attributed to contaminated poultry (Skarp et al., 2016; Lee et al., 2017; Oh et al., 2017). However, Lynch et al. (2011), reported that most *Campylobacter* in their study were isolated from beef (36% prevalence), pork (22%), and chicken (16%). *Campylobacter* has also been found in unpasteurised milk and untreated water. Because of the common nature of this pathogenic threat, a broad-based approach in all segments of the food industry has been taken in the United Kingdom to reduce incidents of campylobacteriosis. Efforts have been made across the supply chain; including farms, processing plants, retailers, and through educating the public. Rejection of poultry carcasses at the slaughter for pathological reasons was it has been reported to be certainly associated also with *Campylobacter* presence (Powell et al., 2012). Collectively, results suggest that *Campylobacter* is often present in broilers in a poor health condition, therefore suggesting that the mortality rate could be an efficiency marker for farm management practices and biosecurity. However, because there is not a linear trend in the data, it suggests that when greater mortality rates are recorded, then antibiotics are prescribed which impacts upon *Campylobacter* colonization and or contamination.

Powell et al. (2012) surveyed 25 United Kingdom abattoirs which represented 88% of the total United Kingdom poultry production, reported that 75% of chicken carcasses were contaminated with *Campylobacter* from feces, and that 87% of this contamination occurred at slaughter. In the most recent survey by Public Health England (2015), the prevalence of *Campylobacter* spp. In the United Kingdom, within fresh chicken at the retail level was 73.3%. A significant proportion (19.4%) of the positive chicken carcasses had levels of >1000 CFU/g of chicken skin. Also, a higher percentage of positive chickens were contaminated with higher levels of *Campylobacter* spp. during the summer compared to winter period. Speciation revealed that *C. jejuni* was identified in most of the positive chicken skin samples (76.6%) whereas *C. coli* was present in only 13.9% of samples. It was suggested by Powell et al. (2012) that a 2-log reduction in the number of *Campylobacter* on chicken carcasses would lead to a 30-fold drop in human cases connected to contaminated chicken meat. *Campylobacter* are able to survive in different farm environments and have been found in the air, soil, water, dust, and abiotic surfaces (Bull et al., 2006; Ellis-Iversen et al., 2009).

Most transmission to poultry occurs from the environment, as well as through horizontal transmission between flock mates, but once *Campylobacter* colonizes a broiler flock, the spread is fast making an eradication approach impossible. A well-designed and planned biosecurity approach at farm level have been established as a fundamental method to counteract colonization of flocks (Georgiev et al., 2017). The present review will focus on the avian management practices on poultry farms and what effect these have on *Campylobacter* prevalence.

BIOSECURITY

Biosecurity is an important measure for the control of *Campylobacter* because once colonization occurs in a poultry flock the horizontal transmission can be rapid (Battersby et al., 2016). A study by Gibbens et al. (2001) demonstrated that well-implemented disinfection protocols could lead to a decrease in *Campylobacter* prevalence from 80 to less than 40% in broilers (Gibbens et al., 2001). Installing hygienic barriers between internal and external environments, controlling the entry of personnel, strict hygiene rules (handwashing and sanitizing hands), changing boots and overalls before entering have been shown to be effective (Silva et al., 2011). A literature review conducted from 1980 to 2008 concluded that high standards in biosecurity measures should contribute to the reduction of flock *Campylobacter* colonization (Newell and Fearnley, 2003). Risk factors with respect to increased *Campylobacter* colonization include: deprived farm hygiene, a reduced flock replacement period, the presence of other farm animals, rodents and insects, seasonal changes and partial depopulation (Russa et al., 2005). Hygiene and physical barriers are specific biosecurity measures which should be efficient in protecting poultry against *Campylobacter*. Increased biosecurity measures are always associated with absence of *Campylobacter* in poultry however, the impact is difficult to measure (Lin, 2009). Controlling *Campylobacter* on the farm is complex as the pathogen can persist in a variety of environments and hosts that can be found on the farm (Lynch et al., 2011).

Campylobacter can be carried into the broiler house via boots, clothes and equipment. The use of dedicated footwear has proven to be the most important risk factor in a Danish study (Newell and Fearnley, 2003). Other factors that affected biosecurity measures were the greater number of people working on the farm increased the chances of biosecurity breaches (Newell et al., 2011). Biosecurity measures are to include equipment such as sanitizing of buckets used to lift dead birds and any equipment brought into the house (Newell and Fearnley, 2003).

Hygiene measures by on farm personnel include handwashing, use of separate boots for each house, use of footbath disinfectants, limited access to key personnel only, pest control and staff training (Sahin et al., 2015). The entrance of the catching crew and catching equipment is often a serious breach of biosecurity because external sources posing a 21% contamination rate, this is a vehicle for introducing contamination, particularly during depopulation (Ellis-Iversen et al., 2012). Farm emergency procedures should also consider the biosecurity measures to ensure these are adhered consistently. Staff training is key to the successful implementation of these biosecurity measures at all times, but is most especially impactful in an emergency (Sahin et al., 2015). An observational study in Canada reported that an average of four biosecurity breaches occurred per farm visit. Out of all the breaches, 61% of faults were linked to cross contamination of clean and contaminated zones, 14% related to improper use of footwear, 11% inadequate hand washing, and 7% unclean overalls. This high error rate may be attributed to lack of training or lack of understanding of consequences amongst other reasons (Battersby et al., 2016).

The efficient use of boot baths increased the interval to infection considerably. The presence of boot baths at the entrance of the broiler house is considered a risk factor (Borck Hog et al., 2016) and for these to be efficient, high maintenance with disinfection replenishment being done at regular intervals. If not well-maintained the footbaths will increase the risk rather than act as a defensive barrier. Disinfectants should be replaced weekly or if the dilution is reduced (due to rain water) or if there is organic matter build up at the bottom of the foot bath, they must be replenished (Evans and Sayers, 2000).

Strict biosecurity and farm hygiene measures have been shown to be linked with reduced *Campylobacter* positive flocks (Newell et al., 2011). However, the most severe biosecurity controls are not most efficient in preventing the presence of *Campylobacter* (Russa et al., 2005) because they are dependent on personnel following the correct protocols at all times. Even when the most stringent controls are followed, there may still be contamination of door handles and other contact areas (fomites) that are often misunderstood. This may result in the contamination of a new flock during chick placement, which ensures that the pathogen has a long time to spread horizontally within a new "clean" flock during the grow out phase. Biosecurity measures are also not always cheap to implement, and the purchase cost may be prohibitive. This includes both the cost of equipment to have the biosecurity barriers as well as the cost of time for farm personnel in implementing these measures. On evaluating the cost effectiveness of *Campylobacter* interventions, van Wagenberg et al. (2016) concluded that the most cost effective procedure was to apply barriers in each house and to utilize dedicated tools for each house to minimize the cross-contamination risk; however, the most cost effective intervention was the ban of partial depopulation and introducing the all-in/all-out system at approximately day 35. Hygiene barriers proved effective to some extent in preventing *Campylobacter* infection in broiler flocks. These results suggested that expanding the hygiene barriers to include gates, vehicle disinfection, respecting biosecurity measures during catching would increase *Campylobacter* reduction, however the value (cost effectiveness) of these additional efforts would not be detectable in the final results (Hald et al., 2000). Preventing farm staff from direct contact with the broilers has been shown to protect the broilers from *Campylobacter* infection (Battersby et al., 2016). A study by Ridley et al. (2011) found *Campylobacter* matching the subsequent flock type found in the catcher lunch bags, further emphasizing the risk of employees bringing personal items onto the farm (Ridley et al., 2011).

Harmonization, benchmarking, and implementation of universal controls for biosecurity measures across different countries is difficult due to the different farm practices, housing, production stages, and equipment designs (Sahin et al., 2015). A study conducted on Danish farms concluded that factors like the oldness of the poultry house, rodent control, the age of broiler at slaughter, storage of whole wheat, number of chimneys on the broiler house, the location of the broiler farm in relation to cattle density are very important in controlling *Campylobacter* presence. However, it must also be taken in account that these observations are specific to Danish farm practices and may differ from one country to the next, however, they are a valuable place to begin understanding the value of proper biosecurity appreciation and implementation, as well as some of the challenges that underpin implementation on every farm world-wide (Sommer et al., 2013).

Ridley et al. (2011) carried out a study to compare *Campylobacter* prevalence in normal farm practices versus enhanced biosecurity farm practices. Findings revealed that although there was a reduction in *Campylobacter* presence with enhanced biosecurity measures (up to 35% reduction in vehicles), the enhanced biosecurity measures were insufficient to prevent flock colonization. Unfortunately, once a flock is infected, it is very difficult to eliminate *Campylobacter* in a flock, because chickens (along with other species of animals) are carriers and major reservoirs of *Campylobacter*. They act as passive transmitters and amplifiers via the spread of fecal contamination. Following the entry of *Campylobacter* into a newly occupied house all the birds can become positive within a week based on cloacae swabs, demonstrating that *Campylobacter* colonization spreads very rapidly amongst housed broiler chickens (Evans and Sayers, 2000). Results of a questionnaire study by Hald et al. (2000) indicated that other animals located in the intermediate vicinity of the broiler house posed a significant risk to broiler flocks in terms of *Campylobacter* colonization. It is strongly suggested that a farmer tending both cattle and poultry on the same farm transmitted *Campylobacter* from cattle to poultry (and vice versa) on his/her boots (van de Giessen et al., 1998) Farm personnel and equipment (e.g., feed trucks) can carry *Campylobacter* between broiler houses and onto subsequent or neighboring farms (Newell et al., 2011). In the absence of infected neighbors in 2 km radius of susceptible farm, in the same month, showed a significant protective effect in comparison with presence of infected neighbors in the same distance and time (Chowdhury et al., 2012). Livestock and broiler farms with flocks positive for *Campylobacter* spp. within a few kilometers distance, as well as heavy rainfall events constitute significant risks for colonization in broiler flocks (Jonsson et al., 2012).

Rainfall events may also result in unintentional and unrecognized breaches of biosecurity measures, increasing the routes by which *Campylobacter* can colonize broiler flocks.

PARTIAL DEPOPULATION

Thinning of a flock is a significant threat to biosecurity and increases the risk of *Campylobacter* contamination by compromising isolation of birds that are not depopulated within a house or a flock. Most of previously declared as *Campylobacter* free broilers can be rapidly contaminated during the process of thinning (Sahin et al., 2015). A study conducted in Ireland revealed that 85% of flocks were positive at depopulation, and their results identified thinning as a significant risk factor for *Campylobacter* introduction and the authors provided the suggestion was that partial depopulation should be discontinued (Smith et al., 2016). The importance of thinning was also emphasized in a trial conducted by Ellis-Iversen et al. (2012) which showed that 83% of the *Campylobacter* positive flocks are associated with this process of partial depopulation and only 43% are identified as positive in the absence of thinning. Age of birds and depopulation are closely associated, making it difficult to be certain which of these two factors affects *Campylobacter* prevalence most significantly, but more recently it has been shown that seasonality is also an important factor on the prevalence that *Campylobacter* in broiler flocks that have not been thinned (Jorgensen et al., 2011). Russa et al. (2005) suggested that there was a link between age at depopulation and *Campylobacter* prevalence. Although the method used in their investigation is not clear, the results show that the proportion of *Campylobacter* positive flocks increased with increasing age. They also detected a link between the proportion of *Campylobacter* positive flocks to weather where higher numbers were seen in the autumn (Russa et al., 2005). Live bird crates that were contaminated with *Campylobacter* from previous (or other) flocks are reintroduced on the farm during catching, and quite often these crates undergo inadequate washing at the slaughter house (Newell et al., 2011). Crates can carry identical genotypes of microorganisms which originate from broiler flocks and abattoirs, which suggests that transport crates are responsible for contamination during transport to slaughter or they could contribute to the *Campylobacter* colonization of broiler houses (Hastings et al., 2011). Research has shown that *Campylobacter* can survive on crates post-sanitization (Hansson et al., 2005; Allen et al., 2008). Results from the survey by Powell et al. (2012) showed company specific risk factors or probable recurrence of strains within a company, this warrants further investigation.

CLEANING AND DISINFECTION

Due to the intensive cleaning and disinfection that is often between flocks it is difficult to predict *Campylobacter* infection from the status of previous flocks. When farms remove litter between grow-out periods, it is often found that negative flocks follow positive ones. The presence of colonized flocks was linked to the turnaround time in a house. Periods of over 14 days can decrease the possibility of residual bacterial contamination (Newell et al., 2011). The benefit of longer turnaround periods is also supported by Battersby et al. (2016) who state that rapid flock turnover contributes to *Campylobacter* carry over with increased risk being reported if houses are restocked within 9 days of depopulation. A study by Jonsson et al. (2012) also investigated the effect of the length of time the house was empty. Based on a small data set, the study showed that keeping the broiler house empty for less than 9 days would increase the risk for *Campylobacter* spp. Also, if the empty time is extended the risk of introducing *Campylobacter* into the houses is kept low only if the biosecurity and hygiene levels are maintained optimal (Borck Hog et al., 2016). It is well-known that an external reservoir can host multiple *Campylobacter* strains, during the empty period, which will allow colonization of the new flock (Ellis-Iversen et al., 2012).

Using real time PCR Battersby et al. (2016) first detected *Campylobacter* before chick arrival in both internal and external broiler house samples but many of the flocks were negative for *Campylobacter* prior to slaughter. These results indicate that only *Campylobacter* DNA was detected or *Campylobacter* were present in a viable but non-culturable (VBNC) state. In this VBNC state *Campylobacter* can survive for at least 7 months (Lázaro et al., 1999). Further research is required to understand the role of VBNC cells in *Campylobacter* cross contamination.

When looking at persistent external reservoirs, Ellis-Iversen et al. (2012) found that contaminated shed entrances, anterooms and drinkers and shedding of *Campylobacter* by other animals (e.g., cattle, dogs, rodents) have been found to be linked to positive flocks. In order to reduce the risk of *Campylobacter* introduction into the shed they have suggested disinfecting the surroundings of the poultry shed around day 25 of the cycle. Other reservoirs of contamination include, vehicles, equipment used by catchers and catching crews (Ellis-Iversen et al., 2012).

ANTIBIOTIC USAGE

Antibiotics are widely utilized in poultry production around the world to improve production efficiency, but they can also impact the microbial population of the gut, including populations of pathogenic bacteria. Campylobacteriosis is a zoonotic foodborne illness, therefore presence of antibiotic resistant *Campylobacter* strains could also impact on human health. It has been indicated that the usage of fluoroquinolones in poultry farms is associated with increased resistance in chicken and human *Campylobacter* isolates (Wieczorek and Osek, 2013).

Most campylobacteriosis patients will not require specific treatment other than fluid replacements but there are situations in which antibiotics, such as tetracycline, fluoroquinolones were used (Silva et al., 2011). The resistance of *Campylobacter* strains to these antibiotics compromises the effectiveness of human treatments. In countries where the use of antibiotics in broiler production well-controlled it is unlikely to have a high prevalence of resistant strains (Norstrom et al., 2007). However, it has been shown that the use of these antibiotics as the first line

of treatment in humans led to the development of significant resistance and their efficacy should be re-analyzed (Shobo et al., 2016).

In the E.U. in 2013, it has been reported that ciprofloxacin resistance among human *Campylobacter* isolates ranged from 23% in Denmark to 92%, in Spain, however, the resistance among broiler isolates ranged from 0% in Finland to 90% in Spain (European Food Safety Authority and European Centre for Disease Prevention and Control, 2015). A promising strategy in reducing *Campylobacter*, at gut level, could be achieved by the supplementation with additives in feed that could reduce pathogen caecal colonization by exacting an effect on *Campylobacter* themselves or by altering the chicken caecal microbiome toward a composition that will not favor *Campylobacter* growth and/or survival. Essential oils and organic acids are known as effective in reducing the colonization levels in broiler chickens. According to Grilli et al. (2013) a blend of propionic and sorbic acid, and eugenol and thymol significantly reduced *C. jejuni* in slaughter-age chickens even at low doses (0.1%). Hermans et al. (2010) also investigated the effect of caproic, caprylic, and capric acids against *C. jejuni.* Although, *in vitro* results showed promising antimicrobial activity, *in vivo* testing showed that there was not a reduction in *C. jejuni* when they were included in the feed, 3 days before they were sacrificed. Thibodeau et al. (2015) investigated the chicken caecal microbiome to establish the effect of a non-antibiotic feed additive (mixture of short chain organic acids and phenolic essential oils on *C. jejuni* colonization). Their study concluded that the microbiome is not extensively disturbed when colonized by *C. jejuni.* The investigation also concluded that the feed additive used could significantly reduce *C. jejuni* colonization without significantly affecting the gut microbiome of the chicken.

SEASONALITY OF INFECTIONS OR CARRIAGE

The public health burden due to campylobacteriosis cases necessitates the characterization of the seasonal patterns of *Campylobacter* contamination since the possibility of illness increases with increasing levels of contamination. The prevalence of *Campylobacter* in chickens has been found to be associated with seasonality (Taylor et al., 2013; Friedrich et al., 2016). In western countries with temperate climates seasonal peaks of human campylobacteriosis are observed between July and August. The summer peaks in human infection are consistent with higher *Campylobacter* isolation levels from chickens in the summer period, compared to winter, with the human infection peaks preceding the chicken one suggesting a link between the two (Skarp et al., 2016). The reasons behind *Campylobacter* seasonality are not clear yet. However, an increase in pathogen reservoirs, changes in human behavior and climate can influence the shedding and transmission of the pathogen. There is a clear risk level of acquiring campylobacteriosis between rural and urban regions and this risk must be taken in consideration (Deckert et al., 2014; Williams et al., 2015).

Research has also shown that the sources of environmental exposure are season dependent with flies being a common vehicle of transmission between the environment and food (Ekdahl et al., 2005). The use of fly screens ventilation openings was recently described as an efficient method to reduce the number of *Campylobacter* positive flocks (Sahin et al., 2015). These findings confirm that flies serve as a vector particularly during the summer months when temperatures are high (Sahin et al., 2015). In a study conducted in Denmark, the use of fly screens reduced the prevalence of *Campylobacter* from 41.4 to 10.3% in 10 farms. A cluster of farms within 4 km from contaminated flocks were also positive. This is believed to be due to carriers such as flies, however, flies caught from four ante-rooms in houses with *Campylobacter* free chickens were always negative (Berndtson et al., 1996). Four fly samples from positive flocks in the same study were positive. *Campylobacter* spp., have been isolated from beetles originating form poultry farms (Hald et al., 2000). The isolation of *Campylobacter* from the small intestine and the caeca also exhibited a seasonal pattern with a specific increase during the summer months. This pattern was also confirmed in a review of performed over a period of 10 years in Europe in six countries (Jore et al., 2010). Approximately, 2.1–3.5% of annual human campylobacteriosis are associated with wild birds or wild bird infected sources (Cody et al., 2015). This issue is also very much related to seasonality as it has been shown that 50% of the wild bird fecal samples, collected during winter, were contaminated with *C. jejuni* weather none of the samples collected in summer months were contaminated (Craven et al., 2000).

Temperature is correlated with *Campylobacter* spp., colonization of broilers in a study by Jonsson et al. (2012). Daily mean temperatures, above 6°C, had an important role in the colonization with a more significant effect accentuated by increasing temperatures. The same study also showed that below 0°C reduces the probability for a chicken flock to be positive for *Campylobacter*. *Campylobacter* spp. are known as sensitive to low temperatures which could explain the incremental effect caused by the increase in environmental temperatures.

WATER

Campylobacter has a high survival rate in water and thus can contaminate water reservoirs following translocation from pastures of grazing animals. Water chlorination appears to be very effective against *Campylobacter* (Newell and Fearnley, 2003; Hutchison et al., 2004). Only 18.8% campylobacters strains isolated from surface water in Louxembourg can be attributed to poultry, 61% to wild birds and 20.2% to other farm animals (Mughini-Gras et al., 2016). Spreading of animal feces on land was not correlated to increased presence of Campylobacter in surface waters (Sterk et al., 2016). Contamination of water in the broiler house usually follows colonization of a flock indicating that this is caused by contamination of water lines with microorganisms excreted from the birds. Water treatments need to consider the resistance of water borne protozoa such as *Tetrahymena pyriformis* which act as reservoirs for *C. jejuni* (Newell et al., 2011). The addition of organic acids has been

examined and observations suggest that these provide partial effect in *Campylobacter* colonization and transmission. They can be used as part of ahurdle approach in conjunction with other measures (Sahin et al., 2015).

Drinker systems with nipples without cups were shown to be better than nipples with cups and bells in reducing the presence of *Campylobacter* in both Danish and Norwegian broiler flocks. *Campylobacter* may be introduced into the reservoirs of drinker systems with cups and bells via colonized birds or their droppings, allowing the water to become a dissemination vehicle (Borck Hog et al., 2016). Using municipal sources of water was deemed to be the best as opposed to using wells or boreholes. Private water supply was significantly linked to higher risk of *Campylobacter* contamination compared to the public water supply (Jonsson et al., 2012), however, this was not always the case (Hald et al., 2000).

VERTICAL TRANSMISSION AND LAG PHASE

Most of the investigations to date have focused on horizontal transmission and farm management practices have been the primary focus of investigations aimed at finding solutions to reduce *Campylobacter*. There is sufficient evidence to support the vertical transmission of *Salmonella* (which can be transmitted via the eggs) however in the case of *Campylobacter* this evidence is absent (Newell and Fearnley, 2003). This statement is supported by the lack of *Campylobacter* colonization during the first weeks in the life of broilers hatched form eggs originated from breeder flocks infected with *Campylobacter*. This is further supported by the different genotypes in broilers in comparison to breeders that the eggs have been hatched from. However, *C. jejuni* can penetrate egg shells, indicating that contact with fecal material could contaminate the shells (Allen and Griffiths, 2001). Research has shown that *C. jejuni* can colonize egg contents by both oviduct colonization and fecal contamination of egg shells (Cox et al., 2012).

Further research is required to understand vertical transmission risks and measures required to reduce these risks throughout the supply chain. Chicks identified as negative on hatching remain negative until the lag phase (around 10 days of age). It is thought that there are other protective factors such as immunity that protect the chicks up to this stage. Further investigation into the physiological changes of the birds and the effect of changes in farm practices such as changes in feed composition, is required during this lag phase (Newell et al., 2011). Berndtson et al. (1996) have shown that *Campylobacter* are not detected in samples from newly hatched, 1 week old chicks or their drinkers. Artificial infection of 1 day old chicks with an invasive strain can cause diarrhea within 24 h (Berndtson et al., 1996). As described above the appearances of gastrointestinal disorders are associated with the age of the host as infection at 3 days of age with 10^9 organisms failed to produce any detectable clinical change. This has also been confirmed in a longitudinal study showing the effect of age on colonization. The risk of flock infection increased with age. Several reports suggest that the immune support in the first 2 weeks plays a part in this. The environmentally stressed campylobacters are poor colonizers and they normally require an *in vivo* passage to enhance their colonization potential (Evans and Sayers, 2000).

Samples collected from the environment, air, feed, water, meconium and the fecal pools, of 2 and 7 old chickens, have always been identified to be negative by Damjanova et al. (2011). In contrast all flocks became colonized by *Campylobacter* between the third and sixth week of rearing. All finished flocks were found to be colonized.

LOWER RISK FACTORS

Feed is not seen as a high-risk *Campylobacter* contaminant within the broiler house. This is because the low water activity of the dry feed does not permit *Campylobacter* survival. The feed however can be a vehicle for horizontal transmission into the broiler house (Silva et al., 2011). Hald et al. (2000) showed that the incidence of Campylobacter was lower in farms that fed home grown wheat compared to farms that are depended of external supplies (Hald et al., 2000).

Chickens are known to ingest litter and research has shown that a significant of litter is consumed form the floor by the animals (Svihus et al., 2009). A study Torok et al. (2009) has shown that the caecal microbiota of chickens farmed on reused litter differed from that of chickens farmed on any of the other litter materials. Fresh litter is deemed to be low risk due to the low moisture content (Newell et al., 2011). In recent studies to show a controlled comparison, 60% of chickens in reused litter were positive for *Campylobacter*, compared to 33% in fresh litter. This observation suggests that used litter can act as a pool and source of *Campylobacter* (Sahin et al., 2015).

CONCLUSION

The understanding of epidemiology and ecology of *Campylobacter* in poultry has been significantly improved over the past years. A minimum of 40 cells can constitute a successful infectious dose in chickens (Chen et al., 2006). Horizontal transmission is considered the main source of *Campylobacter* infection in poultry. *Campylobacter* are ubiquitous in the environment and can be transferred into the poultry farm in several different ways. The potential role of climatic factors as well as routine flock management practices have also to be taken under consideration. Increased biosecurity to minimize *Campylobacter* contamination should be of paramount importance during the summer period and when chicken flocks are thinned. The high prevalence of *Campylobacter* positive flocks and human cases of campylobacteriosis suggest that current biosecurity procedures are inadequate in ensuring *Campylobacter* negative flocks. Pre-harvest control measures in farms can help reduce *Campylobacter* dissemination in the environment, on farms and the food chain. Since no single

contamination vehicle or route is responsible for *Campylobacter* contamination the different vehicles or routes should be tackled simultaneously. An integrated system that incorporates multiple pre- and post-harvest interventions as well as flock management interventions is necessary in order to reduce the risk from *Campylobacter* infections linked to consumption and/or handling of contaminated poultry meat. Maintaining such control measures on the farm can be difficult and it should be reinforced with farm worker education and incentives.

AUTHOR CONTRIBUTIONS

NS, AM, AR, SR, TC, AS, OG, and NC wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work has been funded through a research grant awarded to NC by Moy Park.

REFERENCES

Allen, K. J., and Griffiths, M. W. (2001). Use of luminescent *Campylobacter jejuni* ATCC 33291 to assess eggshell colonization and penetration in fresh and retail eggs. *J. Food Prot.* 64, 2058–2062. doi: 10.4315/0362-028X-64.12.2058

Allen, V. M., Burton, C. H., Wilkinson, D. J., Whyte, R. T., Harris, J. A., Howell, M., et al. (2008). Evaluation of the performance of different cleaning treatments in reducing microbial contamination of poultry transport crates. *Br. Poult. Sci.* 49, 233–240. doi: 10.1080/00071660802094206

Battersby, T., Whyte, P., and Bolton, D. J. (2016). The pattern of Campylobacter contamination on broiler farms; external and internal sources. *J. Appl. Microbiol.* 120, 1108–1118. doi: 10.1111/jam.13066

Berndtson, E., Danielsson-Tham, M. L., and Engvall, A. (1996). *Campylobacter* incidence on a chicken farm and the spread of Campylobacter during the slaughter process. *Int. J. Food Microbiol.* 32, 35–47. doi: 10.1016/0168-1605(96)01102-6

Bhaduri, S., and Cottrell, B. (2004). Survival of cold-stressed *Campylobacter jejuni* on ground chicken and chicken skin during frozen storage. *Appl. Environ. Microbiol.* 70, 7103–7109. doi: 10.1128/AEM.70.12.7103-7109.2004

Borck Hog, B., Sommer, H. M., Larsen, L. S., Sorensen, A. I., David, B., Hofshagen, M., et al. (2016). Farm specific risk factors for *Campylobacter* colonisation in Danish and Norwegian broilers. *Prev. Vet. Med.* 130, 137–145. doi: 10.1016/j.prevetmed.2016.04.002

Bull, S. A., Allen, V. M., Domingue, G., Jørgensen, F., Frost, J. A., Ure, R., et al. (2006). Sources of *Campylobacter* spp. colonizing housed broiler flocks during rearing. *Appl. Environ. Microbiol.* 72, 645–652. doi: 10.1128/AEM.72.1.645-652.2006

Cean, A., Stef, L., Simiz, E., Julean, C., Dumitrescu, G., Vasile, A., et al. (2015). Effect of human isolated probiotic bacteria on preventing *Campylobacter jejuni* colonization of poultry. *Foodborne Pathog. Dis.* 12, 122–130. doi: 10.1089/fpd.2014.1849

Chen, L., Geys, H., Cawthraw, S., Havelaar, A., and Teunis, P. (2006). Dose response for infectivity of several strains of *Campylobacter jejuni* in chickens. *Risk Anal.* 26, 1613–1621. doi: 10.1111/j.1539-6924.2006.00850.x

Chowdhury, S., Sandberg, M., Themudo, G. E., and Ersbøll, A. K. (2012). The effect of presence of infected neighbouring farms for the *Campylobacter* infection status in Danish broiler farms. *Spat. Spatiotemporal Epidemiol.* 3, 311–322. doi: 10.1016/j.sste.2012.06.001

Cody, A. J., Mccarthy, N. D., Bray, J. E., Wimalarathna, H. M., Colles, F. M., Jansen Van Rensburg, M. J., et al. (2015). Wild bird-associated *Campylobacter jejuni* isolates are a consistent source of human disease, in Oxfordshire, United Kingdom. *Environ. Microbiol. Rep.* 7, 782–788. doi: 10.1111/1758-2229.12314

Corcionivoschi, N., Alvarez, L. A. J., Sharp, T. H., Strengert, M., Alemka, A., Mantell, J., et al. (2012) Mucosal reactive oxygen species decrease virulence by disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host Microbe* 12, 47–59. doi: 10.1016/j.chom.2012.05.018

Cox, N. A., Richardson, L. J., Maurer, J. J., Berrang, M. E., Fedorka-Cray, P. J., Buhr, R. J., et al. (2012). Evidence for horizontal and vertical transmission in Campylobacter passage from hen to her progeny. *J. Food Prot.* 75, 1896–1902. doi: 10.4315/0362-028.JFP-11-322

Craven, S. E., Stern, N. J., Line, E., Bailey, J. S., Cox, N. A., and Fedorka-Cray, P. (2000). Determination of the incidence of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal droppings. *Avian Dis.* 44, 715–720. doi: 10.2307/1593118

Damjanova, I., Jakab, M., Farkas, T., Meszaros, J., Galantai, Z., Turcsanyi, I., et al. (2011). From farm to fork follow-up of thermotolerant campylobacters throughout the broiler production chain and in human cases in a Hungarian county during a ten-months period. *Int. J. Food Microbiol.* 150, 95–102. doi: 10.1016/j.ijfoodmicro.2011.07.011

Deckert, A. E., Taboada, E., Mutschall, S., Poljak, Z., Reid-Smith, R. J., Tamblyn, S., et al. (2014). Molecular epidemiology of *Campylobacter jejuni* human and chicken isolates from two health units. *Foodborne Pathog. Dis.* 11, 150–155. doi: 10.1089/fpd.2013.1610

Ekdahl, K., Normann, B., and Andersson, Y. (2005). Could flies explain the elusive epidemiology of campylobacteriosis? *BMC Infect. Dis.* 5:11. doi: 10.1186/1471-2334-5-11

Ellis-Iversen, J., Jorgensen, F., Bull, S., Powell, L., Cook, A. J., and Humphrey, T. J. (2009). Risk factors for *Campylobacter* colonisation during rearing of broiler flocks in Great Britain. *Prev. Vet. Med.* 89, 178–184. doi: 10.1016/j.prevetmed.2009.02.004

Ellis-Iversen, J., Ridley, A., Morris, V., Sowa, A., Harris, J., Atterbury, R., et al. (2012). Persistent environmental reservoirs on farms as risk factors for *Campylobacter* in commercial poultry. *Epidemiol. Infect.* 140, 916–924. doi: 10.1017/S095026881100118X

European Food Safety Authority and European Centre for Disease Prevention and Control (2015). EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. *EFSA J.* 13:4036. doi: 10.2903/j.efsa.2015.4036

Evans, S. J., and Sayers, A. R. (2000). A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. *Prev. Vet. Med.* 46, 209–223. doi: 10.1016/S0167-5877(00)00143-4

Friedrich, A., Marshall, J. C., Biggs, P. J., Midwinter, A. C., and French, N. P. (2016). Seasonality of Campylobacter jejuni isolates associated with human campylobacteriosis in the Manawatu region, New Zealand. *Epidemiol. Infect.* 144, 820–828. doi: 10.1017/S0950268815002009

Georgiev, M., Beauvais, W., and Guitian, J. (2017). Effect of enhanced biosecurity and selected on-farm factors on Campylobacter colonization of chicken broilers. *Epidemiol. Infect.* 145, 553–567. doi: 10.1017/S095026881600251X

Gibbens, J. C., Pascoe, S. J., Evans, S. J., Davies, R. H., and Sayers, A. R. (2001). A trial of biosecurity as a means to control Campylobacter infection of broiler chickens. *Prev. Vet. Med.* 48, 85–99. doi: 10.1016/S0167-5877(00)00189-6

Grilli, E., Vitari, F., Domeneghini, C., Palmonari, A., Tosi, G., Fantinati, P., et al. (2013). Development of a feed additive to reduce caecal *Campylobacter jejuni* in broilers at slaughter age: from in vitro to in vivo, a proof of concept. *J. Appl. Microbiol.* 114, 308–317. doi: 10.1111/jam.12053

Hald, B., Wedderkopp, A., and Madsen, M. (2000). Thermophilic *Campylobacter* spp. in Danish broiler production: a cross-sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks. *Avian Pathol.* 29, 123–131. doi: 10.1080/03079450094153

Hansson, I., Ederoth, M., Andersson, L., Vågsholm, I., and Olsson Engvall, E. (2005). Transmission of *Campylobacter* spp. to chickens during transport to slaughter. *J. Appl. Microbiol.* 99, 1149–1157. doi: 10.1111/j.1365-2672.2005.02689.x

Hastings, R., Colles, F. M., McCarthy, N. D., Maiden, M. C., and Sheppard, S. K. (2011). Campylobacter genotypes from poultry transportation crates indicate a source of contamination and transmission. *J. Appl. Microbiol.* 110, 266–276. doi: 10.1111/j.1365-2672.2010.04883.x

Hermans, D., Martel, A., Van Deun, K., Verlinden, M., Van Immerseel, F., Garmyn, A., et al. (2010). Intestinal mucus protects *Campylobacter jejuni* in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. *Poult. Sci.* 89, 1144–1155. doi: 10.3382/ps.2010-00717

Hutchison, M. L., Walters, L. D., Moore, A., Crookes, K. M., and Avery, S. M. (2004). Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. *Appl. Environ. Microbiol.* 70, 5111–5118. doi: 10.1128/AEM.70.9.5111-5118.2004

Jonsson, M. E., Chriel, M., Norstrom, M., and Hofshagen, M. (2012). Effect of climate and farm environment on *Campylobacter* spp. colonisation in Norwegian broiler flocks. *Prev. Vet. Med.* 107, 95–104. doi: 10.1016/j.prevetmed.2012.05.002

Jore, S., Viljugrein, H., Brun, E., Heier, B. T., Borck, B., Ethelberg, S., et al. (2010). Trends in *Campylobacter* incidence in broilers and humans in six European countries, 1997-2007. *Prev. Vet. Med.* 93, 33–41. doi: 10.1016/j.prevetmed.2009.09.015

Jorgensen, F., Ellis-Iversen, J., Rushton, S., Bull, S. A., Harris, S. A., Bryan, S. J., et al. (2011). Influence of season and geography on *Campylobacter jejuni* and *C. coli* subtypes in housed broiler flocks reared in Great Britain. *Appl. Environ. Microbiol.* 77, 3741–3748. doi: 10.1128/AEM.02444-10

Lázaro, B., Cárcamo, J., Audícana, A., Perales, I., and Fernández-Astorga, A. (1999). Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Appl. Environ. Microbiol.* 65, 4677–4681.

Lee, S. K., Park, H. J., Lee, J. H., Lim, J. S., Seo, K. H., Heo, E. J., et al. (2017). Distribution and molecular characterization of *Campylobacter* species at different processing stages in two poultry processing plants. *Foodborne Pathog. Dis.* 14, 141–147. doi: 10.1089/fpd.2016.2218

Lin, J. (2009). Novel approaches for *Campylobacter* control in poultry. *Foodborne Pathog. Dis.* 6, 755–765. doi: 10.1089/fpd.2008.0247

Lynch, O. A., Cagney, C., McDowell, D. A., and Duffy, G. (2011). Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol. *Int. J. Food Microbiol.* 150, 171–177. doi: 10.1016/j.ijfoodmicro.2011.07.037

Mughini-Gras, L., Penny, C., Ragimbeau, C., Schets, F. M., Blaak, H., Duim, B., et al. (2016). Quantifying potential sources of surface water contamination with *Campylobacter jejuni* and *Campylobacter coli*. *Water Res.* 101, 36–45. doi: 10.1016/j.watres.2016.05.069

Newell, D. G., Elvers, K. T., Dopfer, D., Hansson, I., Jones, P., James, S., et al. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. *Appl. Environ. Microbiol.* 77, 8605–8614. doi: 10.1128/AEM.01090-10

Newell, D. G., and Fearnley, C. (2003). Sources of Campylobacter colonization in broiler chickens. *Appl. Environ. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Norstrom, M., Johnsen, G., Hofshagen, M., Tharaldsen, H., and Kruse, H. (2007). Antimicrobial resistance in *Campylobacter jejuni* from broilers and broiler house environments in Norway. *J. Food Prot.* 70, 736–738. doi: 10.4315/0362-028X-70.3.736

Oh, J. Y., Kwon, Y. K., Wei, B., Jang, H. K., Lim, S. K., Kim, C. H., et al. (2017). Epidemiological relationships of *Campylobacter jejuni* strains isolated from humans and chickens in South Korea. *J. Microbiol.* 55, 13–20. doi: 10.1007/s12275-017-6308-8

Powell, L. F., Lawes, J. R., Clifton-Hadley, F. A., Rodgers, J., Harris, K., Evans, S. J., et al. (2012). The prevalence of *Campylobacter* spp. in broiler flocks and on broiler carcases, and the risks associated with highly contaminated carcases. *Epidemiol. Infect.* 140, 2233–2246. doi: 10.1017/S0950268812000040

Public Health England (2015). *Survey Report. A Microbiological Survey of Campylobacter Contamination in Fresh whole UK Produced Chilled Chickens at Retail Sale (2014-15)*. Available at: https://www.food.gov.uk/sites/default/files/media/document/Final%20Report%20for%20FS241044%20Campylobacter%20Retail%20survey.pdf

Ridley, A., Morris, V., Gittins, J., Cawthraw, S., Harris, J., Edge, S., et al. (2011). Potential sources of Campylobacter infection on chicken farms: contamination and control of broiler-harvesting equipment, vehicles and personnel. *J. Appl. Microbiol.* 111, 233–244. doi: 10.1111/j.1365-2672.2011.05038.x

Russa, A. D., Bouma, A., Vernooij, J. C., Jacobs-Reitsma, W., and Stegeman, J. A. (2005). No association between partial depopulation and *Campylobacter* spp. colonization of Dutch broiler flocks. *Lett. Appl. Microbiol.* 41, 280–285. doi: 10.1111/j.1472-765X.2005.01751.x

Sahin, O., Kassem, I. I., Shen, Z., Lin, J., Rajashekara, G., and Zhang, Q. (2015). *Campylobacter* in poultry: ecology and potential interventions. *Avian Dis.* 59, 185–200. doi: 10.1637/11072-032315-Review

Shane, S. M. (1992). The significance of *Campylobacter jejuni* infection in poultry: a review. *Avian Pathol.* 21, 189–213. doi: 10.1080/03079459208418836

Shobo, C. O., Bester, L. A., Baijnath, S., Somboro, A. M., Peer, A. K., and Essack, S. Y. (2016). Antibiotic resistance profiles of Campylobacter species in the South Africa private health care sector. *J. Infect. Dev. Ctries.* 10, 1214–1221. doi: 10.3855/jidc.8165

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Skarp, C. P. A., Hänninen, M. L., and Rautelin, H. I. K. (2016). Campylobacteriosis: the role of poultry meat. *Clin. Microbiol. Infect.* 22, 103–109. doi: 10.1016/j.cmi.2015.11.019

Smith, S., Messam, L. L., Meade, J., Gibbons, J., McGill, K., Bolton, D., et al. (2016). The impact of biosecurity and partial depopulation on Campylobacter prevalence in Irish broiler flocks with differing levels of hygiene and economic performance. *Infect. Ecol. Epidemiol.* 6:31454. doi: 10.3402/iee.v6.31454

Sommer, H. M., Heuer, O. E., Sorensen, A. I., and Madsen, M. (2013). Analysis of factors important for the occurrence of Campylobacter in Danish broiler flocks. *Prev. Vet. Med.* 111, 100–111. doi: 10.1016/j.prevetmed.2013.04.004

Sterk, A., Schijven, J., De Roda Husman, A. M., and De Nijs, T. (2016). Effect of climate change on runoff of Campylobacter and Cryptosporidium from land to surface water. *Water Res.* 95, 90–102. doi: 10.1016/j.watres.2016.03.005

Svihus, B., Hetland, H., Mikkelsen, L., and Wu, S. (2009). *Role of Voluntary Litter Consumption by Broiler Chickens on Gut Function and Gut Health*. Final Report Project No 06–18. Armidale: Poultry CRC.

Tam, C. C., and O'Brien, S. J. (2016). Economic cost of campylobacter, Norovirus and rotavirus disease in the United Kingdom. *PLoS One* 11:e0138526. doi: 10.1371/journal.pone.0138526

Taylor, E. V., Herman, K. M., Ailes, E. C., Fitzgerald, C., Yoder, J. S., Mahon, B. E., et al. (2013). Common source outbreaks of Campylobacter infection in the USA, 1997-2008. *Epidemiol. Infect.* 141, 987–996. doi: 10.1017/S0950268812001744

Thibodeau, A., Fravalo, P., Yergeau, É., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS ONE* 10:e0131978. doi: 10.1371/journal.pone.0131978

Torok, V. A., Hughes, R. J., Ophel-Keller, K., Ali, M., and MacAlpine, R. (2009). Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poult. Sci.* 88, 2474–2481. doi: 10.3382/ps.2008-00381

Ugarte-Ruiz, M., Dominguez, L., Corcionivoschi, N., Wren, B. W., Dorrell, N., and Gundogdu, O. (2018). Exploring the oxidative, antimicrobial and genomic properties of *Campylobacter jejuni* strains isolated from poultry. *Res. Vet. Sci.* 119, 170–175. doi: 10.1016/j.rvsc.2018.06.016

van de Giessen, A. W., Tilburg, J. J., Ritmeester, W. S., and van der Plas, J. (1998). Reduction of campylobacter infections in broiler flocks by application of hygiene measures. *Epidemiol. Infect.* 121, 57–66. doi: 10.1017/S0950268898008899

van Wagenberg, C. P. A., van Horne, P. L. M., Sommer, H. M., and Nauta, M. J. (2016). Cost-effectiveness of Campylobacter interventions on broiler farms in six European countries. *Microb. Risk Anal.* 2–3, 53–62. doi: 10.1016/j.mran.2016.05.003

Wieczorek, K., and Osek, J. (2013). Antimicrobial resistance mechanisms among Campylobacter. *Biomed Res. Int.* 2013:340605. doi: 10.1155/2013/340605

Williams, M. S., Golden, N. J., Ebel, E. D., Crarey, E. T., and Tate, H. P. (2015). Temporal patterns of Campylobacter contamination on chicken and their relationship to campylobacteriosis cases in the United States. *Int. J. Food Microbiol.* 208, 114–121. doi: 10.1016/j.ijfoodmicro.2015.05.018

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: 04 September 2018
doi: 10.3389/fmicb.2018.02092

Prevalence and Characteristics of *Campylobacter* Throughout the Slaughter Process of Different Broiler Batches

*Xiaoyan Zhang, Mengjun Tang, Qian Zhou, Jing Zhang, Xingxing Yang and Yushi Gao**

Jiangsu Institute of Poultry Sciences, Supervision, Inspection and Testing Centre for Poultry Quality, Ministry of Agriculture, Yangzhou, China

Handling and consumption of chicken meat are risk factors for human campylobacteriosis. This study was performed to describe the *Campylobacter* population in broiler carcasses and environmental samples throughout the slaughter process. Moreover, the genetic diversity and antimicrobial resistance of the *Campylobacter* strains were evaluated. Cloacal swabs, samples from carcasses at different stages, and environmental samples were collected thrice from the different flocks at the same abattoir located in Central Jiangsu, China. *Campylobacter* isolated from the three batches (n = 348) were identified as *Campylobacter jejuni* (n = 117) and *Campylobacter coli* (n = 151) by multiplex PCR. Characterization by multilocus sequence typing revealed a specific genotype of *Campylobacter* for each batch. Antimicrobial sensitivity to 18 antibiotics were analyzed for all selected strains according to the agar diffusion method recommended by the Clinical and Laboratory Standards Institute. Antibiotic susceptibility tests indicated that the majority of the tested isolates were resistant to quinolones (>89.7%). Less resistance to macrolide (59.8%), gentamicin (42.7%), amikacin (36.8%) was observed. Results showed that 94.0% of the tested strains demonstrated multidrug resistance.

Keywords: *Campylobacter*, broiler, slaughter process, prevalence, MLST, antibiotic susceptibility

OPEN ACCESS

Edited by:
Michael J. Rothrock, United States Department of Agriculture, United States

Reviewed by:
Aude Locatelli, Georgia Department of Agriculture, United States
Jeferson Menezes Lourenco, University of Georgia, United States

***Correspondence:**
Yushi Gao
gaoys100@sina.com

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

Received: *19 June 2018*
Accepted: *16 August 2018*
Published: *04 September 2018*

Citation:
Zhang X, Tang M, Zhou Q, Zhang J, Yang X and Gao Y (2018) Prevalence and Characteristics of Campylobacter Throughout the Slaughter Process of Different Broiler Batches. Front. Microbiol. 9:2092. doi: 10.3389/fmicb.2018.02092

INTRODUCTION

Campylobacter is a leading cause of bacterial foodborne infections in developed countries. This infection has surpassed *Salmonella* several years ago and caused a significant economic burden (EFSA and ECDC, 2016). Although new species of *Campylobacter* have recently been discovered, human campylobacteriosis are dominated by two main species, *Campylobacter jejuni* and *Campylobacter coli* (Tresse et al., 2017). *Campylobacter* infection causes watery diarrhea, abortion, human acute enteritis, and several complications, such as Guillain-Barré syndrome and Reiter's syndrome, in severe cases. Handling and consumption of poultry are the major sources for human infection (Boysen et al., 2014). Reducing the prevalence or number of *Campylobacter* in broilers at the primary stage could be an effective way to protect public health from *Campylobacter* infections (European Food Safety Authority [EFSA], 2010). However, despite the many biosecurity interventions at the farm, *Campylobacter* has not been well controlled in broiler flocks after the rearing period (Newell et al., 2011).

During slaughter, many opportunities may facilitate cross-contamination and spread of bacteria despite good hygiene (Althaus et al., 2017). Poultry meat from *Campylobacter*-negative flocks may be contaminated by previously slaughtered *Campylobacter*-positive flocks (Miwa et al., 2003; Allen et al., 2007). *Campylobacter* can be spread to the poultry meat in the slaughter line, especially after evisceration or from dirty surfaces (Corry and Atabay, 2001; Melero et al., 2012). The prevalence of *Campylobacter* within positive flocks at slaughter are high (approximately 80%) (Colles et al., 2010; European Food Safety Authority [EFSA], 2010). However, in China, few studies have been conducted on the contamination of broiler carcasses throughout the production chain. During slaughter, *Campylobacter* could also be recovered in the processing equipment and environmental samples (Berrang et al., 2000; Cason et al., 2007; Ellerbroek et al., 2010). Studies have shown that some *Campylobacter* strains recovered from the slaughterhouse environment can contaminate carcasses when several batches of poultry are slaughtered (Peyrat et al., 2008; Melero et al., 2012). Previous studies only assessed the slaughtering performance to identify operations that increase or decrease the contamination of carcasses (Habib et al., 2012; Seliwiorstow et al., 2015). Moreover, the possibility of *Campylobacter* in plants as a continuous source of contamination is still ambiguous (García-Sánchez et al., 2017).

Campylobacter outbreaks are sporadic and caused by cross-contamination, and these characteristics hamper the determination of the sources of contamination. Molecular methods play an important role in the epidemiological study of tracing sources and routing of pathogen transmission. Pulsed-field gel electrophoresis and multilocus sequence typing (MLST) have been employed successfully for the epidemiological study of *Campylobacter* from different sources and outbreaks (Dingle et al., 2005; Michaud et al., 2005; Thakur et al., 2009). The resistance of *Campylobacter* to antibiotics has also been a persistent issue generally related to the indiscriminate use of antibiotics for therapy or as a growth promoter (Chen et al., 2010; Rozynek et al., 2013). Determining the drug resistance of *Campylobacter* strains is important to control and prevent human infection. In China, molecular subtyping and antimicrobial susceptibilities of *Campylobacter* strains from different sources have been conducted (Zhang et al., 2014; Zeng et al., 2016). However, limited data are available on the comprehensive molecular characterization and antibiotic susceptibility of *Campylobacter* from broilers during slaughter.

This study was performed to determine the prevalence of *Campylobacter* in the slaughter and poultry processing environment. Moreover, the genotype characteristics and antibiotic susceptibility of these strains were assessed.

MATERIALS AND METHODS

Sample Collection

Samples were collected from a slaughterhouse located in Central Jiangsu, China. This slaughterhouse processes 20,000 broilers per day. We selected three batches (birds from one flock slaughtered at the same day) in different farms from April 2017 to November 2017. During each visit, cloacal samples were individually collected using sterile swabs before slaughter. Then, samples from the broiler carcasses were collected after plucking, evisceration, washing, and chilling. Water from the cleaning pool and swabs from the operating table and workers' gloves were also collected as environmental samples. Furthermore, sample collection was performed consecutively during 1 h of the slaughter process. All cloacal swabs were placed in Cary-Blair medium. Swab samples taken at different points were collected using cotton swabs moistened with sterile saline and stored in aseptic bags. Water samples were placed in sterile plastic containers. All samples were transported to the laboratory under cool conditions within 3 h and analyzed the same day. The number of samples collected at different points are shown in **Table 1**.

Campylobacter Isolation and Identification

All cloacal swab samples were placed in 1 mL of PBS (phosphate buffer saline) for full immersion. Then, the swabs were removed

TABLE 1 | Contamination ratio of *Campylobacter* during slaughter process in three batches.

Sample site	No. of *Campylobacter* positive samples /total no. of samples (%)					
	Batch 1 (Farm 1)		Batch 2 (Farm 2)		Batch 3 (Farm 3)	
	C. jejuni	*C.coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*
Cloacal swabs	19/30 (63.3%)	0	0	20/20 (100.0%)	0	3/22 (13.6%)
After plucking	22/30 (73.3%)	0	0	18/21 (85.7%)	4/15 (26.7%)	10/15 (66.7%)
After evisceration	24/30 (80.0%)	0	0	17/22 (77.3%)	0	12/15 (80.0%)
After washing	23/30 (76.7%)	0	0	20/21 (95.2%)	3/20 (15.0%)	11/20 (55.0%)
After chilling	7/8 (87.5%)	0	1/10 (10.0%)	9/10 (90.0%)	0	10/10 (100.0%)
Operating table	4/5 (80.0%)	0	0	5/5 (100.0%)	1/10 (10.0%)	9/10 (90.0%)
Workers' gloves	3/5 (60.0%)	0	1/5 (20.0%)	4/5 (80.0%)	2/5 (40.0%)	2/5 (40.0%)
Water	3/3 (100.0%)	0	0	1/3 (33.3%)	0	0/3/0
Total	105/141 (74.5%)		96/107 (89.7%)		67/100 (67.0%)	

TABLE 2 | The statistical contrast across the three batches (*P*-value).

Sample site	Batch (Farm) (No. of *Campylobacter* positive samples/total no. of samples)		*P*-value
Cloacal swabs	1 (19/30)	2 (20/20)	0.317
	1 (19/30)	3 (3/22)	0.000*
	2 (20/20)	3 (3/22)	0.000*
After plucking	1 (22/30)	2 (18/21)	0.295
	1 (22/30)	3 (14/15)	0.118
	2 (18/21)	3 (14/15)	0.480
After evisceration	1 (24/30)	2 (17/22)	0.814
	1 (24/30)	3 (12/15)	1.000
	2 (17/22)	3 (12/15)	0.845
After washing	1 (23/30)	2 (20/21)	0.076
	1 (23/30)	3 (14/20)	0.602
	2 (20/21)	3 (14/20)	0.034*
After chilling	1 (7/8)	2 (10/10)	0.264
	1 (7/8)	3 (10/10)	0.264
	2 (10/10)	3 (10/10)	N

"N" means no statistics are computed; "" resembles $P < 0.05$.*

from the solution. After 10 times dilution, 100 μL of each solution was placed on modified charcoal-cefoperazone-deoxycholate agar (mCCDA) (Oxoid CM0739) with cefoperazone (LKT, C1630), amphotericin B (Wako, 011-1363), and rifampicin (Wako, 185-01003). All plates were incubated at 42°C for 36–42 h in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2).

Cotton swabs from carcasses and environment were enriched in 10 mL of Bolton Broth (BB, CM0983, Oxoid; supplemented with BB Selective Supplement, SR0183E, Oxoid) based on previous reports (Adzitey et al., 2012; Tissier et al., 2012). Water from the cleaning pool were filtered through filters with a 0.22 μm pore diameter. Then the membranes were introduced into 10 mL of BB. After 24 h of incubation at 42°C in glass jars under a microaerobic atmosphere, 10 μL of the resulting solutions were streaked onto mCCDA plates, and the plates were incubated at 42°C for 48 h.

After incubation, the suspected colonies were picked and subcultured in Mueller–Hinton (MH) agar (Difco, MD) with blood and incubated for 24–48 h at 42°C under microaerobic conditions. Multiplex PCR tests were used to confirm and identify whether the strains were *Campylobacter jejuni* or *Campylobacter coli* according to a previous study (Huang et al., 2009).

Antibiotic Susceptibility Testing

Susceptibility of *Campylobacter* to 18 antibiotics (Oxoid) from six classes of antibiotics was determined using the following antimicrobial impregnated disks (Oxoid, England, United Kingdom): β-lactams (ampicillin, AMP, 10 μg; amoxicillin, AML, 30 μg; cefotaxime, CEX, 10 μg; and ceftriaxone, CRO, 30 μg); aminoglycosides (streptomycin, S, 10 μg; gentamicin, GEN, 10 μg; kanamycin, K, 30 μg; amikacin, AMK, 30 μg; and tobramycin, TOB, 10 μg); quinolones (norfloxacin, NOR, 10 μg; ciprofloxacin, CIP, 5 μg; ofloxacin, OFX, 5 μg; nalidixic acid, NA, 30 μg; and enrofloxacin, ENR, 5 μg); macrolide (erythromycin, E, 15 μg; and azithromycin, AZM, 15 μg); tetracycline (TE, 30 μg); and clindamycin (DA, 2 μg). This process was carried out according to the Kirby-Bauer disk diffusion method (Bauer et al., 1966) and as recommended by the Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2012). Some isolates were revived from the glycerol stocks using BB with 10% lysed sheep

TABLE 3 | Number and percentages of resistance of *Campylobacter* isolates from three batches.

Antibiotic group	Antibiotic name	No. of resistant *Campylobacter* isolates (%)			
		Batch 1	Batch 2	Batch 3	Total
β-Lactams	AMP	39/39 (100%)	31/38 (81.6%)	7/40 (17.5%)	77/117 (65.8%)
	AML	39/39 (100%)	31/38 (81.6%)	7/40 (17.5%)	77/117 (65.8%)
	CEX	39/39 (100%)	34/38 (89.5%)	40/40 (100%)	113/117 (96.6%)
	CRO	39/39 (100%)	20/38 (52.6%)	40/40 (100%)	99/117 (84.6%)
Aminoglycosides	S	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	GEN	39/39 (100%)	5/38 (13.2%)	6/40 (15.0%)	50/117 (42.7%)
	K	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	AMK	39/39 (100%)	2/38 (5.7%)	2/40 (5.0%)	42/117 (36.8%)
	TOB	39/39 (100%)	15/38 (39.5)	40/40 (100%)	94/117 (80.3%)
Quinolones	NOR	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	CIP	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	OFX	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	NA	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
	ENR	39/39 (100%)	26/38 (68.4)	40/40 (100%)	105/117 (89.7%)
Macrolide	E	0	31/38 (81.6%)	40/40 (100%)	70/117 (59.8%)
	AZM	0	31/38 (81.6%)	40/40 (100%)	70/117 (59.8%)
Tetracyclines	TE	39/39 (100%)	31/38 (81.6%)	40/40 (100%)	109/117 (93.2%)
Clindamycin	DA	39/39 (100%)	31/38 (81.6%)	37/40 (92.5%)	106/117 (90.6%)

TABLE 4 | Resistance spectra of 117 *Campylobacter* to various antibiotic combinations.

MAR index	No. of *Campylobacter* isolates			Antibiotic
	Batch 1	Batch 2	Batch 3	
0	0	7	0	–
0.72	0	1	0	AMP, AML, CEX, CRO, S, K, NOR, CIP, NA, E, AZM, TE, DA
0.78	0	2	0	AMP, AML, CEX, CRO, S, K, NOR, CIP, OFX, NA, E, AZM, TE, DA
0.78	0	2	0	AMP, AML, CEX, S, K, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.78	0	0	33	CEX, CRO, S, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.83	0	5	0	AMP, AML, CEX, S, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.83	0	2	0	AMP, AML, CEX, CRO, S, K, TOB, NOR, CIP, NA, ENR, E, AZM, TE, DA
0.83	0	4	0	AMP, AML, CEX, CRO, S, K, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.83	0	0	2	AMP, AML, CEX, S, GEN, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE,
0.83	0	1	0	AMP, AML, CEX, S, GEN, K, TOB, NOR, CIP, OFX, NA, E, AZM, TE, DA
0.89	0	9	0	AMP, AML, CEX, CRO, S, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.89	0	1	0	AMP, AML, CEX, GEN, K, AMK, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.89	0	1	0	CEX, CRO, S, GEN, K, AMK, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
0.89	0	0	2	AMP, AML, CEX, CRO, S, GEN, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE
0.89	39	0	0	AMP, AML, CEX, CRO, S, GEN, K, AMK, TOB, NOR, CIP, OFX, NA, ENR, TE, DA
0.94	0	3	1	AMP, AML, CEX, CRO, S, GEN, K, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA
1.00	0	0	2	AMP, AML, CEX, CRO, S, GEN, K, AMK, TOB, NOR, CIP, OFX, NA, ENR, E, AZM, TE, DA

blood. Then, these isolates were incubated for 36 h at 42°C under microaerobic conditions. Revived cultures were streaked using sterile cotton swabs on MH (Mueller Hinton) agar plates (Oxoid) supplemented with 10% sheep blood for another 36 h of incubation. The bacteria were scrapped to PBS, and the turbidity of the suspension was adjusted to 0.5 McFarland standard (Clinical and Laboratory Standards Institute, 2012). The suspension was stacked onto MH agar plates for 36 h of incubation under microaerophilic conditions. *Escherichia coli* ATCC 25922 and *C. jejuni* NCTC 11168 were used as reference strains.

The diameters of the inhibition zones around the antibiotic disk were measured. The breakpoints used to categorize isolates as susceptible, intermediate, and resistant–were based on the Clinical and Laboratory Standards Institute recommendations (Clinical and Laboratory Standards Institute, 2012). Isolates resistant to $\geq$3 unrelated antibiotic classes were classified as isolates with multidrug resistance (MDR).

MAR (Multiple Antimicrobial Resistance) was used to quantify the multi-resistance of *Campylobacter* isolates. MAR index = a/b. In this formula, "a" indicated the number of antibiotics to which the isolate was resistant and "b" indicated the total number of antibiotics to which the isolate was tested (Krumperman, 1983).

Multilocus Sequence Typing for *Campylobacter*

DNA was extracted from some representative strains using a commercial DNA Kit (Tiangen Biotech Inc., Beijing, China). MLST was conducted using primer sequences obtained from http://pubmlst.org/ *Campylobacter* as previously described (Dingle et al., 2001). The nucleotide sequences of the amplicons were determined by GenScript, Inc. (Nanjing, China). Allele numbers and STs (sequence types) were assigned using the *Campylobacter* PubMLST database.

Data Analysis

The difference in the prevalence levels across the batches in cloacal swabs and various sample points obtained after plucking, evisceration, washing and chilling were analyzed using a nonparametric test (Chi-square test) using SPSS software (version 17.0). $P < 0.05$ were considered statistically significant.

Sequence analysis and ST determinations of clonal complexes were performed using the PubMLST database[1] for ST designation. Consensus tree was constructed using UPGMA cluster analysis based on the seven housekeeping gene sequences.

RESULTS

Prevalence of *Campylobacter* in the Slaughter Process

The isolation rates of *Campylobacter* and the samples collected from each point are listed in **Table 1**. All strains isolated in batch 1 were *C. jejuni* (105/105). However, in batches 2 and 3, *C. coli* was the predominant isolated strain (94/96 and 57/67, respectively). The contamination rate of *Campylobacter* on the carcasses at every point during slaughter was relatively high even after chilling (87.5–100%). As shown in **Table 2**, compared with batches 1 and 2, *Campylobacter* infection rate in the cloacal swabs in batch 3 was significantly lower ($P < 0.05$). However, significant correlations

[1]http://pubmlst.org/campylobacter/

TABLE 5 | Distributions of STs for 117 *Campylobacter* isolates.

Batch	species	ST	ST-CC	Source	Number
1	*C. jejuni*	8089	UA	Cloacal swab	7
		8089	UA	After plucking	7
		8089	UA	After evisceration	8
		8089	UA	After washing	4
		8089	UA	After chilling	7
		8089	UA	Operating table	2
		8089	UA	Workers' gloves	2
		8089	UA	Water	2
2	*C. jejuni*	6186	UA	After chilling	1
	C. coli	6186	UA	Cloacal swab	3
		5511	828	Cloacal swab	1
		6186	UA	After plucking	6
		6186	UA	After evisceration	3
		5511	828	After washing	1
		825	828	After washing	1
		6186	UA	After washing	4
		NEW1	–	After washing	2
		825	828	After chilling	2
		860	828	After chilling	3
		6186	UA	After chilling	3
		872	828	Operating table	2
		860	828	Operating table	1
		872	828	Workers' gloves	4
		6186	UA	Water	1
3	*C. jejuni*	860	828	Workers' gloves	2
	C. coli	860	828	Cloacal swab	3
		860	828	After plucking	6
		830	828	After plucking	1
		860	828	After evisceration	6
		860	828	After washing	5
		6186	828	After washing	1
		860	828	After chilling	7
		830	828	After chilling	1
		825	828	After chilling	1
		860	828	Operating table	4
		825	828	Operating table	2
		860	828	Workers' gloves	1

"ST-CC" means ST clonal complex; "UA" means unassigned.

of infection rates were not observed after evisceration among different batches.

At the batch level, we compared the contamination rates of *Campylobacter* at four sampling points (after plucking, evisceration, washing, and chilling) and found no significant difference except the point of after washing between batches 2 and 3 ($P = 0.034$) (**Table 2**). A total of 268 isolates were obtained from 348 samples (77.0%), including 117 *C. jejuni* and 151 *C. coli* isolates.

Campylobacter isolates were recovered from all environmental samples. The cotton swab samples from the operating table and workers' gloves in the 3 batches showed relatively high contamination levels. However, *Campylobacter* species were not isolated from the cooled water in batch 3.

Antimicrobial Resistance

We selected 39 isolates (*C. jejuni*) from batch 1, 38 isolates (2 *C. jejuni* and 36 *C. coli*) from batch 2, and 40 isolates (1 *C. jejuni* and 39 *C. coli*) from batch 3 for antimicrobial resistance testing. A total of 18 antimicrobials classified under six antimicrobial groups were employed to test the selected *Campylobacter* isolates. Except for seven samples from batch 2, all isolates were resistant to at least one or more antimicrobials (**Table 4**). The majority of the tested isolates were resistant to quinolones (≥89.7%). Less resistance to macrolide (59.8%), gentamicin (42.7%), and amikacin (36.8%) was observed. The resistance to other antibiotics in this study was greater or equal to 65.0%. Resistance to macrolide was not detected from batch 1, but 81.6 and 100% from batches 2 and 3, respectively, were resistant to this antimicrobial (**Table 3**).

The MAR (Multiple Antimicrobial Resistance) indices of the tested isolates from the current study are indicated in **Table 4**. MDR (resistance to three or more antimicrobial families) was observed in the majority of the isolates. A total of 17 different antibiotic resistance patterns with MAR index ranging from 0 to 1.00 were observed. Multiple resistances were common with resistance from 14 to 16 to 18 antibiotics (MAR index 0.78–0.89). This characteristic was observed in most of the 117 Campylobacter strains. Strains from batch 1 showed resistance to 16 antibiotics (except AZM and E). The resistance spectra of the strains from batches 2 and 3 were more diverse than that of the strains from batch 1. A total of 94.0% of the tested strains demonstrated MDR.

MLST Analysis of *Campylobacter* Isolated From the Slaughter Process and the Environment

Isolates from cloacal swabs, environmental samples, and carcasses at different slaughter points were selected and subjected to MLST analysis. The STs, species, sources, and numbers of bacteria are summarized in **Table 5**. A total of eight STs, including one novel type, were observed from the 117 isolates. Three *C. jejuni* STs and 7 *C. coli* STs were identified. One clonal complex CC828 (55 isolates) was generated from these isolates, but 62 isolates could not be assigned to any of the defined CCs.

All 39 *C. jejuni* isolates from batch 1 were identified as ST8089. Compared with batch 1, batches 2 and 3 showed more diversity in STs. In batches 2 and 3, ST6186 (21/38, 55.3%) and ST860 (33/40, 82.5%) were the most frequently observed STs. This result was similar to the isolates from each cloacal sample. In addition, the STs from the environmental samples in each batch were highly consistent with those from the carcasses and cloacal swabs. In batch 2, strains after washing contained three traditional and one new ST types. All identified STs were further analyzed using UPGMA (**Figure 1**). Eight identified STs were classified into three clonal groups. All *C. jejuni* isolates from batch 1 belonged to group 1, and most of the *Campylobacter* isolates in batches 2 and 3 belonged to groups 2 and 3, respectively.

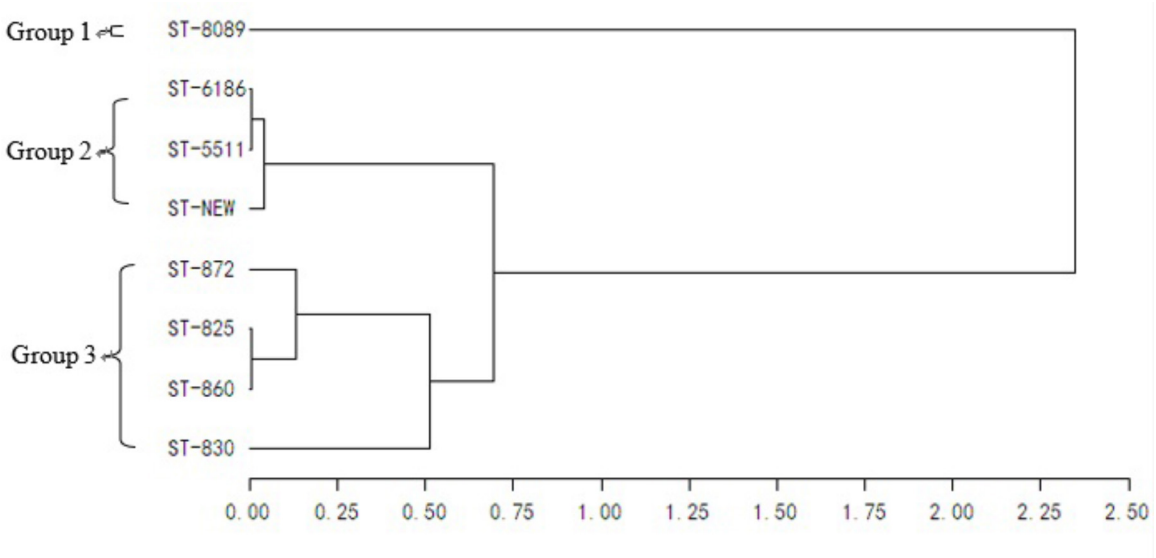

FIGURE 1 | Genetic relationships of the isolates based on the MLST. The consensus tree was developed using UPGMA cluster analysis.

DISCUSSION

Campylobacter infection is considered one of the leading causes of bacterial gastroenteritis in developed and developing countries (Wei et al., 2014; Kaakoushm et al., 2015; Nohra et al., 2016). Several studies have associated the risk of human *Campylobacter* infection with highly contaminated broiler carcasses (Callicott et al., 2008; Nauta and Havelaar, 2008). Although China is one of the largest poultry producers worldwide, data on this pathogen are limited, especially during broiler production. In the present study, we showed the contamination rate of *Campylobacter* in samples collected along the slaughter line, that is, from the cloacal swabs in live birds to post chiller carcasses. The prevalence of *Campylobacter* after chilling was high (87.5–100%). This result was consistent with the finding of Seliwiorstow and collaborators, who compared the carcass contaminations before washing and after chilling in the slaughterhouse and found that the contamination rates were not reduced (Seliwiorstow et al., 2015). Accordingly, improved hygiene during slaughtering may reduce the number of *Campylobacter* in the carcasses, but the infection rates cannot be reduced because of cross-contamination. Despite the significantly low *Campylobacter* infection rate of cloacal swabs in batch 3 ($P < 0.05$), significant correlations of infection rates after evisceration among the different batches were not observed. These data explained that in the case of batch 3, which had lower positive cloacal numbers, the cross-contamination coming from the intestinal content of other flocks or its own flock still existed.

The investigation was conducted in 2012 in a commercial poultry production in Shanghai, China. A total of 23 *C. jejuni* (17.0%) and 33 *C. coli* (24.4%) were isolated from 135 broiler carcasses from the slaughterhouse (Ma et al., 2014). Wang and collaborators investigated the prevalence of *Campylobacter* from broiler chickens in slaughter houses in five Chinese provinces during 2008–2014, and isolated 977 *C. jejuni* (18.1%) and 1021 *C. coli* (19.0%) from 5,385 chickens (Wang et al., 2016). In southern Brazil, samples from the broiler slaughtering process were analyzed to directly count *Campylobacter* and results showed that 72 and 38% were *C. jejuni* and *C. coli*, respectively (Gonsalves et al., 2016). Similar results were demonstrated in the European Union, where 60.8% of broiler samples tested positive for *C. jejuni*, and 41.5% tested positive for *C. coli* (European Food Safety Authority [EFSA], 2010). In the present study, a total of 117 *C. jejuni* (33.6%) and 151 *C. coli* (43.4%) were isolated from 348 samples collected in the slaughterhouse. The isolation rates were higher than those obtained in previous years in China. More samples are needed for further study of the prevalence of *Campylobacter* species found in the different steps of the slaughter process.

Campylobacter isolates were recovered from all environmental samples. *Campylobacter* can form biofilms to survive outside the host and protect against chemical products, physical cleaning processes, and environmental stress, and these processes are proposed as a survival mechanism (Hanning et al., 2008). Thus, this mechanism may explain the presence of *Campylobacter* in the cool water despite the chemical treatment of the water. Defeathering and evisceration are considered as critical contamination steps in poultry processing (Sasaki et al., 2013; Gruntar et al., 2015). Samples from the operating table and workers' gloves showed a high contamination level in this study. This result suggested an important cross-contamination rate between carcasses and processing equipment. Therefore, it is recommended that contaminated broiler flocks should be slaughtered at the end of the working day to reduce the cross-contamination among the flocks.

Antibiotic resistance is a persistent issue in veterinary medicine and human medical treatment because of the indiscriminate use of antibiotics in therapy or as a growth

promoter. Although most *Campylobacter* infections are self-limiting and do not require any antibiotic treatment, antimicrobial treatment is necessary for some severe and prolonged cases. Fluoroquinolones and macrolides are usually administered to treat human campylobacteriosis in China (Zhang et al., 2014). Our results showed that more than 89.7% of the tested isolates were resistant to quinolones and 59.8% of the tested isolates were resistant to macrolide. This result was consistent with those of previous reports (Li et al., 2018). All tested isolates of *C. jejuni* from batch 1 were susceptible to macrolide (AZM and E), compare with 81.6 and 100% of the tested isolates of *C. coli* from batches 2 and 3, respectively, resistant to this antimicrobial. These data were in accordance with recent reports (Ruzauskas et al., 2011; Haruna et al., 2012; Fraqueza et al., 2014; Pergola et al., 2017), which indicated that *C. jejuni* was predominantly susceptible to erythromycin while *C. coli* was resistant. However, another possible reason for the results in the current study is that *Campylobacter* isolated from the same batch may have primarily the same antibiotic resistance profile.

MDR was observed in the majority of the tested isolates (94%) in this study. Higher frequency of MDR was also noted in *C. coli* isolates from different sources (99%) in China (Zhang et al., 2014). In the present study, 17 different antibiotic resistance patterns with MAR index ranging from 0 to 1.00 were observed. The majority of the MAR index calculated ranged from 0.78 to 0.89 among the 117 selected *Campylobacter* strains. Strains with a MAR index >0.2 have been identified from animals frequently treated with antimicrobials (Marian et al., 2012). In contrast, significantly lower resistance rates of ciprofloxacin were observed in *Campylobacter* from poultry meat in countries with strict antimicrobial controls (Miflin et al., 2007; Zhao et al., 2010). Hence, severe MDR, which may be a threat to public safety in China, should be given proper attention.

Campylobacter isolates from one farm showed primarily the same genotype and the same antibiotic resistance profile as previously reported (Pergola et al., 2017). Some studies have supported the hypothesis that the contamination of *Campylobacter* in broiler carcasses is mainly from the processed *Campylobacter*-positive birds within a batch (Rasschaert et al., 2006; Sasaki et al., 2014). In the present study, the composition of the MLST was relatively stable within a batch because of the predominance of certain MLST types. In each batch, the most frequently observed STs (ST8089, ST6186, and ST860) were similar to the STs of the isolates from each cloacal sample. **Figure 1** shows the close genetic relationship with the dominant STs in each batch. These results may indicate that *Campylobacter* in slaughterhouses originated mainly from the farms. Thus, minimizing the *Campylobacter* colonization in the incoming broiler flock is important to reduce the public health risk.

Several isolates collected in this study shared identical genotypes (ST6186, ST825, ST830, ST860, and ST872) with those isolates from the feces of a diarrheal patient in China (Zhang et al., 2014). The consistency of STs in the environmental and carcass samples suggested that some environmental samples, such as those from the operating table and workers' gloves, may reflect the potential source of contamination. This result further indicated the importance of good hygienic practices during the slaughter process.

AUTHOR CONTRIBUTIONS

QZ, JZ, and XY performed the collection of samples. XZ and QZ did the *Campylobacter* detection and identification. MT and XZ performed the MLST and antibiotic susceptibility tests. QZ and MT did the data analysis. XZ prepared the manuscript. YG supervised and assisted in the manuscript preparation.

FUNDING

This work was supported by the National Natural Science Foundation of China grant (31700005), Yangzhou Social Development Project (YZ2016058).

ACKNOWLEDGMENTS

We thank Changzhou Lihua food company for their help with the sample collection.

REFERENCES

Adzitey, F., Rusul, G., Huda, N., Cogan, T., and Corry, J. (2012). Prevalence, antibiotic resistance and RAPD typing of *Campylobacter* species isolated from ducks, their rearing and processing environments in Penang, Malaysia. *Int. J. Food Microbiol.* 154, 197–205. doi: 10.1016/j.ijfoodmicro.2012.01.006

Allen, V. M., Bull, S. A., Corry, J. E., Domingue, G., Jørgensen, F., Frost, J. A., et al. (2007). *Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonisation. *Int. J. Food Microbiol.* 113, 54–61. doi: 10.1016/j.ijfoodmicro.2006.07.011

Althaus, D., Zweifel, C., and Stephan, R. (2017). Analysis of a poultry slaughter process: influence of process stages on the microbiological contamination of broiler carcasses. *Ital. J. Food Saf.* 6:7097. doi: 10.4081/ijfs.2017.7097

Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45, 493–496. doi: 10.1093/ajcp/45.4_ts.493

Berrang, M. E., Buhr, R. J., and Cason, J. A. (2000). *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poult. Sci.* 79, 286–290. doi: 10.1093/ps/79.2.286

Boysen, L., Rosenquist, H., Larsson, J. T., Nielsen, E. M., Sørensen, G., Nordentoft, S., et al. (2014). Source attribution of human campylobacteriosis in Denmark. *Epidemiol. Infect.* 142, 1599–1608. doi: 10.1017/S0950268813002719

Callicott, K. A., Harğardóttir, H., Georgsson, F., Reiersen, J., Friğriksdóttir, V., Gunnarsson, E., et al. (2008). Broiler *Campylobacter* contamination and human campylobacteriosis in Iceland. *Appl. Environ. Microbiol.* 74, 6483–6494. doi: 10.1128/AEM.01129-08

Cason, J. A., Hinton, A. Jr., Northcutt, J. K., Buhr, R. J., Ingram, K. D., Smith, D. P., et al. (2007). Partitioning of external and internal bacteria carried by broiler chickens before processing. *J. Food Prot.* 70, 2056–2062. doi: 10.4315/0362-028X-70.9.2056

Chen, X., Naren, G. W., Wu, C. M., Wang, Y., Dai, L., Xia, L. N., et al. (2010). Prevalence and antimicrobial resistance of *Campylobacter* isolates in broilers from China. *Vet. Microbiol.* 144, 133–139. doi: 10.1016/j.vetmic.2009.12.035

Clinical and Laboratory Standards Institute (2012). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M11-S22*. Wayne, PA: CLSI.

Colles, F. M., McCarthy, N. D., Sheppard, S. K., Layton, R., and Maiden, M. C. (2010). Comparison of *Campylobacter* populations isolated from a free-range broiler flock before and after slaughter. *Int. J. Food Microbiol.* 137, 259–264, doi: 10.1016/j.ijfoodmicro.2009.12.021

Corry, J. E., and Atabay, H. I. (2001). Poultry as a source of *Campylobacter* and related organisms. *Symp. Ser. Soc. Appl. Microbiol.* 30, 96S–114S. doi: 10.1046/j.1365-2672.2001.01358.x

Dingle, K. E., Colles, F. M., Falush, D., and Maiden, M. C. (2005). Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. *J. Clin. Microbiol.* 43, 340–347. doi: 10.1128/JCM.43.1.340-347.2005

Dingle, K. E., Colles, F. M., Wareing, D. R., Ure, R., Fox, A. J., Bolton, F. E., 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 and ECDC (2016). European Food Safety Authority, European Centre for Disease Prevention and Control: the European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA J.* 13:4329. doi: 10.2903/j.efsa.2015.4329

Ellerbroek, L. I., Lienau, J. A., and Klein, G. (2010). *Campylobacter* spp. in broiler flocks at farm level and the potential for cross-contamination during slaughter. *Zoonoses Public Health* 57, e81–e88. doi: 10.1111/j.1863-2378.2009.01267

European Food Safety Authority [EFSA] (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. *EFSA J.* 8, 1503–1602.

Fraqueza, M. J., Martins, A., Borges, A. C., Fernandes, M. H., Fernandes, M. J., Vaz, Y., et al. (2014). Antimicrobial resistance among *Campylobacter* spp. strains isolated from different poultry production systems at slaughterhouse level. *Poult. Sci.* 93, 1578–1586. doi: 10.3382/ps.2013-03729

García-Sánchez, L., Melero, B., Jaime, I., Hänninen, M. L., Rossi, M., and Rovira, J. (2017). *Campylobacter jejuni* survival in a poultry processing plant environment. *Food Microbiol.* 65, 185–192. doi: 10.1016/j.fm.2017.02.009

Gonsalves, C. C., Borsoi, A., Perdoncini, G., Rodrigues, L. B., and do Nascimento, V. P. (2016). *Campylobacter* in broiler slaughter samples assessed by direct count on mCCDA and Campy-Cefex agar. *Braz. J. Microbiol.* 47, 764–769. doi: 10.1016/j.bjm

Gruntar, I., Biasizzo, M., Kušar, D., Pate, M., and Ocepek, M. (2015). *Campylobacter jejuni* contamination of broiler carcasses: population dynamics and genetic profiles at slaughterhouse level. *Food Microbiol.* 50, 97–101. doi: 10.1016/j.fm.2015.03.007

Habib, I., Berkvens, D., De Zutter, L., Dierick, K., Van Huffel, X., Speybroeck, N., et al. (2012). *Campylobacter* contamination in broiler carcasses and correlation with slaughterhouses operational hygiene inspection. *Food Microbiol.* 29, 105–112. doi: 10.1016/j.fm.2011.09.004

Hanning, I., Jarquin, R., and Slavik, M. (2008). *Campylobacter jejuni* as a secondary colonizer of poultry biofilms. *J. Appl. Microbiol.* 105, 1199–1208. doi: 10.1111/j.1365-2672.2008.03853.x

Haruna, M., Sasaki, Y., Murakami, M., Ikeda, A., Kusukawa, M., Tsujiyama, Y., et al. (2012). Prevalence and antimicrobial susceptibility of *Campylobacter* in broiler flocks in Japan. *Zoonoses Public Health* 59, 241–245. doi: 10.1111/j.1863-2378.2011.01441.x

Huang, J. L., Xu, H. Y., Bao, G. Y., Zhou, X. H., Ji, D. J., Zhang, G., et al. (2009). Epidemiological surveillance of *Campylobacter jejuni* in chicken, dairy cattle and diarrhoea patients. *Epidemiol. Infect.* 137, 1111–1120. doi: 10.1017/S0950268809002039

Kaakoushm, N. O., Castaño-Rodríguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi: 10.1128/CMR.00006-15

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* 46, 165–170.

Li, Y., Zhang, S., He, M., Zhang, Y., Fu, Y., Liang, H., et al. (2018). Prevalence and molecular characterization of *Campylobacter* spp. isolated from patients with diarrhea in Shunyi, Beijing. *Front. Microbiol.* 9:52. doi: 10.3389/fmicb.2018.00052

Ma, L., Wang, Y., Shen, J., Zhang, Q., and Wu, C. (2014). Tracking *Campylobacter* contamination along a broiler chicken production chain from the farm level to retail in China. *Int. J. Food Microbiol.* 181, 77–84. doi: 10.1016/j.ijfoodmicro.2014.04.023

Marian, M. N., Sharifah Aminah, S. M., Zuraini, M. I., Son, R., Maimunah, M., Lee, H. Y., et al. (2012). MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. *Food Control* 28, 309–314. doi: 10.1016/j.foodcont.2012.05.030

Melero, B., Juntunen, P., Hänninen, M. L., Jaime, I., and Rovira, J. (2012). Tracing *Campylobacter jejuni* strains along the poultry meat production chain from farm to retail by pulsed-field gel electrophoresis, and the antimicrobial resistance of isolates. *Food Microbiol.* 32, 124–128. doi: 10.1016/j.fm.2012.04.020

Michaud, S., Ménard, S., and Arbeit, R. D. (2005). Role of real-time molecular typing in the surveillance of *Campylobacter* enteritis and comparison of pulsed-field gel electrophoresis profiles from chicken and human isolates. *J. Clin. Microbiol.* 43, 1105–1111. doi: 10.1128/JCM.43.3.1105-1111.2005

Miflin, J. K., Templeton, J. M., and Blackall, P. J. (2007). Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the south-east Queensland region. *J. Antimicrob. Chemother.* 59, 775–778. doi: 10.1093/jac/dkm024

Miwa, N., Takegahara, Y., Terai, K., Kato, H., and Takeuchi, T. (2003). *Campylobacter jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing in a Japanese slaughterhouse. *Int. J. Food Microbiol.* 84, 105–109. doi: 10.1016/S0168-1605(02)00398-7

Nauta, M. J., and Havelaar, A. H. (2008). Risk-based standards for *Campylobacter* in the broiler meat chain. *Food Control* 19, 372–381. doi: 10.1016/j.foodcont.2007.04.016

Newell, D. G., Elvers, K. T., Dopfer, D., Hansson, I., Jones, P., James, S., et al. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. *Appl. Environ. Microbiol.* 77, 8605–8614. doi: 10.1128/AEM.01090-10

Nohra, A., Grinberg, A., Midwinter, A. C., Marshall, J. C., Collins-Emerson, J. M., and French, N. P. (2016). Molecular epidemiology of *Campylobacter coli* strains isolated from different sources in New Zealand between 2005 and 2014. *Appl. Environ. Microbiol.* 82, 4363–4370. doi: 10.1128/AEM.00934-16

Pergola, S., Franciosini, M. P., Comitini, F., Ciani, M., De Luca, S., Bellucci, S., et al. (2017). Genetic diversity and antimicrobial resistance profiles of *Campylobacter coli* and *Campylobacter jejuni* isolated from broiler chicken in farms and at time of slaughter in central Italy. *J. Appl. Microbiol.* 122, 1348–1356. doi: 10.1111/jam.13419

Peyrat, M. B., Soumet, C., Maris, P., and Sanders, P. (2008). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol.* 124, 188–194. doi: 10.1016/j.ijfoodmicro.2008.03.030

Rasschaert, G., Houf, K., Van Hende, J., and De Zutter, L. (2006). *Campylobacter* contamination during poultry slaughter in Belgium. *J. Food Prot.* 69, 27–33. doi: 10.4315/0362-028X-69.1.27

Rozynek, E., Maćkiw, E., Kamińska, W., Tomczuk, K., Antos-Bielska, M., Dzierżanowska-Fangrat, K., et al. (2013). Emergence of macrolide-resistant *Campylobacter* strains in chicken meat in Poland and the resistance mechanisms involved. *Foodborne Pathog. Dis.* 10, 655–660. doi: 10.1089/fpd.2012.1333

Ruzauskas, M., Virgailis, M., Siugzdiniene, R., Zienius, D., and Mockeliunas, R. (2011). Differences in antimicrobial resistance of *Campylobacter jejuni* isolated from broiler intestines and drumsticks in Lithuania. *J. Food Saf.* 31, 379–385. doi: 10.1111/j.1745-4565.2011.00310.x

Sasaki, Y., Haruna, M., Mori, T., Kusukawa, M., Murakami, M., Tsujiyama, Y., et al. (2014). Quantitative estimation of *Campylobacter* cross-contamination in carcasses and chicken products at an abattoir. *Food Control* 43, 10–17. doi: 10.1016/j.foodcont.2014.02.015

Sasaki, Y., Maruyama, N., Zou, B., Haruna, M., Kusukawa, M., Murakami, M., et al. (2013). *Campylobacter* cross-contamination of chicken products at an abattoir. *Zoonoses Public Health* 60, 134–140. doi: 10.1111/j.1863-2378.2012.01509.x

Seliwiorstow, T., Baré, J., Van Damme, I., Uyttendaele, M., and De Zutter, L. (2015). *Campylobacter* carcass contamination throughout the slaughter process

of *Campylobacter*-positive broiler batches. *Int. J. Food Microbiol.* 194, 25–31. doi: 10.1016/j.ijfoodmicro.2014.11.004

Thakur, S., White, D. G., McDermott, P. F., Zhao, S., Kroft, B., Gebreyes, W., et al. (2009). Genotyping of *Campylobacter coli* isolated from humans and retail meats using multilocus sequence typing and pulsed-field gel electrophoresis. *J. Appl. Microbiol.* 106, 1722–1733. doi: 10.1111/j.1365-2672.2008.04142.x

Tissier, A., Denis, M., Hartemann, P., and Gassilloud, B. (2012). Development of a rapid and sensitive method combining a cellulose ester microfilter and a real-time quantitative PCR assay to detect *Campylobacter jejuni* and *Campylobacter coli* in 20 liters of drinking water or low-turbidity waters. *Appl. Environ. Microbiol.* 78, 839–845. doi: 10.1128/AEM.06754-11

Tresse, O., Alvarez-Ordóñez, A., and Connerton, I. F. (2017). Editorial: about the foodborne pathogen *Campylobacter*. *Front. Microbiol.* 8:1908. doi: 10.3389/fmicb.2017.01908

Wang, Y., Dong, Y., Deng, F., Liu, D., Yao, H., Zhang, Q., et al. (2016). Species shift and multidrug resistance of *Campylobacter* from chicken and swine, China, 2008-14. *J. Antimicrob. Chemother.* 71, 666–669. doi: 10.1093/jac/dkv382

Wei, B., Cha, S. Y., Kang, M., Roh, J. H., Seo, H. S., Yoon, R. H., et al. (2014). Antimicrobial susceptibility profiles and molecular typing of *Campylobacter jejuni* and *Campylobacter coli* from ducks in South Korea. *Appl. Environ. Microbiol.* 80, 7604–7610. doi: 10.1128/AEM.02469-14

Zeng, D., Zhang, X., Xue, F., Wang, Y., Jiang, L., and Jiang, Y. (2016). Phenotypic characters and molecular epidemiology of *Campylobacter jejuni* in East China. *J. Food Sci.* 81, 106–113. doi: 10.1111/1750-3841.13146

Zhang, M., Liu, X., Xu, X., Gu, Y., Tao, X., Yang, X., et al. (2014). Molecular subtyping and antimicrobial susceptibilities of *Campylobacter coli* isolates from diarrheal patients and food-producing animals in China. *Foodborne Pathog. Dis.* 11, 610–619. doi: 10.1089/fpd.2013.1721

Zhao, S., Young, S. R., Tong, E., Abbott, J. W., Womack, N., Friedman, S. L., et al. (2010). Antimicrobial resistance of *Campylobacter* isolates from retail meat in the United States between 2002 and 2007. *Appl. Environ. Microbiol.* 76, 7949–7956. doi: 10.1128/AEM.01297-10

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 Nutrition

ORIGINAL RESEARCH
published: 05 September 2018
doi: 10.3389/fnut.2018.00079

Feed Choice Led to Higher Protein Intake in Broiler Chickens Experimentally Infected With *Campylobacter jejuni*

Christian Visscher[1], Linus Klingenberg[1], Julia Hankel[1], Ralph Brehm[2], Marion Langeheine[2] and Ariane Helmbrecht[3]*

[1] Institute for Animal Nutrition, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, [2] Institute for Anatomy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany, [3] Evonik Nutrition & Care GmbH, Hanau, Germany

In 2016, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union with 246,307 reported cases. Of these cases, 83.6% were *Campylobacter jejuni*. The objective of the present study was to investigate to what extent an infection with *C. jejuni* alters the feed intake behavior of broiler chicks in terms of protein intake. This was done to see if, conversely, measures of control could be derived. In total, 300 commercial broilers of the Ross 308 line were allocated to four different groups, including five replications of 15 chickens each. In two groups, a conventional diet [216 g CP/kg dry matter (DM)] and in the two choice diet groups, diets with different levels of crude protein (286 and 109 g CP/kg DM, respectively) were fed between day 14 and day 42. An intake of both choice diets at a ratio of 3:2 resulted in a composition of consumed feed identical to that of the control concerning composition, energy and nutrient content. One group of each feeding concept was infected artificially with *C. jejuni* at day 21 by applying an oral *C. jejuni*-suspension containing 5.26 ± 0.08 $\log_{10}$ colony forming units of *C. jejuni* to three out of 15 chickens. No significant differences concerning *C. jejuni* prevalence and excretion could be seen. Broilers infected with *C. jejuni* chose a higher amount of the high protein choice diet in comparison to *C. jejuni* negative broilers. This resulted in a significantly ($p < 0.0001$) higher content of crude protein in the consumed diet (198 ± 3.09 g CP/kg DM and 208 ± 8.57 g CP/kg DM, respectively). Due to *C. jejuni* infection, a significant increase in crude mucin in excreta at day 42 was seen in experimentally infected groups (62.6 ± 4.62 g/kg DM vs. 59.6 ± 6.21 g/kg DM, respectively; $p = 0.0396$). There were significantly deeper crypts in infected birds (256 ± 71.6 vs. 234 ± 61.3 μm). In summary, *C. jejuni* infections significantly alter the feed intake behavior of broiler chickens regarding higher protein intake. Therefore, targeted manipulation of protein supply could be tested for limiting the spread of infection.

Keywords: broiler, *Campylobacter jejuni*, feed choice, protein requirements, performance

OPEN ACCESS

Edited by:
Michael J. Rothrock, United States Department of Agriculture, United States

Reviewed by:
Guillermo Tellez, University of Arkansas, United States
Cristiano Bortoluzzi, University of Georgia, Georgia

****Correspondence:***
Christian Visscher
christian.visscher@tiho-hannover.de

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

Received: *05 July 2018*
Accepted: *16 August 2018*
Published: *05 September 2018*

Citation:
Visscher C, Klingenberg L, Hankel J, Brehm R, Langeheine M and Helmbrecht A (2018) Feed Choice Led to Higher Protein Intake in Broiler Chickens Experimentally Infected With Campylobacter jejuni. Front. Nutr. 5:79. doi: 10.3389/fnut.2018.00079

INTRODUCTION

Campylobacteriosis is one of the world's most important diarrheal diseases (1). In 2016, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) and has been the case since 2005 (2). The number of reported confirmed cases of human *campylobacteriosis* was 246,307, with an EU notification rate of 66.3 per 100,000 population (2). This represented an increase of 6.1% compared with 2015 (2). Of confirmed cases reported in the EU, 83.6% were *Campylobacter jejuni* [*C. jejuni*; (2)].

Carbohydrates are only a minor substrate for the metabolism of *Campylobacter*. They are used only to a very limited extent (3). This is different than most other intestinal bacteria. The metabolism of *C. jejuni* relies on utilizing amino acids (3). Serine, aspartate, glutamate and proline are the preferred amino acids (4, 5). These amino acids are essential for the formation of the mucin-glycoproteins of the intestinal mucus layer (6, 7). Thus, they are most frequently included in both, the mucus layer and the excreta of poultry (8). In principle, threonine is of structural importance in the mucin protein backbone (9). The main constituents of the intestinal mucus layer are the mucins, which are produced by the goblet cells (10, 11). *C. jejuni* possesses very successful strategies to invade and colonize the mucus layer (12). The presence of mucins appears to be essential for the survival and growth of *C. jejuni* (12, 13). The crude protein content in the diet is relevant for mucin synthesis (14). Therefore, the crude protein supply is in parts responsible for the thickness and composition of the intestinal mucus layer (14, 15). Reduced crude protein content leads to reduced mucin production as well as a decrease in its release into the intestinal tract of broilers (14). In the context of enteral infections, there is an increased production of mucins associated with high amino acid demand (7, 16, 17). In addition to this demand for amino acids, the immune cells themselves assume high glucose consumption for immunological mechanisms (18). The mobilization of fat deposits provides energy reserves (19, 20), while metabolites of amino acid breakdowns can be used for enhanced gluconeogenesis (18, 20). This high variability in the demand for amino acids also affects the feed intake behavior of animals (21–24). There is much to be said for the homeostatic regulation of amino acid uptake, which is based on an extremely complex but not yet well-researched interplay of different mechanisms (24). In addition to this ability to detect an unbalanced amino acid composition of the diet, broilers can find out the component that best suits their needs (22, 25–27). In empirical scientific work, this ability is used to investigate the influence of different external and internal factors on the feed intake behavior of the animals in feeding experiments (election experiments) with a choice between different animal feeds (26, 28, 29). However, it should be noted that in feeding trials dealing with the level of protein intake that feed intake essentially depends on the energy content of the diet, whereby the total feed intake is negatively correlated with the energy content (26). In growing poultry, the protein requirement remains relatively constant, while the energy requirement increases to some degree (22). Broilers tend to meet their energy needs by minimizing total feed intake (22).

The hypothesis in the present experiment was that broilers show different dietary intake behavior depending on *C. jejuni* infection. Therefore, targeted manipulation of the protein supply and amino acid pattern could be tested for limiting the spread of infection in further studies.

MATERIALS AND METHODS

The aim of the investigations was to analyze the influence of an experimental *C. jejuni* infection in broilers on feed intake behavior. In control groups, a compound feed regime (SP-diet) was used. In the choice experiment, birds were given the option to select between a low protein diet (CD^{CP-}-diet) and a high protein diet (CD^{CP+}-diet). At the same time, potential effects of the experimental *C. jejuni* on the spread of infection, the level of *C. jejuni* excretion, performance of birds, histology of the ileum and the mucin content in excreta were of interest. The artificial infection was done in a seeder model. Therefore, animal experiments were performed in accordance with the German rules and regulations and approved by the Ethics Committee of Lower Saxony for Care and Use of Laboratory Animals, LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit; reference: 33.19-42502-05-15A540).

Animals and Housing

Day of hatching chicks (day 0 = d 0) of both sexes ($N = 300$; ROSS 308) were obtained from a commercial hatchery (BWE-Brüterei Weser Ems, PHW Gruppe/LOHMANN & Co. AG, Visbek, Germany). For the first 14 days, the birds were housed in four identical floor pens littered with wood shavings. The temperature profile in the pen started with a temperature of about 34–36°C. During the trial, the temperature was lowered by about 1°C every 2 days, reaching a minimum temperature of about 20°C. The photoperiod beginning from d 4 was 16 h of light and 8 h of darkness during the whole trial which complies with the local regulations on keeping chickens (Tierschutz-Nutztierhaltungsverordnung), which provides for 6 h of dark time plus dimming periods of light.

After a fourteen-day rearing phase, the animals were transferred in-house to the experimental unit (security level 2, this means standard biosecurity and institutional safety procedures). Following this, the animals were randomly subdivided into 20 subgroups in a 2 × 2 factorial design with two different diets (SD-diet, CD-diet consisting of the components CD^{CP+} and CD^{CP-}) and a different infection modus (CN, *Campylobacter* Negative, CP, *Campylobacter* Positive) and the following combinations thereof (SDCN-Standard Diet, *Campylobacter* Negative; CDCN-Choice Diet, *Campylobacter* Negative; SDCP- Standard Diet, *Campylobacter* Positive; CDCP-Choice Diet, *Campylobacter* Positive). All birds in the study were individually tagged with wing-tags.

The animals were kept in modified boxes (AviMax, Big Dutchman AG, Vechta, Germany) with solid flooring littered with wood shavings (1 kg/m^2) in groups of 15 animals ("subgroup"). Each subgroup had an unrestricted available floor

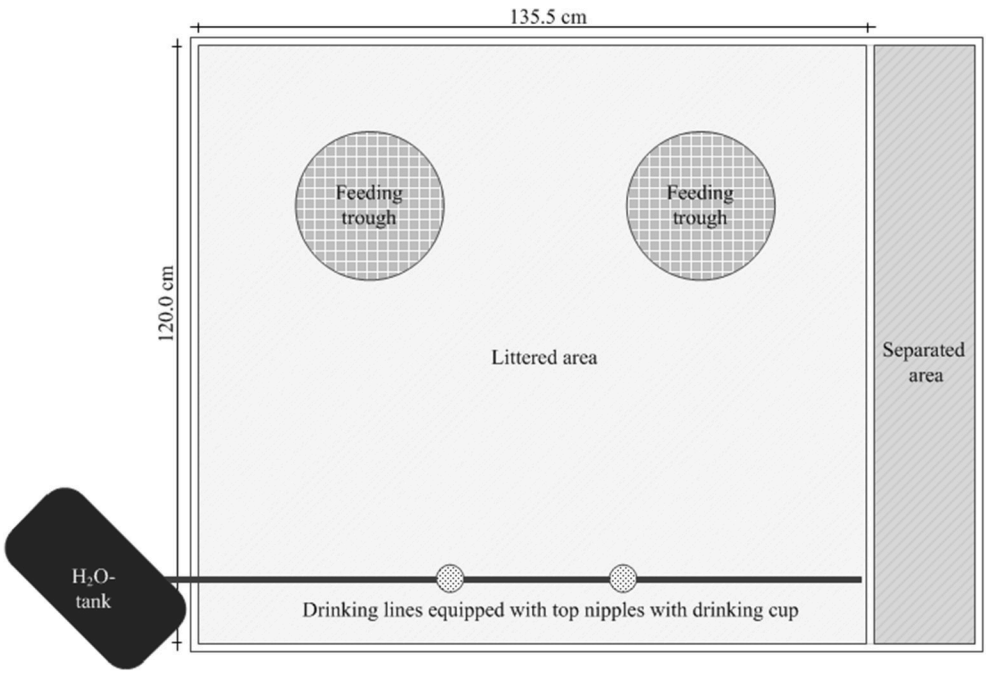

FIGURE 1 | Presentation of the arrangement of the feeding troughs and the water lines for control and experimental groups in a box.

space (total area minus the area under the trough) of 1.45 m². Rearing took place up to the end of the 4-week experimental period (beginning at d 42) or rather up to dissection (d 43–45; **Figure 1**).

Feeding Regime and Feed Analysis

In the rearing phase (d 0–13) a conventional pelleted starter diet (starter) was fed for 1 week, followed by a subsequent 7-day phase with a commercially available pelleted grower diet (grower; Best 3 Geflügelernährung GmbH, Twistringen, Germany). Diets for the main experimental period (d 14 onwards) were produced in cooperation with Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany). A manufacturer for trial diets (Research Diet Services BV, Wijk bij Duurstede, the Netherlands; **Table 1**) produced the different diets. The standard diet (SD-diet) was designed in accordance with a commercially available standard fattening diet concerning ingredients and composition (**Tables 1**–**3**). The energy and nutrient content to a certain amount exceeded the recommendations for the energy and nutrient supply of the laying hens and fowls (broilers) of the Committee for Needs Standards of the Society for Nutritional Physiology (30). The other two groups were given the opportunity to freely choose between two different diets. This choice diet consisted of a high protein component (CD^{CP+}-diet: 286 CP/kg DM) and a low protein component (CD^{CP-}-diet: 109 g CP/kg DM). These two compound feeds were based on the SD-diet, modified only in their content of wheat and soybean meal. Due to the specifically formulated composition of the two components of the CD-diet (CD^{CP+}-diet, CD^{CP-}-diet; **Table 1**), a bird consuming three parts CD^{CP+}-diet (60%) and two parts CD^{CP-}-diet (40%) ingested a feed mixture that referred to an identical botanically and chemically to the standard feed (SD-diet). All diets were offered *ad libitum*. Circular feeding troughs, two for each box, were used during the entire trial (Crown Poultry Feeders, New Zealand). In the control groups (SDCN, SDCP), both troughs contained an identical diet. In the experimental groups (CDCN, CDCP), each trough contained one of the two CD-diets (CD^{CP+}-diet, CD^{CP-}diet). Water was offered *ad libitum* in double-cylinder plastic bell drinkers in the rearing phase, later via drinking lines equipped with Top Nipples with a drinking cup (Big Dutchman International GmbH, Vechta-Calveslage, Germany). The water was treated with chlorine-oxygen preparation at a concentration of 0.3 mg/L to kill any *C. jejuni* in the drinking water (Virbac Clean Pipe, VIRBAC Tierarzneimittel GmbH, Bad Oldesloe, Germany). Water and feed samples were tested for *C. jejuni*. Both substrates were originally *C. jejuni* negative.

Diets were analyzed by standard procedures in accordance with the official methods of the VDLUFA (31). The dry matter content (DM) was determined by drying to the weight constancy at 103°C, whereas the raw ash was analyzed by means of incineration in the muffle furnace at 600°C for 6 h. The total nitrogen content was determined in accordance with the DUMAS combustion method by means of the analyzer Vario Max® (Elementar, Hanau, Germany). The crude fat content was determined in the soxleth apparatus and the content of crude fiber was analyzed after washing in dilute acids and alkalis. Starch determination was carried out polarimetrically (Polatronic E, Schmidt und Haensch GmbH & Co., Berlin, Germany). The sugar content was analyzed by the method in

TABLE 1 | Ingredient composition of the standard protein diet and high protein diet and low protein diet of the choice feeding concept for the main experimental period (days 21–42).

Ingredients (in %)	SP-diet[a]	CD^{CP+} -diet	CD^{CP-} -diet	CD^{CP+}/CD^{CP-} -diet [60/40]
Wheat	41.4	25.3	65.5	41.4
Corn	25.0	25.0	25.0	25.0
Soybean meal[b]	24.1	40.2	0.00	24.1
Soybean oil	5.16	5.16	5.16	5.16
Monocalciumphosphate	1.37	1.37	1.37	1.37
Calcium carbonate	1.28	1.28	1.28	1.28
Premix "Blank Poultry"[c]	0.50	0.50	0.50	0.50
L-Lysin-HCl®[d]	0.26	0.26	0.26	0.26
Sodium bicarbonate	0.25	0.25	0.25	0.25
MetAMINO®[e]	0.25	0.25	0.25	0.25
Sodium Chloride	0.20	0.20	0.20	0.20
ThreAMINO®[f]	0.10	0.10	0.10	0.10
L-Isoleucin	0.06	0.06	0.06	0.06
ValAMINO®[g]	0.06	0.06	0.06	0.06

SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}-diet [60/40], Composition of the choice diet with 60% CD^{CP+}-diet and 40% CD^{CP-}diet.

[a]The sum of all the ingredients does not equal 100 due to rounding differences.

[b]48% crude protein.

[c]Carrier: cornflour; content per kg: iron (16,000 mg), copper (2,400 mg), manganese (17,000 mg), zinc (12,000 mg), iodine (160 mg), selenium (30 mg), vitamin A (2,000,000 IU), vitamin D3 (500,000 IU), vitamin E (10,000 mg), vitamin K3 (300 mg), vitamin B1 (400 mg), vitamin B2 (1,500 mg), vitamin B6 (700 mg), vitamin B12 (4,000 µg), niacin (7,000 mg), D-pantothenic acid (2,400 mg), choline chloride (92,000 mg), folic acid (200 mg), biotin [40 mg].

[d]78.0% L-Lysine.

[e]99.0% DL-Methionine.

[f]98.5% L-Threonine.

[g]98.0% L-Valine.

TABLE 2 | Concentrations of ingredients and energy content after chemical analysis in the standard protein diet and in the high protein diet and low protein diet of the choice feeding in the experimental period (days 21–42).

Item	SP-diet[1]	CD^{CP+} -diet	CD^{CP-} -diet	CD^{CP+}/CD^{CP-}-diet [60/40]
Dry matter [g/kg diet]	883	888	877	884
Crude ash [g/kg DM]	53.0	60.9	41.1	53.0
Crude fat	80.6	76.5	78.4	77.2
Crude fiber	28.9	28.9	25.1	27.4
Crude protein	216	286	109	215
Nitrogen free extract[a]	621	547	747	627
Starch	460	366	618	467
Sugar	46.1	56.8	27.5	45.1
Calcium	9.42	9.93	9.39	9.71
Phosphorus	7.78	8.37	6.83	7.75
Potassium	8.32	12.3	4.11	9.00
Sodium	1.86	1.77	1.98	1.85
Chloride	2.74	2.61	2.88	2.72
Magnesium	1.85	2.27	1.22	1.85
Sulfur	2.96	3.47	2.25	2.98
Metabolisable energy AME_N[b] [MJ/kg DM]	14.4	13.9	15.1	14.4

SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}-diet, Low protein choice diet; CD^{CP+}/CD^{CP-}-diet [60/40], Composition of the choice diet with 60% CD^{CP+}-diet and 40% CD^{CP-}-diet

[a]Nitrogen-free extract, DM - (crude ash + crude fat + crude fiber + crude protein).

[b]Metabolisable energy AME_N (MJ/kg DM), 0.1551×% crude protein+0.3431×% crude fat+0.1669×% starch+0.1301×% sugar.

accordance with Luff-Schoorl by titration, whereas the mineral content was determined by atomic absorption spectrometry (Unicam Solaar 116, Thermo, Dreieich, Germany). Amino acids were determined by ion-exchange chromatography (AA analyser LC 3000, Biotronic, Maintal, Germany).

Experimental Infection and Sampling

Prior to *Campylobacter* challenge, there was a two-step procedure to ensure that there was no *Campylobacter* colonization prior to experimental infection. This was done at d 18 in five animals per subgroup. Three days later, at d 21, this was performed for each individual animal ($N = 300$). Samples were taken by means of a cloacal swab (Cary Blair smear test system, Süsse Labortechnik GmbH & Co. KG, Gudensberg, Germany).

At d 21, in each subgroup, three of 15 broilers in a box ($n = 5$ for groups SDCP and CDCP each) were administered orally with a *C. jejuni* suspension. A field strain of *C. jejuni* was used for experimental infection (32). This isolate had been identified as *C. jejuni* both culturally and mass-spectrometrically (MALDI-TOF MS; AniCon Labor GmbH, Höltinghausen, Germany). Preparation of the conserved strain for experimental infection was done as previously described (33). The infection strain was used in its stationary growth phase (24–48 h) for preparing the inoculum. *C. jejuni* was resuspended in isotonic 0.9% sodium chloride solution (∼10,000 CFU/2 mL). Bacterial suspension for infection was administered orally in three out of 15 randomly selected animals. A button cannula (single-button cannula, sterile, 1.0 × 100 mm, Meiser Medical GmbH, Neuenstein, Germany) was used for application. Analogous to the groups with experimental infection, three of 15 animals were randomly selected from the non-infected control groups (SDCN and CDCN). These animals were administered 2 mL of a sterile sodium chloride solution by means of a button cannula.

At days 22, 23, 24, 25, 28, 35, and 42, individual samples in groups SDCP and CDCP were taken in an identical manner ($n = 75$/group) as described above. In the groups SDCN and CDCN, regular spot-checks of five randomly selected animals per subgroup were examined for *C. jejuni* occurrence. Twenty-one days following experimental infection (beginning at d 42), animals from all groups were qualitatively tested concerning their *Campylobacter* status in accordance with DIN EN ISO 10272-1:2006 (see paragraph "Bacterological Analyses"). Quantitative analyses by determining the colony-forming units of *C. jejuni* in excreta of seeder birds in groups SDCP und CDCP were performed at days 23, 32, and 38. For excreta collection, the corresponding animals were individually placed in purified, disinfected 10 L plastic bucket (26.5 cm) in order to collect freshly dropped excreta.

For determining the mucin content samples in fresh excreta ($n = 3$ pooled samples per box) of the birds, samples were collected from each box at d 20 and d 42 in accordance with

TABLE 3 | Amino acid content in the standard protein diet as well as in the high protein diet and low protein diet of the choice feeding in the experimental period (days 21–42).

Item	SP-diet	CD^{CP+} -diet	CD^{CP-} -diet	CD^{CP+}/CD^{CP-}-diet [60/40]
Arginine [g/kg DM]	13.8	19.7	4.72	13.7
Cysteine	4.55	5.12	3.17	4.34
Isoleucine	9.39	13.1	3.96	9.42
Leucine	16.2	21.9	7.72	16.2
Lysine	12.8	18.0	5.37	13.0
Methionine	6.10	6.84	4.90	6.06
Phenylalanine	10.4	14.0	4.68	10.3
Threonine	8.02	11.8	4.38	8.85
Valine	10.3	14.1	4.93	10.4
Alanine	9.18	12.4	4.33	9.17
Aspartic acid	19.9	28.4	5.43	19.2
Glutamic acid	41.2	53.0	24.6	41.7
Glycine	8.56	11.7	4.00	8.63
Histidine	5.44	7.58	2.51	5.55
Proline	13.9	17.7	9.40	14.4
Serine	10.2	14.5	4.80	10.6
Tyrosine	8.02	9.88	3.28	7.24

SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}-diet [60/40], Composition of the choice diet with 60% CD^{CP+}-diet and 40% CD^{CP-}-diet.

established methods (34). Within the infection trial, sampling was omitted to minimize the risk of transmitting infection between boxes. The collected excreta were then removed from each box, thoroughly mixed, and processed in parts for further analysis (mucin content).

Dissection of the animals was performed by standard protocol approved by the Animal Care Committee on three consecutive days (d 43, 44, and 45). The contents of the two ceca were removed under sterile conditions and placed in a screw cup (screw cup 100 mL, PP, Sarstedt AG & Co., Nümbrecht, Germany) for all animals in groups SDCP and CDCP.

Bacteriological Analyses

Bacteriological examination at bird level (qualitative analyses) was based on DIN EN ISO 10272-1:2006, taken from the official collection of analysis methods in accordance with § 64 LFBG. The sample matrix was incubated in a one-to-nine ratio (sample:Bolton-broth) in sterile 5 mL tubes mounted with a vent cap (Sarstedt AG & Co., Nümbrecht, Germany). Incubation lasted 4 h at 37°C, followed by 44 ± 4 h at 41.5°C under a microaerobic atmosphere (oxygen content of 5 ± 2%, carbon dioxide content of 10 ± 3%; DIN EN ISO 10272-1:2006). Enrichment in Bolton-broth was followed by streaking samples onto two solid selective culture media (mCCD agar and Karmali agar; Oxoid Germany GmbH, Wesel, Germany) with sterile inoculation loops. Selective cultures were incubated again for 44 ± 4 h at 41.5°C in a microaerophilic atmosphere. Presence of *Campylobacter* was confirmed by analyzing individual colonies. This was done by phase contrast microscopy (Distelkamp-Electronic, Kaiserslautern, Germany) and biochemical methods (apiCampy, bioMérieux SA, Marcy-LÈtoile, France).

Quantitative bacteriological examination was done by a ten-fold dilution series (0.5 g sample material in 4.5 mL of sterile Phosphate Buffered Saline—PBS) with PBS (Phosphate Buffered Saline, Oxoid Germany GmbH, Wesel, Germany). In duplicate, 100 µL of each dilution was plated onto mCCD agar (Oxoid Germany GmbH, Wesel, Germany). After incubation (microaerophilic atmosphere: 44 ± 4 h at 41.5°C), the colonies were counted and an average value from the two duplicate experiments was taken for calculating the CFU/g intestinal content. In accordance with DIN EN ISO 10272-2:2006, only plates with more than 30 and fewer than 300 colonies were considered.

Analysis of Mucin Content and Histological Investigations

The content of total mucin was determined in birds' pooled excreta. Quantifying the water-soluble and ethanol-precipitable fraction of excreta was carried out in accordance with modified methods (9, 35) as described by Visscher et al. (33).

For histological investigations, an ∼1 cm long piece was removed from the apex of the right cecum, ∼1 cm proximal to the apex, and fixed in 4% formaldehyde for 48 h. After fixation, tissue samples were embedded in paraffin using standard techniques (36). For histological evaluation, 4 µm sections of all samples were stained with HE using established protocols (36). For determining the number of goblet cells in the cecal crypts, sections were viewed with a Zeiss axioscope (Carl Zeiss Jena GmbH, Jena, Germany). Sample analysis was done in accordance with established methods (9) with slight modifications. The depth of five complete vertically oriented crypts was measured in each of the blinded samples. The number of goblet cells of the crypt was counted. The number of goblet cells of the individual, measured crypts of each sample was converted to a standard crypt depth of about 250 µm for comparison between groups.

Performance Parameters

The body weight of the birds was recorded individually at the beginning of days 7, 14, 21, and 42 (PCE TB 30, PCE Instruments, Meschede, Germany). The animal losses were taken into account for all analyzed parameters. The feed and water intake were recorded at the level of the box (20 boxes in total; $n = 5$ subgroups/boxes per group). The feed conversion ratio (FCR) reflected feed consumption per kilogram of body weight gain. Protein efficiency was calculated as the increase in body weight per kilogram of crude protein intake. The feed-water ratio was calculated as the ratio of water intake to feed intake. At the time of dissection (d 43, 44, and 45), the slaughtering weight (body with feathers and without head, feet, gastrointestinal tract, liver, gallbladder, spleen, heart) and the slaughtering exploitations were calculated as a quotient of slaughter weight to the body mass (in %).

Statistical Analyses

The statistical analysis of the collected data was performed using the Statistical Analysis System for Windows the SAS® Enterprise Guide®, version 9.3 (SAS Institute Inc., Cary, USA). The normal distribution of the residues was tested first with a Shapiro–Wilk test before comparing mean values. Normally distributed data were examined in parts for differences in means by a two-factorial analysis of variance with "diet" (SD-diet, CD-diet) and "infection" (CN, CP) as independent variables as well as multiple pairwise comparisons between combinations of variables (Fisher's smallest significant difference). Due to the study design, the factors "diet" and "infection" were not always to be classified as classic independent factors. The SD-Diet was fixed for energy and nutrient composition. Therefore, the effect of the infection on the parameters related to the choice of feed was not done by a two-factorial evaluation because the animals in the SDCP subgroup had no choice. Here the focus was then placed on the comparison between combined factors. Furthermore, half of the subgroups were not experimentally infected with *C. jejuni*. Therefore, a two factorial analysis of the quantitative excretion of the bacteria was not necessary.We sometimes were forced to make pairwise comparison of the fixed combination of CD groups (CDCN, CDCP) in comparison to the fixed parameters from the SD groups. Non-normalized data were processed with a Wilcoxon signed-rank test in pairs to investigate differences in the mean values. For comparing a sample with a constant named above or for comparisons according to *C. jejuni*, a one-sample *t*-test was used for normal distributed data. For uniform distribution of the sample, two-dimensional frequency distributions of categorial features were checked for dependency with the Pearson's Chi square homogeneity test. Otherwise, the Fisher's exact test was used. Correlation analyses were carried out on normal distributed data using Pearson's correlation coefficient. Non-normalized data were analyzed using Spearman's rank correlation coefficient. At $p < 0.05$, differences in the mean values, dependence of the frequency distribution or a correlation were regarded as significant.

RESULTS

The experiment ran completely without complications. Mortality was 1.33%. Three out of 300 broilers used in the experiment died during the experiment, one had to be euthanized (losses in %: SD-diet: 1.33%, CD-diet: 1.33%; CN: 0.67%; CP: 2.00%; SDCN: 1.33%, CDCN: 0.00%, SDCP: 1.33%, CDCP: 2.66%).

Campylobacter Excretion

Before and at the time of the experimental infection, all animals in the experiment were *Camplylobacter* spp. negative in cloacal swab samples ($N = 300$).

Experimental infection in groups SDCP and CDCP took place at d 21. The inoculum for the experimental infection contained an average of 5.26 ± 0.08 $\log_{10}$ CFU *C. jejuni* per bird (2 mL). Already 1 day after this infection, an excretion of *C. jejuni* could be seen with cultural techniques (**Table 4**). Seven days after experimental infection in both groups, 100% of animals were *C. jejuni* positive in excreta. During the trial, there were no significant differences between groups.

TABLE 4 | Prevalence of *C. jejuni* in cloacal swabs of all animals and counts of *C. jejuni* in the excreta of the seeder birds ($n = 15$ per group) at days 23, 32, and 38 as well as total counts of *C. jejuni* in the caecal content of all animals on the day of dissection.

	Diet				
	SP-diet		CD^{CP+}/CD^{CP-}-diet		*P*-value
Item	% positive	n (pos./total)	% positive	n (pos./total)	
PREVALENCE ON GROUP LEVEL					
Day 18	0.00	(0/25)	0.00	(0/25)	1.0000
Day 21	0.00	(0/75)	0.00	(0/75)	1.0000
Day 22	1.33	(1/75)	8.00	(6/75)	0.1162
Day 23	18.7	(14/75)	17.3	(13/75)	0.8317
Day 24	38.7	(29/75)	42.7	(32/75)	0.6180
Day 25	70.7	(53/75)	77.3	(58/75)	0.3520
Day 28	100	(75/75)	100	(75/75)	1.0000
Day 35	100	(75/75)	100	(75/75)	1.0000
Day 39	100	(75/75)	100	(73/73)	1.0000
Day 42	100.0[a]	(75/75)	100.0[a]	(73/73)	1.0000
QUANTITATIVE COUNTS *C. jejuni* Excreta Seeder Birds ($\log_{10}$ cfu/g)					
	Mean	SD	Mean	SD	P-value
Day 23	4.11	1.44	3.75	2.12	0.5906
Day 32	5.00	0.75	5.06	0.79	0.8551
Day 38	4.15	0.82	3.58	1.55	0.2454
QUANTITATIVE COUNTS *C. jejuni* Caecal Content ($\log_{10}$ cfu/g)					
Dissection	7.02	0.85	6.87	1.12	0.6878

SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}-diet, Choice diet; [a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

The mean values of $\log_{10}$ CFU *C. jejuni* in the excreta of the seeder animals (15 animals per group, three animals per subgroup) as well as the mean counts of *C. jejuni* in the cecal content did not differ between the SDCP and CDCP groups (**Table 4**). The SDCN and CDCN groups remained *C. jejuni* negative up to the end of the trial.

Feed Intake and Performance Parameters

The SD-diet groups were given a diet with an energy content of 14.4 MJ ME/ kg DM throughout the experimental phase. The energy density in the recorded CD-diets of the experimental groups showed maximum deviation of 0.17 MJ ME/ kg DM (+1.18%) in the experimental group CDCN between d 14 and d 21. By comparison, the CP-content in the ingested diet of the experimental group CDCN was 13% lower than the CP-content of the SD diet during the same period (**Table 5**).

Between d 21 and d 42, the animals in the CD-diet groups ingested a diet with an average CP-content of 203 ± 8.00 g/ kg DM. This value was significantly lower than in the groups with SD-diets ($p < 0.0001$). Looking at the combined effects, it turned out that the CDCN group preferred, a bit more than common, the CD^{CP-}-diet component. Therefore, birds

TABLE 5 | Energy and crude protein concentration in diets as well as feed and nutrient intake.

Item	CN				CP			
	SP-diet		CD^{CP+}/CD^{CP-}-diet		SP-diet		CD^{CP+}/CD^{CP-}-diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ME-CONTENT [MJ ME/kg DM]								
Day 14–20	14.4^{b}	0.00	14.6^{a}	0.07	14.4^{b}	0.00	14.5^{a}	0.05
Day 21–27	14.4^{a}	0.00	14.4^{a}	0.03	14.4^{a}	0.00	14.4^{a}	0.07
Day 28–34	14.4^{b}	0.00	14.5^{a}	0.05	14.4^{b}	0.00	14.4^{b}	0.05
Day 35–41	14.4^{b}	0.00	14.6^{a}	0.06	14.4^{b}	0.00	14.5^{ab}	0.09
Day 21–41	14.4^{b}	0.00	14.5^{a}	0.02	14.4^{b}	0.00	14.4^{b}	0.06
CP-CONTENT [g/kg DM]								
Day 14–20	216^{a}	0.00	188^{b}	10.0	216^{a}	0.00	192^{b}	7.47
Day 21–27	216^{a}	0.00	210^{b}	3.83	216^{a}	0.00	220^{a}	10.0
Day 28–34	216^{a}	0.00	198^{b}	6.94	216^{a}	0.00	211^{a}	7.22
Day 35–41	216^{a}	0.00	190^{b}	8.69	216^{a}	0.00	197^{b}	13.6
Day 21–41	216^{a}	0.00	198^{b}	3.09	216^{a}	0.00	208^{a}	8.57
Ratio CD^{CP+}/CD^{CP-}-diet day 21–41	1.50^{a}	0.00	1.01^{b}	0.07	1.50^{a}	0.00	1.28^{a}	0.24
DM-INTAKE [g/animal and day]								
Day 14–20	81.7^{ab}	4.72	76.4^{b}	4.60	83.6^{a}	2.13	79.2^{ab}	4.35
Day 21–27	123^{ab}	4.09	118^{b}	4.22	125^{a}	4.78	120^{ab}	5.57
Day 28–34	162^{ab}	5.89	156^{b}	2.16	166^{a}	7.61	157^{b}	5.51
Day 35–41	180^{a}	3.79	175^{a}	8.65	177^{a}	6.01	180^{a}	5.93
Day 21–41	155^{ab}	4.09	150^{b}	4.12	156^{a}	5.28	152^{ab}	3.20
CP-INTAKE [g/animal and day]								
Day 14–20	17.7^{a}	1.02	14.4^{b}	1.38	18.1^{a}	0.46	15.2^{b}	0.68
Day 21–27	26.7^{a}	0.89	24.8^{b}	0.95	27.1^{a}	1.03	26.3^{a}	0.40
Day 28–34	34.9^{ab}	1.27	30.9^{c}	1.40	36.0^{a}	1.65	33.2^{ab}	1.15
Day 35–41	38.9^{a}	0.82	33.2^{b}	2.24	38.3^{a}	1.30	35.3^{b}	2.71
Day 21–41	33.5^{a}	0.88	29.6^{c}	0.73	33.8^{a}	1.14	31.6^{b}	0.76
AA_{GL} intake	9.36^{a}	0.25	8.37^{c}	0.21	9.43^{a}	0.32	8.97^{b}	0.25
AA_{CM} intake	13.2^{a}	0.35	11.8^{c}	0.29	13.3^{a}	0.45	12.6^{b}	0.30

CN, without experimental C. jejuni infection; CP, with experimental C. jejuni infection; SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}diet, Choice diet; AA_{GL}, Sum "growth limiting" amino acids like arginine, isoleucine, lysine, methionine, threonine and valine; AA_{CM}, Sum of C. jejuni metabolisable amino acids like aspartic acid, glutamic acid, proline and serine; [a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

consumed nearly identical proportions of both diets (relationship between CD^{CP+}/CD^{CP-}diets: 1.01 ± 0.07; $p < 0.0001$; to compare: theoretical CD^{CP+}/CD^{CP-}-ratio in the SDCN group was 1.50). This was fare away from the ratio three parts CD^{CP+}-diet and two parts CD^{CP-}-diet, if choice would have let to an identical botanical and chemical composition as found in the SD-diet. This shift in feed intake toward the protein-poor CD^{CP-}-diet led to a significant reduction in average protein content of the ingested CD-diet. At 198 ± 3.09 g CP/ kg DM, the recorded crude protein content was significantly lower in the CDCN group ($p = 0.0002$) than in the SDCN group (216 g CP/ kg DM). Compared to the SDCP group (216 g CP/ kg DM), the animals in the experimental group infected with *C. jejuni* (CDCP) showed no significant difference concerning the crude protein content in the ingested feed (208 ± 8.57 g CP/ kg DM).

The differentiation at weekly level showed that in the period prior to the experimental infection (d 14–d 20), offering the choice diets led to ingestion of a diet with a significantly lower protein content (SD-diet: 216 g CP/ kg DM; CD-diet: 190 ± 8.68 g CP/kg DM; $p < 0.0001$). Comparing both choice diet groups (CDCN and CDCP), these groups had both a significantly lower ($p = 0.0032$ and $p = 0.0020$) crude protein density in diets (188 ± 10.0 g CP/ kg DM and 192 ± 7.47 g CP/ kg DM, respectively) in comparison to the feeding groups offered the SD-diet (216 g CP/ kg DM).

In the first week after experimental infection (d 21–d 27), a numerically increased uptake of the high-protein CD^{CP+}-diet was observed in both groups with choice option. Therefore, there was no difference depending the factor diet on weekly basis (SD-diet: 216 g CP/ kg DM; CD-diet: 215 ± 8.89 g CP/ kg DM; $p = 0.5970$; **Table 5**, **Figure 2**). For combined factors, the crude protein content of the ingested feed of the non-experimentally infected experimental group (CDCN) was lower (210 ± 3.83 g CP/kg DM; $p = 0.0188$) than in the SDCN group. The experimentally infected group (CDCP), on the other hand, chose a diet with a numerically higher crude protein content than

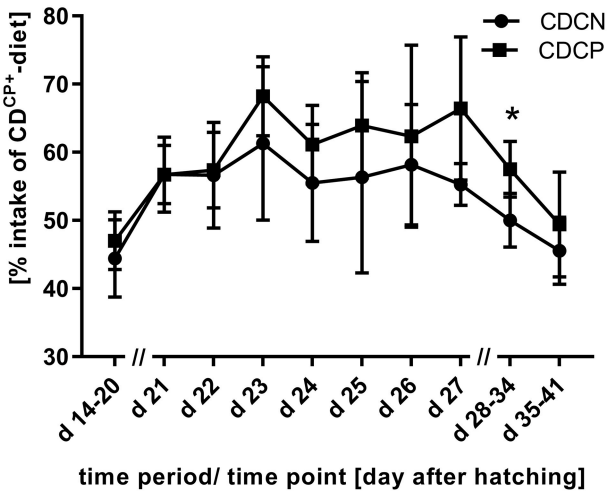

FIGURE 2 | Proportion of uptake of the compound feed with high protein content (CD^{CP+}-diet) in the experimental phase (experimental *C. jejuni* infection at the beginning of d 21; 60% intake of CD^{CP+}-diet would mean a comparable diet composition to SD-diet); *Values differ significantly at $p < 0.05$.

in the infected group offered the SD-diet (220 ± 10.0 g CP/kg DM vs. 216 g/kg DM). In the two subsequent weeks a decline in intake of the protein-rich CD^{CP+}-diet was seen in both experimental groups with choice (d 28–d 34, SD-diet: 216 g CP/kg DM; CD-diet: 204 ± 9.65 g CP/ kg DM; $p = 0.0020$; d 35–d 41, SD-diet: 216 g CP/kg DM; CD-diet: 193 ± 11.3 g CP/ kg DM; $p < 0.0001$). In the period from d 28–d 34, the share of intake of the CD^{CP+}-diet was significantly lower in the CDCN group than in CDCP (**Figure 2**). Additionally, in both groups with choice diets, in the last week of the experiment the crude protein uptake was lower than in the groups with the SD-diet (CDCN: 190 ± 8.89; CDCP: 197 ± 13.6 g CP/ kg DM).

The SD-diet fed subgroups (SDCN, SDCP) showed a significantly higher daily feed intake per animal (156 ± 4.50 g) than the subgroups fed the CD-diet (151 ± 3.70 g) in the period from d 21 to d 42 ($p = 0.0198$). In combination with lower crude protein content of the CD-diets in the CDCN and CDCP subgroups, this resulted in a significantly ($p < 0.0001$) lower daily crude protein intake of the subgroups fed the CD-diets (30.6 ± 1.25 g) compared to the subgroups fed the SD-diets (33.7 ± 0.97 g). Week-by-week observation, however, showed that in the first week after infection, crude protein intake was lower in the CDCN group than in any other group. The CDCP group did not differ at this stage, nor in the subsequent week (d 28–34) from the groups which were offered the SD-diet.

The animals were randomly divided into four groups during the rearing phase, each housed in one box. At the end of the rearing phase, animals from each group were allocated to their respective five subgroups. Thus, there was no mixing between the animals of the groups. At the first body weight assessment (d 7), there was no difference in average body weight between the groups (**Table 6**). At d 14, broilers from the CDCN group had an average body weight lower than that in the other groups (average deviation: maximum 12 g/animal). However, at diet level (SD-diets and CD-diets), no statistical differences in the body weight of the animals were seen before the start of the experiment at d 14 (SD-diet: 494 ± 47.2 g, CD-diet: 488 ± 49.0 g). After the adaptation phase to the diet (d 14–d 20) and before experimental infection, the body weight of the SP-diet fed animals (987 ± 96.9 g) was higher than the body weight of CD-diet fed birds (908 ± 106 g; $p < 0.0001$). This difference was able to prevail during the experimental phase. At the day of dissection, the body weight of the feeding subgroups with SD-diets (3504 ± 428 g) was significantly higher than the body weight of the feeding subgroups with CD-diets (3,338 ± 465 g; $p = 0.0013$). The dressing percentage was also significantly more favorable in subgroups fed with the SD-diets (83.0 ± 1.18%) than in subgroups fed with the CD-diets (82.3 ± 1.21%; $p < 0.0001$).

Rating the body weight as a function of the main factor "infection" with *C. jejuni,* there were comparable starting conditions of non-infected and infected subgroups before the experimental infection at d 21 (940 ± 110 and 955 ± 108 g, respectively). At the end of the experiment, a significantly lower body weight of the non-infected subgroups (3,155 ± 400 g) in comparison to infected subgroups (3,275 ± 405 g; $p = 0.0150$) could be observed.

Histology of the Intestine

This histological examination showed a significantly deeper crypt depth in experimentally infected subgroups compared to the non-infected subgroups (256 ± 71.6 vs. 234 ± 61.3 µm; $p = 0.0011$). Regarding the main factor "diet" there were no significant differences. The feeding concept itself, however, had no effect on crypt depth (**Table 7**). The number of goblet cells was significantly higher in the CDCP subgroups.

Mucins in Excreta

The total mucin content in the excreta showed a continuous increase from d 21 to d 42. An exception was the CDCN group (**Table 8**). In this group, the mucin levels slightly declined.

There was no difference in the total mucin content between the feeding concepts at d 21 (SD-diet: 55.2 ± 5.52 g/kg DM; CD-diet: 55.3 ± 5.19 g/kg DM). At d 42, however, a significantly higher total mucin content of groups fed the SD-diet (63.7 ± 5.30 g/kg DM) in comparison to groups fed the CD-diet was observed (58.5 ± 4.74 g/kg DM; $p = 0.0002$).

At d 21, the excreta of birds in groups which were later not experimentally infected, contained a significantly higher (56.8 ± 4.67) total mucin content compared to those experimentally infected with *C. jejuni* (53.7 ± 5.51 g/kg DM; $p = 0.0188$) at a later stage. By contrast, at d 42, the total mucin content of the *C. jejuni* infected groups was significantly higher (62.6 ± 4.62 g/kg DM) than in the non-infected groups (59.6 ± 6.21 g/ kg DM; $p = 0.0396$).

TABLE 6 | Performance data of broilers depending on experimental infection with *C. jejuni* and diet as well as feed choice.

Item	CN				CP			
	SP-diet		CD^{CP+}/CD^{CP-}-diet		SP-diet		CD^{CP+}/CD^{CP-}-diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BODY WEIGHT [in g]								
Day 7	178[a]	20.3	175[a]	23.7	181[a]	19.9	181[a]	18.8
Day 14	491[ab]	46.0	479[b]	50.0	497[a]	49.0	497[a]	47.0
Day 21	984[a]	97.8	896[b]	105	990[a]	96.5	919[b]	108
Day 42	3273[a]	369	3037[b]	397	3307[a]	391	3242[a]	419
Dissection	3511[a]	436	3262[b]	456	3496[a]	423	3416[a]	465
Carcass weight[1]	2917[a]	359	2683[b]	380	2898[a]	350	2813[a]	388
Dressing %-age	83.1[a]	1.10	82.2[b]	1.39	82.9[a]	1.25	82.3[b]	0.984
WFR (g/g)	1.67[ab]	0.02	1.61[b]	0.06	1.68[a]	0.05	1.66[ab]	0.06
FCR (g/g)	1.61[b]	0.04	1.66[a]	0.03	1.60[b]	0.03	1.56[b]	0.05
CPE (kg/kg DM)	3.25[b]	0.08	3.44[a]	0.09	3.27[b]	0.06	3.49[a]	0.04

CN, without experimental C. jejuni infection; CP, with experimental C. jejuni infection; SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}diet, Choice diet; WFR, water-feed ratio in kg water intake / kg diet intake as fed; FCR, feed conversion ratio in kg diet intake as fed / kg body weight gain between Days 21–42; CPE, crude protein efficiency in kg body weight gain / kg crude protein intake;
[1]after exsanguation, evisceration and without head and legs, incl. feathers; [a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

TABLE 7 | Crypt depth and the number of goblet cells in the caeca of broiler chickens.

Item	CN				CP			
	SP-diet		CD^{CP+}/CD^{CP-}-diet		SP-diet		CD^{CP+}/CD^{CP-}-diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Crypt depth [μm; $n = 25$ per group]	235[b]	66.1	233[b]	56.4	263[a]	65.7	249[ab]	76.6
Goblet cells per standard crypt[a] [$n = 25$ per group]	15.6[b]	5.88	15.4[b]	6.01	15.6[b]	5.48	17.2[a]	6.30

CN, without experimental C. jejuni infection; CP, with experimental C. jejuni infection; SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}diet, Choice diet; [1]Determined goblet cell number to 250 μm crypt depth;
[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

DISCUSSION

In a 2 × 2 factorial design, the effects of an experimental *C. jejuni* infection (without/with) on feed intake preference were analyzed in 300 broiler chickens allocated to 20 subgroups. Simultaneously, the course of this experimental infection, the effects on performance, the number of goblet cells in the ceca, and the concentration of mucins in excreta were analyzed. For this purpose, a choice diet set-up was established in broilers. The birds received either a standard diet with moderate protein content or a choice diet consisting of two supplement diets with low or high protein content.

Campylobacter Excretion

Overall, there were only small differences in the excretion and spread of *C. jejuni* between the feeding concepts. The number of positive animals at day 22 in the CDCP group tended to be slightly higher ($p = 0.1162$). Overall, the CD^{CP+}-component in the CD-diet had the highest proportion, with 68% at day 23. The crude protein uptake between the two experimentally infected groups did not differ significantly during the 21-day experimental phase. A significant impact on the dynamics of infection was therefore not expected. At dissection, counts of *C. jejuni* in cecal content were nearly identical between groups (7.02 ± 0.85/6.87 ± 1.12 $\log_{10}$ CFU *C. jejuni* per g (gram) of content in SDCP or rather CDCP birds). The absolute values are in line with data from Humphrey et al. (37), who found *C. jejuni* within the same range in cecal content after experimental infection at a dosage of about 2×10^5 CFU per bird. Therefore, the experiments are generally well suited to test the effects of the infection on the behavioral choices of the animals with respect to the feed.

Feed Intake and Performance

In both experimental groups with choice diets, in the period before the experimental infection, the feeding behavior was nearly identical. In the period after the experimental infection, the protein intake in the experimentally infected *C. jejuni* group was significantly higher between d 21–27 and d 28–34 than in the group without infection. So far, there are no experiments that have tested this effect in broiler chickens. From the experiments carried out by Han et al. (38) one can deduce that at a higher protein content in the diet (broiler feed vs. laying hen feed),

TABLE 8 | Total mucin content of the excreta of broiler chickens.

Mucin content [g/kg DM; $n = 15$ per group and time point]	CN				CP			
	SP-diet		CD^{CP+}/CD^{CP-}-diet		SP-diet		CD^{CP+}/CD^{CP-}-diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Day 21	56.3^{ab}	4.82	57.4^{a}	4.62	54.1^{ab}	6.11	53.2^{b}	5.01
Day 42	62.9^{ab}	6.30	56.4^{c}	4.18	64.5^{a}	4.12	60.7^{b}	4.38

CN, without experimental C. jejuni infection; CP, with experimental C. jejuni infection; SP-diet, Standard protein diet; CD^{CP+}-diet, High protein choice diet; CD^{CP-}diet, Low protein choice diet; CD^{CP+}/CD^{CP-}diet, Choice diet;

[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

the animals showed a higher *C. jejuni* load. In sheep lambs, Kyriazakis et al. (39) analyzed the intake of feed of different crude protein contents for subclinical nematode infections. Compared to negative control animals, lower feed intake was observed in the infected animals, with high protein components being ingested in higher proportions.

The question is what leads to the altered feed intake—the immunological response of the animal to the pathogen and the corresponding nutritional requirements in order to defend the pathogen, or the interaction between pathogens and feed intake regulation mechanisms.

A formation of immunologically important protein compounds and gluconeogenesis from metabolites of amino acid degradation cause an increased energy and nutrient consumption (18, 20, 40). The effect of immune stimulation on feed intake behavior is dependent on the duration of this stimulus (41). Stimulation of the immune system observed in association with *C. jejuni* infections is usually short (42). Rather, after an initial response of the immune system to the pathogen, the cytokine level associated with tolerance of the pathogen by the broiler immune system decreases (42, 43). One has to discriminate between nutrition and the kind of infection. For lysine, antiviral effects are known (44, 45). Collectively, arginine supplementation attenuated the overexpression of pro-inflammatory cytokines in lipopolysaccharide-induced inflammatory response probably through the suppression of the TLR4 pathway and CD14+ cell percentage (46). Furthermore, excessive arginine supplementation (1.76%) suppressed the percentages of circulating and splenic B cells (46). Expression of both TLR4 and TLR21, but not TLR2, is readily increased (6 h post infection) in cecal tissues in response to *C. jejuni* inoculation in 1-day-old birds, whereas in 2- and 4-week-old broiler chicks this is accompanied, however, by only a limited cytokine gene expression (43). In our study, the differences in the individual amino acid contents were attributed to the different amino acid patterns of wheat and soybean meal, which were used at different levels in the two compound feeds of choice. In the CD^{CP+}-diet, there was 19.7 g/kg DM of arginine, whereas in the CD^{CP-}-diet, we had only 4.72 g/kg DM of this amino acid (factor 4.17). However, due to the age of infection (d 21), it seems unlikely that an increased need for pathogen defense primarily led to an altered amino acid uptake.

Referring to a hypothesized interaction between pathogens and feed intake regulation mechanisms, aspartic acid intake could be interesting. The greatest difference between the CD^{CP+}-diet and the CD^{CP-}-diet was found in the content of aspartic acid (factor 5.23). This amino acid is preferred by *C. jejuni* for its growth (4, 13). Accordingly, the pathogen may also benefit from a high intake of the CD^{CP+}-diet. Microbes may do this by way of two potential strategies: First, by generating cravings for feeds that they specialize on, or feeds that suppress their competitors. Second, by inducing dysphoria until the organism eats foods that enhance its fitness (47). The early pattern of brain activation following *C. jejuni* infection is characteristic of visceral sensory challenges, which modulate digestion and ingestive behavior (48). The strain used in this study was not further described in terms of its potential to generate an immunological response that could have altered feed intake behavior. Clinically, however, no evidence of infection-related changes in the animals were visible.

Furthermore, according to Awad et al. (49), the infection with the pathogen induces intestinal histomorphological changes, most prominently including a decrease in villus height, crypt depth and villus surface. Therefore, there is every indication that *Campylobacter* can, indeed, alter absorptive surface area with indirect negative consequences for production efficiency (49). No reduction in performance was found in the present study. In principle, however, a poorer absorption would also explain a preference for the component with a higher proportion of essential amino acids.

The growth performance of the animals in the experimental group (CDCP) during the experimental period of 21 days was comparable to that of the animals in the SDCP and SPCN groups. The body weight gain during the experimental period (d 21–42) was significantly ($p < 0.0001$) greater than the target of the Aviagen Group (1,880 g) in all groups. Also, the body weight on d 42 was significantly ($p < 0.0001$) higher than the target of 2,809 g (50).

Histology of the Intestine

The caeca are the preferred colonization sites in the digestive tract of broiler chickens (51). In the present study, the crypts in the cecum of infected animals receiving a standard diet were significantly deeper than in non-infected animals. Infected animals receiving CD-diets showed no difference in crypt depth

from all other groups, but showed a significantly increased number of goblet cells. These results are in line with those of Beery et al. (51) who found significantly deeper cecal crypts in animals infected with *C. jejuni*. In particular, in the mucous layer of these crypts, a high colonization density by *C. jejuni* is observed (51, 52). Deeper crypts mean a larger surface of the cecal mucosa and thus a larger surface of the intestinal mucous layer. This could potentially benefit *C. jejuni*.

Mucins in the Excreta

In this study, experimental infection with *C. jejuni* had a positive effect on mucin secretion. This increased regardless of the type of feed supply. In the literature, altered secretion of certain mucins is described by the presence of *C. jejuni* (53, 54). (53) were able to demonstrate in the mouse model that infection with *C. jejuni* caused a specific mucin (MUC1) to be released more frequently. Stimulation of the corresponding gene by the presence of *C. jejuni* was also observed in humans (54). This membrane-bound mucin has an important function in defending the host organism against invasion of the pathogen and the release can therefore be regarded as a physiological response of the host organism (53, 55). Furthermore, generally an increase in mucin release is described by inflammatory changes in the intestinal mucosa (56–58). A stimulatory effect by *C. jejuni* on the immune system is known (49, 59–61). Thus, due to certain surface structures (pathogen-associated molecular patterns) of corresponding receptors (pattern recognition receptors) of the intestinal immune system of the broiler, *C. jejuni* is recognized as potentially pathogenic. Furthermore, it induces an increase in proinflammatory cytokines such as IL-1 and IL-6, which function as messenger substances in the organism (59–62). In addition to many other functions, these messenger substances also activate mucin release (56–58). Overall, there was no evidence of there being any effects of *C. jejuni* infection on performance and health. Therefore, the potential effects of inflammation are maximally local in nature.

CONCLUSION

In this study, it could be proven that *C. jejuni* alters feed intake behavior toward higher protein intake. This had a positive effect on performance of the infected animals. Effects of infection on performance did not exist. Future studies should focus on potentially changing the diet composition away from the *C. jejuni*-induced pathway toward an increased protein uptake. For this dietetic concept for *Campylobacter* colonization, further studies are needed.

AUTHOR CONTRIBUTIONS

CV was the initiators of the idea. CV and AH designed the study. LK, JH, RB, ML, and CV performed the study and made the analyses. LK and CV did the statistics. CV wrote the paper. All authors read and approved the final manuscript.

FUNDING

The study was financially supported by Evonik Nutrition & Care GmbH. The role of the funding body was merely financial. They did not have any role in collecting, interpreting, analyzing, or writing up the data. This publication was supported by Deutsche Forschungsgemeinschaft and University of Veterinary Medicine Hannover, Foundation within the funding programme Open Access Publishing.

ACKNOWLEDGMENTS

We would like to thank Frances Sherwood-Brock for proof reading the manuscript to ensure correct English.

REFERENCES

1. Devleesschauwer B, Bouwknegt M, Mangen MJJ, Havelaar AH. Health and economic burden of *Campylobacter*. In: Klein G, editor. *Campylobacter*. London, Academic Press (2017). p. 27–40. doi: 10.1016/B978-0-12-803623-5.00002-2
2. EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* (2017) 15:e05077. doi: 10.2903/j.efsa.2017.5077
3. Epps SVR, Harvey RB, Hume ME, Phillips TD, Anderson RC, Nisbet DJ. Foodborne *Campylobacter*: infections, metabolism, pathogenesis and reservoirs. *Int J Environ Res Pub Health* (2013) 10:6292–304. doi: 10.3390/ijerph10126292
4. Guccione E, Leon-Kempis MD, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, et al. Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol Microbiol.* (2008) 69:77–93. doi: 10.1111/j.1365-2958.2008.06263.x
5. Wright JA, Grant AJ, Hurd D, Harrison M, Guccione EJ, Kelly DJ, et al. Metabolite and transcriptome analysis of *Campylobacter jejuni in vitro* growth reveals a stationary-phase physiological switch. *Microbiology* (2009) 155(Pt 1):80–94. doi: 10.1099/mic.0.021790-0
6. Ravindran V, Bryden WL. Amino acid availability in poultry - *in vitro* and *in vivo* measurements. *Austr J Agric Res.* (1999) 50:889–908. doi: 10.1071/AR98174
7. Adedokun SA, Adeola O, Parsons CM, Lilburn MS, Applegate TJ. Factors affecting endogenous amino acid flow in chickens and the need for consistency in methodology. *Poult Sci.* (2011) 90:1737–48. doi: 10.3382/ps.2010-01245
8. Parsons CM. Influence of caecectomy and source of dietary fibre or starch on excretion of endogenous amino acids by laying hens. *Br J Nutr.* (1984) 51:541–8. doi: 10.1079/BJN19840059
9. Horn NL, Donkin SS, Applegate TJ, Adeola O. Intestinal mucin dynamics: response of broiler chicks and White Pekin ducklings to dietary threonine. *Poult Sci.* (2009) 88:1906–14. doi: 10.3382/ps.2009-00009
10. Allen A. Structure and function of gastrointestinal mucus. In: Johnson LR, editor. *Physiology of the Gastrointestinal Tract,* Vol 1. New York, NY: Raven Press (1981). p. 617–639.
11. Kim JJ, Khan WI. Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens* (2013) 2:55–70. doi: 10.3390/pathogens2010055
12. Van Deun K, Pasmans F, Ducatelle R, Flahou B, Vissenberg K, Martel A, et al. Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut. *Vet Microbiol.* (2008) 130:285–97. doi: 10.1016/j.vetmic.2007.11.027

13. Hugdahl MB, Beery JT, Doyle MP. Chemotactic Behavior of *Campylobacter-jejuni*. *Inf Immun*. (1988) 56:1560–6.
14. Ravindran V, Morel PCH, Rutherfurd SM, Thomas DV. Endogenous flow of amino acids in the avian ileum as influenced by increasing dietary peptide concentrations. *Br J Nutr*. (2009) 101:822–8. doi: 10.1017/S0007114508039974
15. Lemme A, Ravindran V, Bryden W. Ileal digestibility of amino acids in feed ingredients for broilers. *Worlds Poult Sci J*. (2004) 60:423–38. doi: 10.1079/WPS200426
16. McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol*. (2011) 9:265–78. doi: 10.1038/nrmicro2538
17. Alemka A, Corcionivoschi N, Bourke B. Defense and adaptation: the complex inter-relationship between *Campylobacter jejuni* and mucus. *Front Cell Infect Microbiol*. (2012) 2:15. doi: 10.3389/fcimb.2012.00015
18. Lochmiller RL, Deerenberg C. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* (2000) 88:87–98. doi: 10.1034/j.1600-0706.2000.880110.x
19. Hammond, J. Physiological factors affecting birth weight. *Proc Nutr Soc*. (1944) 2:8–12.
20. Elsasser TH, Caperna TJ, Li CJ, Kahl S, Sartin JL. Critical control points in the impact of the proinflammatory immune response on growth and metabolism. *J Anim Sci*. (2008) 86:E105–125. doi: 10.2527/jas.2007-0634
21. Ranft U, Hennig A. Zur Regulation von Futteraufnahme und -auswahl unter besonderer Berücksichtigung des Geflügels. *Arch Tierernaehr*. (1991) 41:563–81. doi: 10.1080/17450399109428502
22. Forbes JM, Shariatmadari F. Diet Selection for Protein by Poultry. *World Poult Sci J*. (1994) 50:7–24. doi: 10.1079/WPS19940002
23. Kyriazakis I. Is anorexia during infection in animals affected by food composition? *Anim Feed Sci Technol*. (2010) 156:1–9. doi: 10.1016/j.anifeedsci.2010.01.001
24. Morrison CD, Reed SD, Henagan TM. Homeostatic regulation of protein intake: in search of a mechanism. *Am J Physiol Reg Integrat Comp Physiol*. (2012) 302:R917–28. doi: 10.1152/ajpregu.00609.2011
25. Rose S, Kyriazakis I. Diet selection of pigs and poultry. *Proc Nutr Soc*. (1991) 50:87–98. doi: 10.1079/PNS19910014
26. Forbes JM. *Voluntary Food Intake and Diet Selection in Farm Animals*. Oxon: Cab International (2007).
27. Kim JH. Diet selection for protein quality by growing broiler chickens. *Int J Poult Sci*. (2014) 13:461. doi: 10.3923/ijps.2014.461.466
28. Emmans G. Diet selection by animals: theory and experimental design. *Proc Nutr Soc*. (1991) 50:59–64. doi: 10.1079/PNS19910010
29. Rogers PJ, Blundell JE. Mechanisms of diet selection: the translation of needs into behaviour. *Proc Nutr Soc*. (1991) 50:65–70. doi: 10.1079/PNS19910011
30. GfE. *Recommendations for the Energy and Nutrient Supply of Laying Hens and Broilers*. Frankfurt am Main: DLG-Verlag (1999).
31. Naumann C, Bassler R. *Methoden der landwirtschaftlichen Forschungs- und Untersuchungsanstalt, Biochemische Untersuchung von Futtermitteln. Methodenbuch III (einschließlich der achten Ergänzungen)*. Darmstadt: VDLUFA (2012).
32. Jansen W, Reich F, Klein G. Large-scale feasibility of organic acids as a permanent preharvest intervention in drinking water of broilers and their effect on foodborne *Campylobacter* spp. before processing. *J Appl Microbiol*. (2014) 116:1676–87. doi: 10.1111/jam.12490
33. Visscher C, Klingenberg L, Hankel J, Brehm R, Langeheine M, Helmbrecht A. Influence of a specific amino acid pattern in the diet on the course of an experimental *Campylobacter jejuni* infection in broilers. *Poult Sci*. (2018). doi: 10.3382/ps/pey276. [Epub ahead of print].
34. Abd El-Wahab A, Visscher CF, Wolken S, Reperant JM, Beineke A, Beyerbach M, et al. Foot-pad dermatitis and experimentally induced coccidiosis in young turkeys fed a diet without anticoccidia. *Poult Sci*. (2012) 91:627–35. doi: 10.3382/ps.2011-01840
35. Lien KA, Sauer WC, Fenton M. Mucin output in ileal digesta of pigs fed a protein-free diet. *Z Ernährungswiss*. (1997) 36:182–190. doi: 10.1007/BF01611398
36. Slaoui M, Fiette L. Histopathology procedures: from tissue sampling to histopathological evaluation. *Methods Mol Biol*. (2011) 16:19–27. doi: 10.1007/978-1-60761-849-2_4
37. Humphrey S, Lacharme-Lora L, Chaloner G, Gibbs K, Humphrey T, Williams N, et al. Heterogeneity in the infection biology of *Campylobacter jejuni* isolates in three infection models reveals an invasive and virulent phenotype in a ST21 isolate from poultry. *PLoS ONE* (2015) 10:e0141182. doi: 10.1371/journal.pone.0141182
38. Han ZF, Pielsticker C, Gerzova L, Rychlik I, Rautenschlein S. The influence of age on *Campylobacter jejuni* infection in chicken. *Dev Comp Immunol*. (2016) 62:58–71. doi: 10.1016/j.dci.2016.04.020
39. Kyriazakis I, Oldham J, Coop R, Jackson F. The effect of subclinical intestinal nematode infection on the diet seletion of growing sheep. *Br J Nutr*. (1994) 72:665–77. doi: 10.1079/BJN19940070
40. Beisel WR. Magnitude of the host nutritional responses to infection - prospective overview to nutrition metabolism classic. *Nutrition* (1992) 8:126–8.
41. Scrimshaw NS, SanGiovanni JP. Synergism of nutrition, infection, and immunity: an overview. *Am J Clin Nutr*. (1997) 66:S464–S477. doi: 10.1093/ajcn/66.2.464S
42. Smith CK, AbuOun M, Cawthraw SA, Humphrey TJ, Rothwell L, Kaiser P, et al. *Campylobacter* colonization of the chicken induces a proinflammatory response in mucosal tissues. *Fems Immunol Medical Microbiol*. (2008) 54:114–21. doi: 10.1111/j.1574-695X.2008.00458.x
43. Hermans D, Pasmans F, Heyndrickx M, Van Immerseel F, Martel A, Deun K, et al. A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut. *Crit Rev Microbiol*. (2012) 38:17–29. doi: 10.3109/1040841X.2011.615298
44. Chen C, Sander JE, Dale NM. The effect of dietary lysine deficiency on the immune response to Newcastle disease vaccination in chickens. *Avian Dis*. (2003) 47:1346–51. doi: 10.1637/7008
45. Li P, Yin YL, Li D, Kim SW, Wu GY. Amino acids and immune function. *Br J Nutr*. (2007) 98:237–52. doi: 10.1017/S000711450769936X
46. Tan JZ, Liu SS, Guo YM, Applegate TJ, Eicher SD. Dietary l-arginine supplementation attenuates lipopolysaccharide-induced inflammatory response in broiler chickens. *Br J Nutr*. (2014) 111:1394–404. doi: 10.1017/S0007114513003863
47. Alcock J, Maley CC, Aktipis C. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *Bioessays* (2014) 36:940–9. doi: 10.1002/bies.201400071
48. Goehler LE, Gaykema RP, Opitz N, Reddaway R, Badr N, Lyte M. Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with *Campylobacter jejuni*. *Brain Behav Immun*. (2005) 19:334–44. doi: 10.1016/j.bbi.2004.09.002
49. Awad WA, Molnár A, Aschenbach JR, Ghareeb K, Khayal B, Hess C, et al. *Campylobacter* infection in chickens modulates the intestinal epithelial barrier function. *Innate Immun*. (2015) 21:151–60. doi: 10.1177/1753425914521648
50. Aviagen (2014). *Ross 308 Broiler Performance Objectives*. Aviagen Group AL. Available online at: http://eu.aviagen.com/tech-center/search?Subject=8&Type=2&Language=7&ResourceGeneration=3&Tag=5&action_doTechDocSearch=Suche
51. Beery JT, Hugdahl MB, Doyle MP. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol*. (1988) 54:2365–70.
52. Meinersmann RJ, Rigsby WE, Stern NJ, Kelley LC, Hill JE, Doyle MP. Comparative study of colonizing and noncolonizing *Campylobacter jejuni*. *Am J Vet Res*. (1991) 52:1518–22.
53. McAuley JL, Linden SK, Png CW, King RM, Pennington HL, Gendler SJ, et al. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest*. (2007) 117:2313–24. doi: 10.1172/JCI26705
54. Linden SK, Florin THJ, McGuckin MA. Mucin dynamics in intestinal bacterial infection. *PLoS ONE* (2008) 3:e3952. doi: 10.1371/journal.pone.0003952
55. Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, et al. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. *Mucosal Immunol*. (2013) 6:557–68. doi: 10.1038/mi.2012.98

56. Forstner G. Signal-Transduction, Packaging and Secretion of Mucins. *Ann Rev Physiol*. (1995) 57:585–605. doi: 10.1146/annurev.ph.57.030195.003101
57. Shekels LL, Ho SB. Characterization of the mouse Muc3 membrane bound intestinal mucin 5' coding and promoter regions: regulation by inflammatory cytokines. *Biochim Biophys Acta* (2003) 1627:90–100. doi: 10.1016/S0167-4781(03)00081-2
58. Smirnova MG, Guo L, Birchall JP, Pearson JP. LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. *Cell Immunol*. (2003) 221:42–9. doi: 10.1016/S0008-8749(03)00059-5
59. Larson CL, Shah DH, Dhillon AS, Call DR, Ahn S, Haldorson GJ, et al. (2008). *Campylobacter jejuni* invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. *Microbiology* (2008) 154(Pt 12), 3835–47. doi: 10.1099/mic.0.2008/021279-0
60. Shaughnessy RG, Meade KG, Cahalane S, Allan B, Reiman C, Callanan JJ, et al. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. *Vet Immunol Immunopathol*. (2009) 132:191–8. doi: 10.1016/j.vetimm.2009.06.007
61. Vaezirad MM, Keestra-Gounder AM, de Zoete MR, Koene MG, Wagenaar JA, van Putten JPM. Invasive behavior of *Campylobacter jejuni* in immunosuppressed chicken. *Virulence* (2017) 8:248–60. doi: 10.1080/21505594.2016.1221559
62. Pielsticker C, Glunder G, Aung YH, Rautenschlein S. Colonization pattern of *C. jejuni* isolates of human and avian origin and differences in the induction of immune responses in chicken. *Vet Immunol Immunopathol*. (2016) 169:1–9. doi: 10.1016/j.vetimm.2015.11.005

Conflict of Interest Statement: AH is an employee of Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany.

The remaining 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 September 2018
doi: 10.3389/fmicb.2018.02139

A Novel Natural Antimicrobial Can Reduce the *in vitro* and *in vivo* Pathogenicity of T6SS Positive *Campylobacter jejuni* and *Campylobacter coli* Chicken Isolates

Filip Sima[1,2,3†], Alexandros Ch. Stratakos[1,3†], Patrick Ward[3], Mark Linton[1], Carmel Kelly[1], Laurette Pinkerton[1], Lavinia Stef[4], Ozan Gundogdu[5], Veronica Lazar[2] and Nicolae Corcionivoschi[1,4]*

[1] Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, United Kingdom, [2] Department of Microbiology and Immunology, Faculty of Biology, University of Bucharest, Bucharest, Romania, [3] Auranta, NovaUCD, Dublin, Ireland, [4] School of Animal Science and Biotechnology, Banat University of Animal Sciences and Veterinary Medicine – King Michael I of Romania, Timisoara, Romania, [5] London School of Hygiene and Tropical Medicine, London, United Kingdom

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Heriberto Fernandez,
Universidad Austral de Chile, Chile
Florence Dubois-Brissonnet,
AgroParisTech, France

***Correspondence:**
Nicolae Corcionivoschi
nicolae.corcionivoschi@afbini.gov.uk

[†] 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: 09 May 2018
Accepted: 21 August 2018
Published: 07 September 2018

Citation:
Sima F, Stratakos ACh, Ward P, Linton M, Kelly C, Pinkerton L, Stef L, Gundogdu O, Lazar V and Corcionivoschi N (2018) A Novel Natural Antimicrobial Can Reduce the in vitro and in vivo Pathogenicity of T6SS Positive Campylobacter jejuni and Campylobacter coli Chicken Isolates. Front. Microbiol. 9:2139. doi: 10.3389/fmicb.2018.02139

Human campylobacteriosis is considered one of the most common foodborne diseases worldwide with poultry identified as the main source of infection accounting for 50–80% of human cases. Highly virulent *Campylobacter* spp., positive for the Type VI secretion system (T6SS), which have an increased ability to adhere to and invade the host gastrointestinal epithelium are highly prevalent in poultry. Multidrug resistant strains of bacteria are rapidly evolving and therefore, new antimicrobials to supplement animal feed that are able to control *Campylobacter* species, are in great need. The work presented herein indicates that a novel phenolic antimicrobial, Auranta 3001, is able to reduce the adhesion and invasion of human intestinal epithelial cells (HCT-8) by two T6SS positive chicken isolates, *C. jejuni* RC039 ($p < 0.05$) and *C. coli* RC013 ($p < 0.001$). Exposure of *C. jejuni* RC039 and *C. coli RC013* to Auranta 3001 downregulated the expression of *hcp* and *cetB* genes, known to be important in the functionality of T6SS. Furthermore, the reduced adhesion and invasion is associated with a significant decrease in bacterial motility of both isolates ($p < 0.05$–$p < 0.001$) *in vitro*. Most importantly our *in vivo* results show that Auranta 3001 is able to reduce cecum colonization levels from log 8 CFU/ml to log 2 CFU/ml for *C. jejuni* RC039 and from log 7 CFU/ml to log 2 CFU/ml for *C. coli* RC013. In conclusion, this novel antimicrobial is able to reduce the pathogenic properties of T6SS campylobacters *in vitro* and also to decrease colonization *in vivo*.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, HCT-8, attachment, invasion, gene expression

INTRODUCTION

The Gram-negative pathogen *Campylobacter* spp. is the most frequent cause of bacterial foodborne disease (EFSA and ECDC, 2017). The bacterium naturally colonizes the avian intestinal tract, where it can persist for the entire lifespan of the birds leading to contamination of poultry carcasses during slaughter, which increases the risk of human exposure to the pathogen (Shortt et al., 2016).

Campylobacter species can cause gastro-intestinal disorders in humans, including fever, nausea, and abdominal pain. In a small group of patients, it can lead to more severe consequences such as Guillain-Barré syndrome, an acute flaccid paralysis, reactive arthritis, and inflammatory bowel disease (Chaisowwong et al., 2012; van Alphen et al., 2012; Hoseinpour et al., 2017).

In order to cause disease *Campylobacter* interacts with the gastrointestinal epithelium and colonizes the host. The full picture of the mechanisms involved is not yet known (Freitag et al., 2017), but initial progress has been made to elucidate these mechanisms (Young et al., 2007). Blocking these initial stages of infection including adhesion, motility, and chemotaxis is essential as they represent key factors for a successful infection (Morooka et al., 1985; Nachamkin et al., 1993; Yao et al., 1997; Hendrixson and DiRita, 2004; Backert and Hofreuter, 2013). More specifically these virulence factors include motility systems (*flaA* and *flaB*), adhesion to fibronectin F (*cadF*), chemotaxis (*cetB*), invasion proteins, and cytolethal distending toxins (*cdtB*), whose production causes progressive cellular distension and cell death leading to enteritis. Therefore, reducing the attachment and invasion of *Campylobacter* sp. on intestinal epithelial cells and reducing production of virulence factors such as adhesins and decreasing motility could potentially control campylobacteriosis. Besides other virulence factors, capsular polysaccharides contribute to antimicrobial resistance (Hendrixson et al., 2001; Guerry et al., 2012).

Treating *Campylobacter* infections in humans with antibiotics (e.g., erythromycin, clarithromycin, ciprofloxacin, levofloxacin, moxifloxacin) is common practice. The increasing resistance of bacteria to conventional chemicals and drugs, the decline in new antibiotic discovery over the last few decades, as well as consumer demands for natural food preservatives have encouraged research for the identification of novel natural antimicrobials (Kovac et al., 2015; Oh and Jeon, 2015a; Stratakos et al., 2018). Plant extracts have been used for decades not only as flavor enhancers, but also to extend the shelf life and microbiological safety of food (Hintz et al., 2015). Their applicability was also proven for the treatment of a range of human and animal diseases, improving human health (Holley and Patel, 2005; Teichmann et al., 2016).

Controlling *Campylobacter* spp. is considered a public health priority. Antimicrobial products are used in general with the aim of bacterial killing but this may have a damaging effect on the host microbiome. Therefore, investigations into the possibility of achieving a reduced virulence rather than a lethal effect would be of interest. Auranta 3001 has been shown to be involved in reduced virulence of *Cryptosporidium hominis* and *Cryptosporidium parvum* by downregulating CpSUB1 gene expression (Stratakos et al., 2017). In order to avoid any effect on bacterial survival and growth, and therefore apply less selective pressure for the development of resistance, sub-inhibitory concentrations of antimicrobials need to be identified. The use of sub-inhibitory concentrations is necessary for the sound investigation of the anti-virulence capacity of antimicrobials/compounds (Kummerer, 2004). Our study aimed to investigate if sub-inhibitory concentrations of a mixture of organic acids and plant extracts (Auranta 3001) can reduce the virulence of T6SS positive *C. jejuni* and *C. coli* isolates in human HCT-8 cell models and colonization of caeca in artificially infected chicken broilers.

MATERIALS AND METHODS

Strains and Culture Conditions

C. jejuni RC039 and *C. coli* RC013 strains were obtained from the AFBI laboratory collection and were grown on Blood Agar Base No. 2 (Oxoid Ltd., United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (Aquilant Scientific N.I.). The strains were grown under microaerophilic conditions at 41.5°C in 85% N_2, 5% O_2, and 10% CO_2 in a Don Whitley MACS-VA500 microaerophilic workstation (Davidson & Hardy Ltd., United Kingdom) for 48 h. To enumerate viable microorganisms, suitable 10-fold dilutions were made in Maximum Recovery Diluent (Oxoid Ltd., United Kingdom). One hundred microliters of each of the 10-fold dilutions were spread on *Campylobacter* Blood-Free Selective Agar Base (Modified CCDA – Preston; Oxoid Ltd., United Kingdom) without any supplement, and plates were incubated under microaerophilic conditions at 41.5°C for 48 h.

Chemicals

The novel antimicrobial (Auranta 3001) was supplied by Auranta – Envirotech Innovative Products Ltd and contains lactic and citric acid. The antimicrobial also contains: glycerine-based emulsifying agent, sodium chloride, sodium hydroxide, citrus extract (6%), oregano extract (1%), grape seed extract (2%).

Infection

The ECACC human ileocecal adenocarcinoma cells (HCT-8) were grown in RPMI 1640 (Lonza, Analab Ltd., United Kingdom) supplemented with 10% fetal bovine serum and 2 mL glutamine. The cells were routinely grown in 75 cm^2 tissue culture flasks (Sigma-Aldrich, Arklow, Ireland, SIAL0641) in a humidified incubator at 37°C with 5% CO_2. The gentamicin protection assay was used to test the role of Auranta 3001 in the ability of *C. jejuni* RC039 and *C. coli* RC013 to adhere to and invade host epithelial cells as previously described (Corcionivoschi et al., 2009). HCT-8 cells were grown (80–90% confluence) for 22 to 24 h in six-well tissue culture plates at a concentration of 5.5×10^5 cells per well. For some experiments, HCT-8 monolayers were preincubated with 0.1 and 0.5% Auranta 3001 for 1–3 h. Plate grown *C. jejuni* RC039 and *C. coli* RC013 were harvested, washed, and re-suspended in tissue culture medium at an OD_{600} of 0.3 and 0.25, respectively. The bacterial isolates were preincubated for 1–3 h in the presence of 0.1 and 0.5% Auranta 3001. The HCT-8 cells were washed with fresh culture media containing 10% FBS, and 2 ml of fresh culture medium was added to each well. Bacteria were added to give a multiplicity of infection of 100. Tissue culture plates were centrifuged at $250 \times g$ for 5 min and incubated for 3 h at 41.5°C in 85% N_2, 5% O_2, and 10% CO_2. To quantify the number of cell-associated bacteria, infected monolayers were washed three times with PBS and

treated with 0.1% Triton X-100 in PBS at 41.5°C for 15 min. Tenfold dilutions of each well were plated onto CCDA agar and colonies enumerated after 2 days of incubation at 41.5°C in 85% N_2, 5% O_2, and 10% CO_2. To quantify the number of bacteria that invaded HCT-8 cells, the infected monolayers were washed with tissue culture medium. Fresh medium (2 ml) containing gentamicin (400 μg/ml) was added to kill bacteria that were not internalized. Medium without gentamicin was added to quantify the number of bacteria that adhered to the epithelial cells. The tissue culture plates were then incubated for a further 3 h at 41.5°C and washed with fresh RPMI 1650 + 10% FBS. HCT-8 cells were lysed by the addition of 1 ml of 0.1% Triton X-100 in PBS and incubated for 15 min at 41.5°C (Corcionivoschi et al., 2009). Because in our study, we have used chicken isolates the incubation temperature was increased from 37 to 41.5°C. Tenfold dilution of the contents of each well was plated onto CCDA agar, and colonies were enumerated after 2 days of incubation. Invasion efficiency was calculated as the percentage of the total number of CFU/total initial inoculum. All assays were conducted in triplicate and repeated independently three times. The significance of differences in adhesion and invasion between samples was determined using the Student's *t*-test. A *P*-value of <0.05 was defined as significant.

Susceptibility to Auranta 3001

The twofold tube dilution method was used to determine the lowest concentration of Auranta 3001 that can inhibit growth of bacteria (MIC) and the lowest concentration that results in bacterial death (MBC) according to Zhu et al. (2016). Auranta 3001 was diluted (8% down to 0.0078% v/v) in Müeller Hinton broth (MHB) and thoroughly vortexed. Individual overnight bacterial cultures were harvested by centrifugation, washed with PBS, and diluted to approximately 1×10^6 CFU/mL in MHB. Each tube was inoculated with approximately 5×10^5 CFU/mL of this bacterial culture (final concentration). Noninoculated tubes containing the same growth medium were used as negative controls and tubes inoculated with individual bacterial cultures in MHB without Auranta 3001 were used as positive controls. Subsequently, the tubes were incubated at 41.5°C for 48 h. Tubes without visible growth were considered as below the MIC. One hundred milliliters were taken from the tubes that showed no growth and inoculated onto MHA plates, the highest dilution with no microbial growth was considered as the MBC. Each assay was repeated three times for each strain. In order to determine the sub-inhibitory concentrations used, the two pathogens were exposed to different concentrations of the antimicrobial. The concentrations that showed no effect on survival and no growth inhibition (same growth kinetics as the control) were used for the subsequent experiments.

Motility Assay

The motility of *C. jejuni* RC039 and *C. coli* RC013 was measured after the two strains were exposed to Auranta 3001 for 1, 2, and 3 h at a concentration of 0.1 and 0.5%. In short, 5 μl of culture (grown on blood agar for 48 h and recovered in 1 ml brain heart infusion – BHI – broth) was inoculated into the center of a 20 ml semi-solid BHI plate (0.4% agar). The radius of the zone of visible growth was measured after 48 h of incubation under microaerophilic conditions at 41.5°C. The experiment was carried out in triplicate, on three different days. The results are expressed as percentage decrease compared to the control.

Capsule Polysaccharide (CPS) Detection

Capsule polysaccharide was prepared from bacteria co-cultured with HCT-8 cells which were pretreated with Auranta 3001 and from bacteria directly exposed to Auranta 3001 following a previously described protocol (Hitchcock, 1983). Bacteria were harvested by centrifugation and suspended in 100 μl of lysis buffer containing 31.25 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 0.025% bromophenol blue, and 20% glycerol. After heating to 100°C for 5 min, 5 μl of 20 mg/ml proteinase K was added to the solution, and the tubes were incubated for 1 h at 50°C. The samples were separated on NuPageNovex 12% bis-Tris gels (Invitrogen, United Kingdom). Following electrophoresis, gels were stained with an Alcian blue (Sigma Aldrich, United Kingdom) solution containing 0.1% Alcian blue, 40% ethanol, and 5% acetic acid (Karlyshev and Wren, 2001).

RNA Extraction and qRT-PCR

Total RNA was isolated from bacteria exposed to Auranta 3001 at a concentration of 0.1 and 0.5% for 1, 2, and 3 h by using the RNeasy®Plus Mini Kit (Qiagen, United Kingdom). The RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche, United Kingdom) according to the manufacturer's protocol. The mRNA levels were determined by quantitative RT-PCR using QuantiNovaSYBR® Green PCR Kit (Qiagen, United Kingdom) on a LightCycler® 96 (Roche, United Kingdom). The primers used (Invitrogen, United Kingdom) are described in **Table 1**, and the conditions for genes *rRNA* 16S consisted of incubating for 10 min at 95°C followed by 45 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 10 s. A total of 5 μl of SYBR Green master mixture was used in each reaction along with 0.5 μl of 10 μM primer mixture, 3 μl of molecular grade water, and 1 μl of DNA sample. For *cetB* (10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 54°C for 20 s, and a final extension at 72°C for 5 min) a total of 5 μl of SYBR Green master mixture was used in each reaction along with 1.4 μl of 20 μM primer mixture, 5.2 μl of molecular grade water, and 2 μl of DNA sample. For *hcp* (2 min at 95°C, followed by 40 cycles

TABLE 1 | List of primer sequences used for qRT-PCR for *C. jejuni* RC039 and *C. coli* RC013 genes associated with virulence.

Gene	Primer sequence – forward	Primer sequence – reverse	Reference
cetB	5′ GCCTTGTTGCTGTTCTGCTC 3′	5′ TTCCGTTCGTCGTATGCCAA 3′	Upadhyay et al., 2017
hcp	5′ CAAGCGGTGCATCTACTCAA 3′	5′ TAAGCTTTGCCCTCTCTCCA 3′	Harrison et al., 2014
rRNA 16S	5′ ATCTAATGGCTTAACCATTAAAC 3′	5′ GGACGGTAACTAGTTTAGTATT 3′	Douglas Inglis and Kalischuk, 2003

TABLE 2 | Chemical composition of basal diet.

Item	Starter 0–10 days	Grower 11–24 days	Finisher 25–35 days
Wheat	54.623	57.553	61.300
Full fat soya	12.000	12.000	12.000
Brazilian GM hipro	25.000	21.000	17.000
Lime bulk	0.717	0.700	0.500
DCP bulk (18.1% p)	1.654	2.000	2.150
Salt bulk	0.200	0.200	0.200
Sod.bi-carbonate	0.199	0.166	0.162
DL methionine	0.487	0.435	0.378
L-lysine	0.373	0.318	0.281
Threonine	0.247	0.128	0.029
Vitamin+mineral premix	0.500	0.500	0.500
Soyabean oil	4.000	5.000	5.500
Calculated composition (%)			
ME Kcal/kg	2999	3081	3133.8
CP	23.12	21.53	20.04
Lys	1.45	1.308	1.17
Met+Cys	1.089	0.996	0.91
Ca	0.97	0.906	0.85
AvP	0.49	0.41	0.409

of 95°C for 5 s, 60°C for 10 s, and a final extension at 72°C for 5 min), a total of 5 μl of SYBR Green master mixture was used in each reaction along with 0.8 μl of 20 μM primer mixture, 7.4 μl of molecular grade water, and 1 μl of DNA sample. Relative quantity of the mRNA was calculated using the ΔCt method. *rARN 16S* gene was used as an endogenous control since it was transcribed in equal rates in both treated and untreated cells.

In vivo Infection Assay

Twenty male chicken broilers (Ross 308) were housed in isolation units on wood shaving bedding. The temperature in the isolation unit was kept between 22 and 25°C and thermostatically controlled. Broilers were fed *ad libitum* with a standard diet as described in **Table 2**. *C. jejuni* RC039 and *C. coli* RC013 were grown as described above and suspended in sterile distilled water at a concentration of 1×10^7 CFU/ml. At the age of 15 days, 10 broilers were inoculated with 0.1 ml of *C. jejuni* RC039 and the other 10 broilers with 0.1 ml *C. coli* RC013. The infected broilers were kept in separated and sterile isolation units. Five chickens from each infected group received for 3 days, *ad libitum*, water containing 0.5% Auranta 3001 with the remaining ones receiving water only. After 3 days of treatment, broilers were euthanized, and *Campylobacter* was enumerated by analyzing the cecum content. All broilers were confirmed as being *Campylobacter* free at the time of infection, using cloacal swabs. These experiments were performed in triplicate on three separate occasions. The experiments were performed according to the legislation in place (Law 471/2002 and government ordinance 437/2002) and under the supervision of National Sanitary Veterinary Agency. The ethics committee of Banat University of Agricultural Sciences and Veterinary Medicine – King Michael I of Romania, approved this work

TABLE 3 | Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) activity of the Auranta 3001.

Strains	Origin	MIC	MBC
Campylobacter jejuni RC039	poultry	1%	1%
Campylobacter coli RC013	poultry	2%	2%

The antimicrobial was tested at concentrations from 8 to 0.0078% (v/v) in three independent experiments.

Transepithelial Resistance of Cellular Tight Junctions (TEER)

Transepithelial resistance measures the integrity of cellular tight junctions and is a suitable method to be used in cell culture monolayers for the purpose of measuring the effect of bacteria during infection *in vitro* (Srinivasan et al., 2015). This methodology measures the electrical resistance, in ohms, as a measure of cellular barrier integrity. For the purpose of TEER measurement, the HCT-8 cells were grown on 0.4 μm and 12 mm pore size transwell inserts (Corning) and selected based on the formation of a confluent monolayer. Our aim was to investigate the effect of Auranta 3001 on the barrier properties of HCT-8 cells by taking TEER measurements at 3 h postinfection (±0.1 and 0.5% Auranta 3001) using an EVOM X meter connected to an Endohm chamber (World Precision Instruments).

Statistical Analysis

All experiments were performed in triplicate and data presented as mean ± SEM. To measure the statistical significance of the infection assays and gene expressions results, we used two tailed Student's *t*-test. A group difference was assumed to be statistically significant when $P? < ?0.05$. All results were expressed as means ± SD.

RESULTS

Identification of the Sub-Inhibitory Concentrations

In order to investigate if the antimicrobial agent has any effect in reducing pathogenicity, by attenuating bacterial virulence factors, we first had to identify the sub-inhibitory concentrations for both isolates (*C. jejuni* RC039 and *C. coli* RC013). Strong antimicrobial activities against both *C. jejuni* RC039 and *C. coli* RC013 were observed. The minimum inhibitory concentrations were 1% for *C. jejuni* RC039 and 2% for *C. coli* RC013, and minimum bactericidal concentrations were recorded at similar concentrations (**Table 3**). We have concluded that concentrations of 0.1 and 0.5% were most appropriate for our next investigations in order to avoid any antimicrobial killing or any effect on bacterial growth

Auranta 3001 Reduces the Motility of *C. jejuni* RC039 and *C. coli* RC013

Having identified the sub-inhibitory concentrations, we have next studied the potential impact on virulence by the reduction of bacterial motility (**Figure 1**). Motility plate assays showed

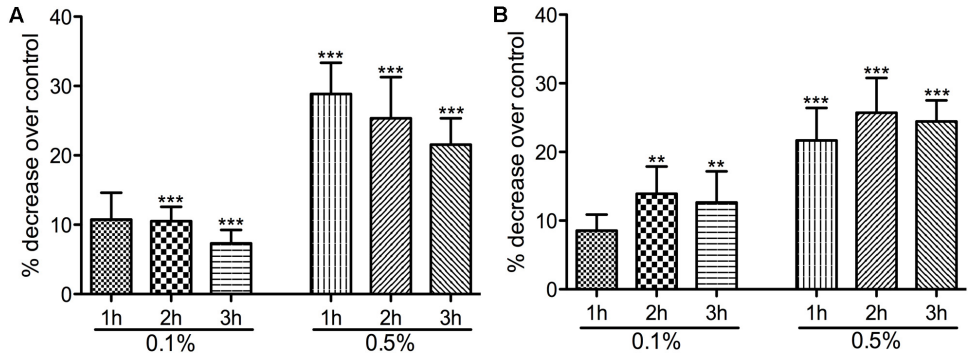

FIGURE 1 | The effect of Auranta 3001 on *C. jejuni* RC039 and *C. coli* RC013 motility. **(A)** shows the percentage decrease of *C. jejuni* RC039 motility and **(B)** of *C. coli* RC013 over control. *Asterisks* indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). *Error bars* represent the standard deviation of means from three different experiments, each containing triplicate samples.

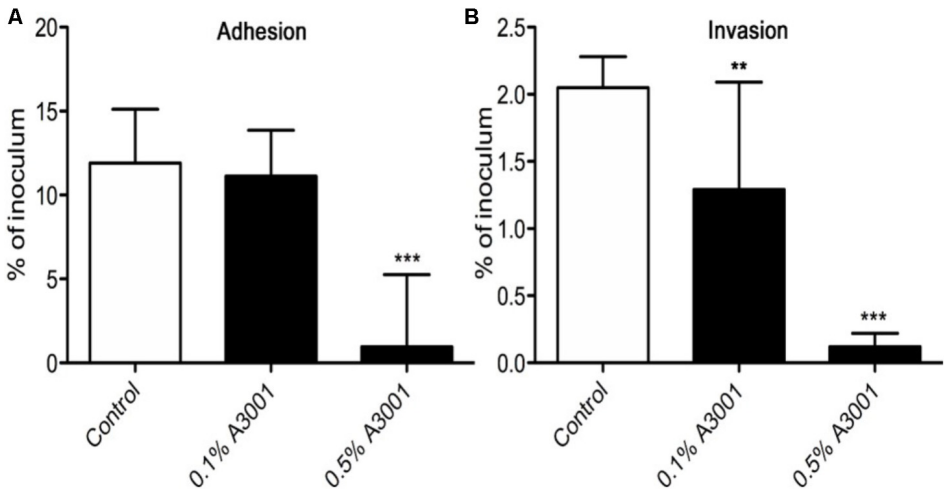

FIGURE 2 | Adhesion and invasion of HCT-8 cells by *C. jejuni* RC039. The adherence **(A)** and invasion **(B)** of HCT-8 cells in the presence of Auranta 3001. Results are expressed as percentages of the initial inoculum. *Asterisks* indicate significant differences (Student's *t*-test *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). *Error bars* represent the standard deviation of means from three different experiments, each containing triplicate samples.

that both strains were highly motile but the pretreatment with 0.1 and 0.5% Auranta 3001 significantly reduced motility in a dose and time dependent manner. As reflected in **Figures 1A,B**, a concentration of 0.1% Auranta 3001 significantly reduced motility after only 2 h for both *C. jejuni* RC039 ($p < 0.01$) and *C. coli* RC013 ($p < 0.05$). A concentration of 0.5% Auranta 3001 reduced bacterial motility by more than 20% ($p < 0.001$) after 1, 2, and 3 h for *C. jejuni* RC039 (**Figure 1A**), and for *C. coli* RC013 (**Figure 1B**) when compared to the control. These results suggested that this antimicrobial can have a negative impact on the motility of *C. jejuni* and *C. coli* T6SS positive isolates.

Auranta 3001 Reduces *C. jejuni* RC039 and *C. coli* RC013 Virulence *in vitro* and Decreases Colonization *in vivo*

To demonstrate if the negative effect observed on motility also translates to reduced virulence we performed *in vitro* infection assays as described in Materials and Methods section. The presence of Auranta 3001 in the culture media throughout the infection assay significantly reduced the adherence of *C. jejuni* RC039 to HCT-8 cells (**Figure 2A**) at a concentration of 0.5%, while the negative effect on invasion (**Figure 2B**) was significant at both concentrations ($p < 0.001$). In the case of *C. coli* RC013, the negative effect on adherence (**Figure 3A**) and invasion (**Figure 3B**) was significant ($p < 0.001$) at both 0.1 and 0.5% Auranta 3001. The invasion ability of *C. jejuni* RC039 and *C. coli* RC013 was significantly diminished ($p < 0.001$) when HCT-8 cells only were exposed to Auranta 3001 suggesting that host related infection pathways are affected by the pretreatment (**Supplementary Figure 1**). Moreover, if the bacteria only are pretreated, prior to infection their invasion capacity is also dramatically reduced (**Supplementary Figure 2**). Overall, these data indicate that the reduced infection could be due to biological changes in both the host and the bacterium. The successful reduction in virulence obtained *in vitro* was extremely

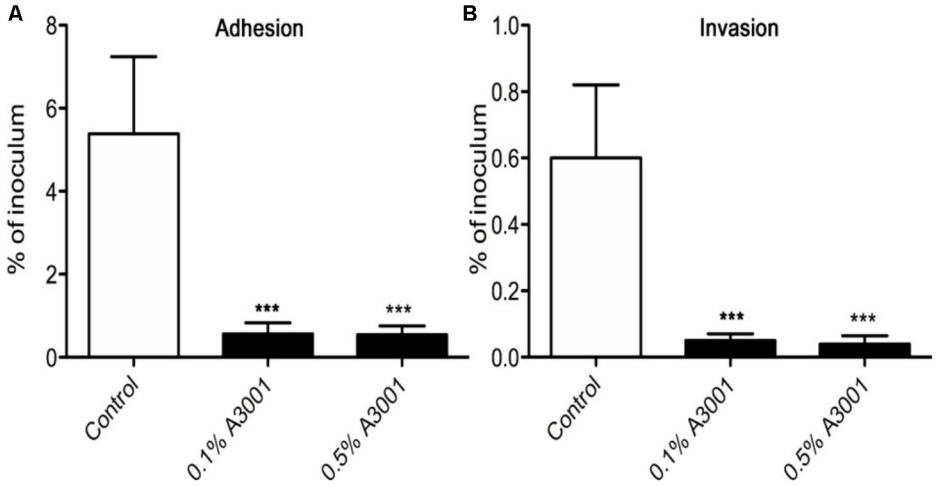

FIGURE 3 | Adhesion and invasion of HCT-8 cells by *C. coli* RC013. The adherence **(A)** and invasion **(B)** of HCT-8 cells in the presence of Auranta 3001. Results are expressed as percentages of the initial inoculum. *Asterisks* indicate significant differences (Student's *t*-test $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). *Error bars* represent the standard deviation of means from three different experiments, each containing triplicate samples.

encouraging and as a consequence we then investigated the effect of Auranta 3001 in preventing cecum colonization of artificially infected chicken broilers. *In vivo*, quantification of viable bacteria in cecum content showed significant decrease ($p < 0.05$) in the ability of *Campylobacter* to colonize the gastrointestinal tract. For *C. jejuni* RC039, the decrease was from log 8 CFU/ml to 2 log CFU/ml and for *C. coli* RC013 from 7 log CFU/ml to 2 log CFU/ml (**Table 4**). In conclusion, the addition of Auranta 3001 to the drinking water significantly reduced the ability of *C. jejuni* RC039 and *C. coli* RC013 to colonize the broiler cecum when compared to controls.

Changes in Capsule Polysaccharide (CPS) Profiles of *C. jejuni* RC039 and *C. coli* RC013 Following Exposure to Auranta 3001

Having observed that exposure of bacteria to the antimicrobial reduces their virulence we then investigated the production of CPS, a major virulence and colonization factor in campylobacters. Our results show that exposure of HCT-8 cells to 0.1% Auranta 3001 prior to infection leads to a significant decrease in the amounts of CPS detected on co-cultured *C. jejuni* RC039 (**Figure 4A**, lanes 3 and 5) compared to control. Interestingly a similar effect was not detected for *C. coli* RC013 in which case no difference was observed when compared to control (**Figure 4B**).

TABLE 4 | Detection of viable campylobacters (log_{10} CFU/ml).

Experiment	*C. jejuni* RC039		*C. coli* RC013	
	+A3001	−A3001	+A3001	−A3001
A	3.9×10^{2a}	7.3×10^{8}	4.2×10^{2a}	5.1×10^{6}
B	2.7×10^{2a}	8.1×10^{9}	2.7×10^{3a}	3.3×10^{7}
C	1.2×10^{2a}	6.6×10^{8}	3.9×10^{2a}	4.7×10^{6}

[a] *Significantly ($P < 0.05$) less than the untreated control.*

A, B, and C represent three replicate experiments performed in three separate occasions.

Auranta 3001 Downregulates *hcp* and *cetB* Gene Expression in *C. jejuni* RC039 and *C. coli* RC013

The effect of Auranta 3001 on the expression of *C. jejuni* RC039 and *C. coli* RC013 *hcp* gene is shown in **Figure 5**. The qRT-PCR results revealed that the antimicrobial agent reduced the transcription levels of *hcp* and the energy chemotaxis related gene (*cetB*). Exposure of *C. jejuni* RC039 to 0.1% Auranta 3001 (**Figure 5A**) resulted in a fivefold reduction of *hcp* gene expression with a 10-fold reduction in *C. coli* RC013 ($p < 0.01$). Interestingly only twofold downregulation of the *hcp* gene (**Figure 5A**) was observed when both isolates were exposed to 0.5% Auranta 3001. In the case of *cetB* gene expression in *C. jejuni* RC039, we show a significant marked increase ($p < 0.01$) in downregulation following exposure from fivefold at 0.1% to 10-fold at 0.5% (**Figure 5B**). For *C. coli* RC013, the downregulation of *cetB* was more significant following exposure to 0.1% Auranta 3001 compared to 0.5% ($p < 0.05$).

Auranta 3001 Prevents Tight Junction Disruption During Infection

As shown in **Figures 2**, **3** Auranta 3001 reduces the virulence of both *C. jejuni* RC039 and *C. coli* RC013 when used at concentrations between 0.1 and 0.5%. We have hypothesized that the effect could be caused by an increase in tight junction resistance. In order to test our hypothesis, TEER was measured at 3 h postinfection in the presence of 0.1 and 0.5% Auranta 3001. By 3 h postinfection, there was a significant increase ($p < 0.01$) in TEER in both uninfected and infected cells when

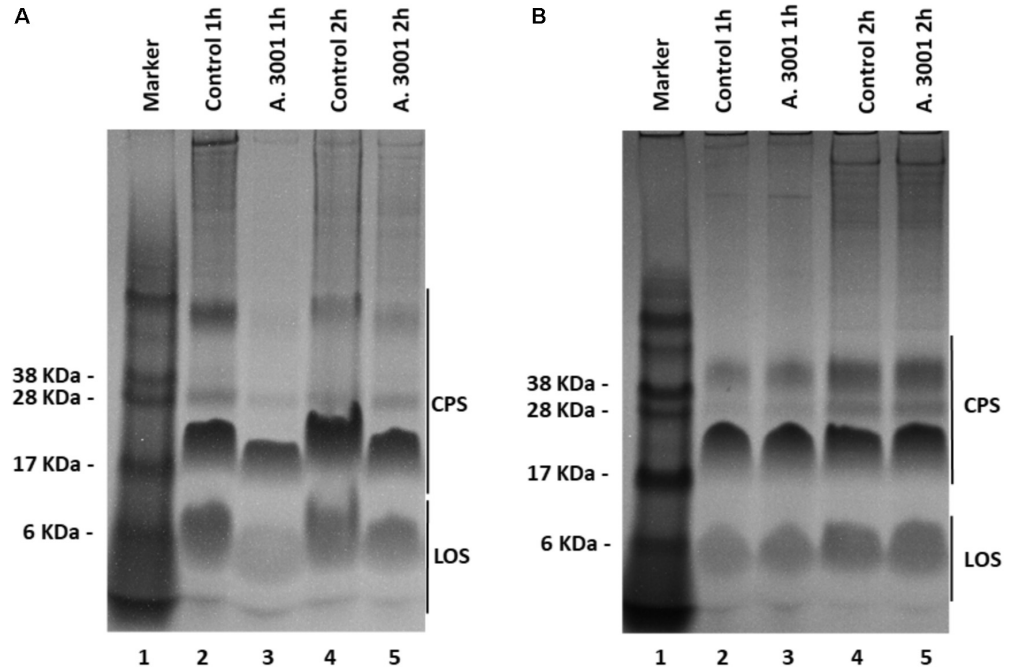

FIGURE 4 | Alcian blue profiles of *Campylobacter jejuni* RC039 **(A)** and *Campylobacter coli* RC013 **(B)** capsule polysaccharides when co-cultured with HCT-8 cells pretreated with 0.1% Auranta 3001.

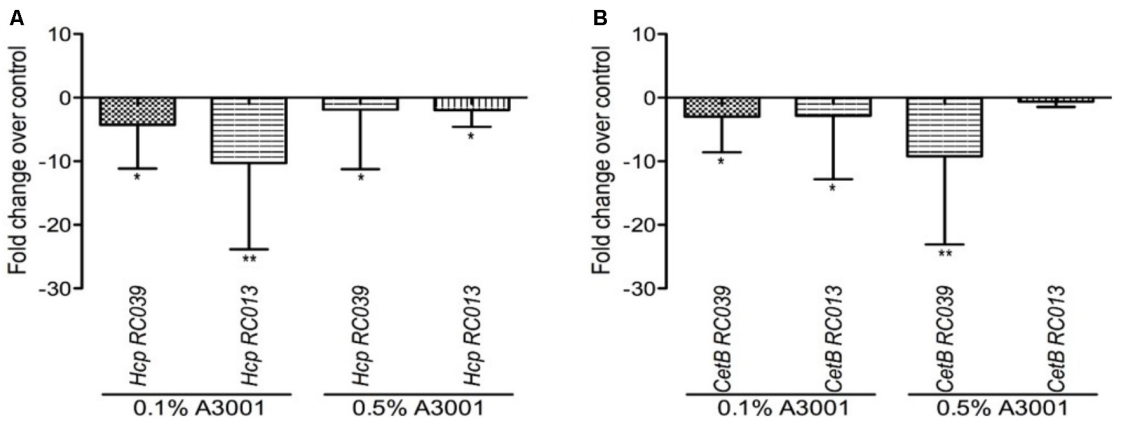

FIGURE 5 | Effect of Auranta 3001 on *C. jejuni* RC039 and *C. coli* RC013 *cetB* **(B)** and *hcp* gene **(A)** expression after 3 h of exposure to 0.1 and 0.5% Auranta 3001. Asterisks indicate significant differences (Student's *t*-test $*p < 0.05$; $**p < 0.01$). *Error bars* represent the standard deviation of means from three different experiments.

Auranta 3001 was present in the infection media (**Figure 6**). The results were similar when 0.1% Auranta 3001 was used (data not shown). These results indicate that Auranta 3001 inhibits the disruption of the tight junctions during infection preventing pathogen translocation and subsequent infection.

DISCUSSION

Campylobacter remains an important microbiological contaminant of chicken products despite substantial efforts to reduce the infection (Hermans et al., 2011a). An effective preharvest control strategy that reduces pathogen colonization in the cecal environment could reduce the risk of fecal shedding and therefore product contamination considering that *Campylobacter* resides primarily in the cecal crypts of birds (Beery et al., 1988). Administration of antimicrobial treatment through feed can be applied as a practical method for controlling pathogen colonization. Plant-derived compounds or phytochemicals represent a large unexploited resource that can serve as a safe and effective alternative for controlling pathogens in birds, considering that there is an increasing number of customers that

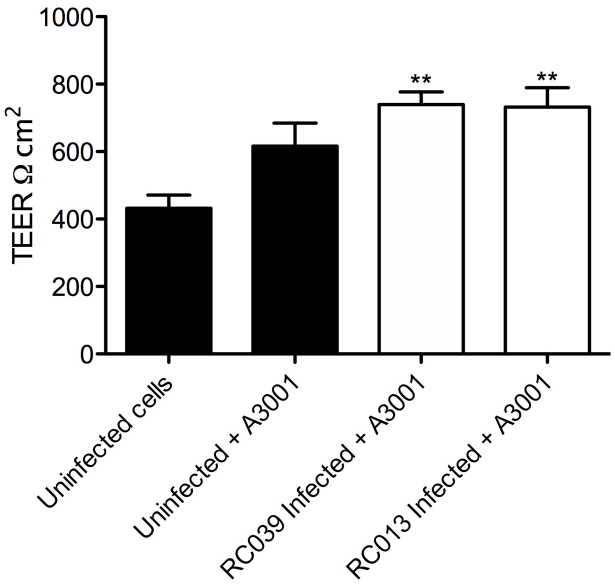

FIGURE 6 | Transepithelial resistance of HCT-8 cells infected with *C. jejuni* RC039 and *C. coli* RC013 at 3 h postinfection using 0.5% Auranta during infection. Asterisk indicates significant difference (**$P < 0.001$) compared to uninfected cells. Error bars indicate standard deviations.

demand antibiotic free chickens. In addition, in the EU the use of antibiotics as growth promotors is not permitted (Hermans et al., 2011b; Wagle et al., 2017).

Bacterial virulence factors such as adhesion are important for colonization; a reduction in this particular attribute could minimize *Campylobacter* ability to attach to the epithelial cells, hence its colonization of poultry (Jin et al., 2001; Hermans et al., 2011a). Previously, Byrne et al. (2007) showed that *Campylobacter* spp. attaches and invades both primary chicken enterocytes and human epithelial cells with similar efficiency. The effect of other natural antimicrobials has been widely investigated in different studies of *Campylobacter* spp. infection. Blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) extracts have been shown to significantly reduce the adhesion and invasion to epithelial cells (Salaheen et al., 2014). Other extracts from *Artemisia ludoviciana, Acacia farnesiana, Cynarascolymu* ssp., *Opuntiaficus indica* (Castillo et al., 2014), as well as β-resorcylic acid, have shown similar effects (Wagle et al., 2017). During the course of this study, we have explored the efficacy of Auranta 3001 (a mixture of organic acids and plant extracts) in reducing the ability of *C. jejuni* and *C. coli*, T6SS positive isolates, to invade epithelial cells *in vitro*. Our results indicate that the antimicrobial agent has a direct effect on the pathogen as following pretreatment of *C. jejuni* RC039 and *C. coli* RC013 a statistically significant reduction in invasiveness was observed ($p < 0.001$). This effect was slightly more pronounced, compared to control, with the increased exposure time and the concentration of Auranta 3001. Moreover, we have observed similar results when Auranta 3001 was used to pretreat the HCT-8 cells before infection, suggesting that the antimicrobial agent may potentially interfere with the host cell metabolic pathways, which subsequently affect the ability of bacteria to infect. This theory is supported by a study, which indicates that carvacrol, the main component of oregano, does not inhibit bacterial growth but significantly reduces the virulence potential of *C. jejuni* and protects against cellular infection of INT-407 cells (van Alphen et al., 2012). Similarly, Citral, which is found in citrus extracts, is able to significantly suppress the attachment and invasion to Caco-2 cells and inhibit the expression of genes involved in the attachment and invasion of host cells by *C. sakazakii* (Shi et al., 2017).

Cellular tight junctions are a physical barrier against pathogen intrusion and strengthening them is key in the development of novel strategies against *Campylobacter* spp., infection (Hatayama et al., 2018). *Campylobacter* ability to disrupt the tight junctions and invade via the basolateral site of eukaryotic cells has been reported in several studies (Monteville and Konkel, 2002; Chen et al., 2006). The increase in TEER, observed in our studies, suggests that the tight junctions of infected cells are strengthened in the presence of Auranta 3001, which could have contributed to the reduction in adhesion and invasion of the two isolates.

To investigate the potential mechanism of action of Auranta 3001, we evaluated the effect of the antimicrobial on various virulence attributes of *C. jejuni* and *C. coli*. Motility is crucial to *Campylobacter* pathogenesis both *in vitro* and *in vivo*, and it has been shown that diminished motility results in reduced ability to invade epithelial cells *in vitro* (Yao et al., 1994; Golden and Acheson, 2002; Stahl et al., 2014). Our study shows that the antimicrobial agent significantly reduces the motility of *C. jejuni* RC039 and *C. coli* RC013 in a time and dose-dependent manner. Similar results were reported in a recent study, which describes that preexposure of *Campylobacter* spp. to different concentrations of organic oils, such as carvacrol, eugenol, and thyme reduced the motility and invasion of *C. jejuni* into intestinal epithelial cells, without affecting the normal function of the cells (Upadhyay et al., 2017). A citric-based disinfectant applied at sub-inhibitory concentrations reduced the motility of *Campylobacter jejuni* as well as interfered with quorum-sensing activity and biofilm formation (Castillo et al., 2015). A similar effect was observed when lactic acid was applied at a concentration of 0.6% in *Salmonella typhimurium* (Burt et al., 2016). Mith et al. (2015) also found that oregano essential oil and carvacrol are able to significantly down-regulate genes involved in motility of *E. coli* O157:H7 (Mith et al., 2015). Therefore, since the antimicrobial is a mixture of organic acids and plant extracts (e.g., oregano), it is likely that the reduced motility observed in *C. jejuni* and *C. coli* is a result of the down-regulation of genes involved in motility.

Motility is an important virulence factor in *Campylobacter jejuni* (Corcionivoschi et al., 2012) that can be affected by structural and quantitative changes in surface polysaccharides (CPS). These changes are very important because similar to other bacterial pathogens, *Campylobacter* spp. expresses capsular polysaccharides as a virulence factor to avoid opsonophagocytosis (Nanra et al., 2013). CPS was also previously shown to play an important role in systemic infections as well as in induction of ovine abortion due to antigenic variation and immune evasion (Sahin et al., 2017). Interestingly, our study shows that changes

were only detected for *C. jejuni* RC039 CPS profiles but absent in *C. coli* RC013. This is possibly caused by the significant diversity in gene content between *C. jejuni* and *C. coli* (Dorrell et al., 2001). These variations for both CPS and LOS could be reflected in variable effects on the bacterial virulence potential.

At gene expression level, it was described previously that sub-inhibitory concentrations of antimicrobials influences gene transcription levels in many bacterial pathogens (Tsui et al., 2004; Donoghue et al., 2015; Oh and Jeon, 2015b). Since the sub-inhibitory concentrations of Auranta 3001 did not inhibit the growth of *C. jejuni* RC039 and *C. coli* RC013, the reduction, observed in the virulence attributes, could be due to the effect of the antimicrobial on the transcription of the virulence genes. Therefore, we used qRT-PCR to determine the effect of Auranta 3001 on the expression of *cetB* and *hcp* genes of *Campylobacter*. *CetB* is involved in energy chemotaxis and has been described to play a role in motility (Konkel et al., 1997; Reuter et al., 2010; Hermans et al., 2011a). The *hcp* gene encodes for a hemolysin-correlated protein and is a key indicator for a functional T6SS (Corcionivoschi et al., 2015; Ugarte-Ruiz et al., 2015). It has been shown that inactivation of *C. jejuni* T6SS resulted in a reduction of adherence to and invasion of *in vitro* cell lines, while over-expression of a hemolysin co-regulated protein (*hcp*), greatly enhanced these processes (Lertpiriyapong et al., 2012). During our study, we observed that Auranta 3001 modulated the transcription level of genes coding energy chemotaxis (*cetB*) and hemolysin correlated protein (*hcp*) thus indicating that the anti-*Campylobacter* colonization effect observed with Auranta 3001 could be mediated via down-regulation of critical colonization genes.

Chicken broilers are still the main source of *Campylobacter*-related infections. Development of novel strategies and products that could be efficient in reducing the colonization levels in poultry is currently becoming a priority for the industry (Johnson et al., 2017). Our *in vivo* results indicate a reduction in colonization up to 6 logs between treated and untreated groups. These results clearly show that the anti-pathogenic effect of Auranta 3001, observed *in vitro*, also translates in less colonization *in vivo*. In conclusion, our results indicate that Auranta 3001 is able to significantly reduce the invasiveness of *C. jejuni* and *C. coli* and protect the host cells *in vitro* and *in vivo* against *Campylobacter* infection and colonization. This effect is expressed by its negative impact on important virulence factors such as motility, adherence, and internalization. Based on these results, Auranta 3001 shows great potential as a method to control campylobacteriosis and provides more information in regards to the mechanistic mode of action of novel antimicrobial products and extracts. Further *in vivo* studies could potentially explain the mechanism of action, by characterizing the genomic, transcriptomic, and proteomic profiles of the pathogens.

AUTHOR CONTRIBUTIONS

FS, AS, CK, ML, LP, and LS conceived the design and performed the experiments. FS, AS, NC, LS, and VL analyzed the data. NC, PW, and LS contributed reagents, materials, and analysis tools. NC, VL, AS, FS, and OG wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work has been funded through a research grant awarded to NC by Auranta, Nova UCD, Belfield Innovation Park, Belfield, Dublin 4, Ireland.

ACKNOWLEDGMENTS

The authors would like to thank John Cullen for his scientific contributions and advice given during this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02139/full#supplementary-material

REFERENCES

Backert, S., and Hofreuter, D. (2013). Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen *Campylobacter jejuni*. *J. Microbiol. Methods* 95, 8–23. doi: 10.1016/j.mimet.2013.06.031

Beery, J. T., Hugdahl, M. B., and Doyle, M. P. (1988). Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54, 2365–2370.

Burt, S. A., Adolfse, S. J., Ahad, D. S., Tersteeg-Zijderveld, M. H., Jongerius-Gortemaker, B. G., Post, J. A., et al. (2016). Cinnamaldehyde, carvacrol and organic acids affect gene expression of selected oxidative stress and inflammation markers in IPEC-J2 cells exposed to *Salmonella* typhimurium. *Phytother. Res.* 30, 1988–2000. doi: 10.1002/ptr.5705

Byrne, C. M., Clyne, M., and Bourke, B. (2007). *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* 153, 561–569. doi: 10.1099/mic.0.2006/000711-0

Castillo, S., Heredia, N., Arechiga-Carvajal, E., and García, S. (2014). Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. *Food Biotechnol.* 28, 106–122. doi: 10.21010/ajtcam.v13i5.10

Castillo, S., Heredia, N., and Garcia, S. (2015). 2(5H)-Furanone, epigallocatechin gallate, and a citric-based disinfectant disturb quorum-sensing activity and reduce motility and biofilm formation of *Campylobacter jejuni*. *Folia Microbiol.* 60, 89–95. doi: 10.1007/s12223-014-0344-0

Chaisowwong, W., Kusumoto, A., Hashimoto, M., Harada, T., Maklon, K., and Kawamoto, K. (2012). Physiological characterization of *Campylobacter jejuni* under cold stresses conditions: its potential for public threat. *J. Vet. Med. Sci.* 74, 43–50. doi: 10.1292/jvms.11-0305

Chen, M. L., Ge, Z., Fox, J. G., and Schauer, D. B. (2006). Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. *Infect. Immun.* 74, 6581–6589. doi: 10.1128/IAI.00958-06

Corcionivoschi, N., Alvarez, L. A., Sharp, T. H., Strengert, M., Alemka, A., Mantell, J., et al. (2012). Mucosal reactive oxygen species decrease virulence by

disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host Microbe* 12, 47–59. doi: 10.1016/j.chom.2012.05.018

Corcionivoschi, N., Clyne, M., Lyons, A., Elmi, A., Gundogdu, O., Wren, B. W., et al. (2009). *Campylobacter jejuni* cocultured with epithelial cells reduces surface capsular polysaccharide expression. *Infect. Immun.* 77, 1959–1967. doi: 10.1128/IAI.01239-08

Corcionivoschi, N., Gundogdu, O., Moran, L., Kelly, C., Scates, P., Stef, L., et al. (2015). Virulence characteristics of hcp (+) *Campylobacter jejuni* and *Campylobacter* coli isolates from retail chicken. *Gut Pathog* 7:20. doi: 10.1186/s13099-015-0067-z

Donoghue, D. J., Arambel, H., Donoghue, A. M., Arsi, K., Upadhyay, A., Woo-Ming, A., et al. (2015). Chitosan supplementation reduces enteric colonization of *Campylobacter jejuni* in broiler chickens and down-regulates expression of colonization genes. *Adv. Food Tech. Nutr. Sci.* 1, 104–111. doi: 10.17140/AFTNSOJ-1-119

Dorrell, N., Mangan, J. A., Laing, K. G., Hinds, J., Linton, D., Al-Ghusein, H., et al. (2001). Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res.* 11, 1706–1715. doi: 10.1101/gr.185801

Douglas Inglis, G., and Kalischuk, L. D. (2003). Use of PCR for direct detection of *campylobacter* species in bovine feces. *Appl. Environ. Microbiol.* 69, 3435–3447. doi: 10.1128/AEM.69.6.3435-3447.2003

EFSA and ECDC (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* 15:5077. doi: 10.1111/cmi.12714

Freitag, C. M., Strijbis, K., and Van Putten, J. P. M. (2017). Host cell binding of the flagellar tip protein of *Campylobacter jejuni*. *Cell Microbiol.* 19, e12714. doi: 10.1111/cmi.12714

Golden, N. J., and Acheson, D. W. (2002). Identification of motility and autoagglutination *Campylobacter jejuni* mutants by random transposon mutagenesis. *Infect. Immun.* 70, 1761–1771. doi: 10.3389/fcimb.2012.00007

Guerry, P., Poly, F., Riddle, M., Maue, A. C., Chen, Y. H., and Monteiro, M. A. (2012). *Campylobacter* polysaccharide capsules: virulence and vaccines. *Front. Cell. Infect. Microbiol.* 2:7. doi: 10.3389/fcimb.2012.00007

Harrison, J. W., Dung, T. T., Siddiqui, F., Korbrisate, S., Bukhari, H., Tra, M. P., et al. (2014). Identification of possible virulence marker from *Campylobacter jejuni* isolates. *Emerg. Infect. Dis.* 20, 1026–1029. doi: 10.3201/eid2006.130635

Hatayama, S., Shimohata, T., Amano, S., Kido, J., Nguyen, A. Q., Sato, Y., et al. (2018). Cellular tight junctions prevent effective *Campylobacter jejuni* Invasion and inflammatory barrier disruption promoting bacterial invasion from lateral membrane in polarized intestinal epithelial cells. *Front. Cell. Infect. Microbiol.* 8:15. doi: 10.3389/fcimb.2018.00015

Hendrixson, D. R., Akerley, B. J., and Dirita, V. J. (2001). Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. *Mol. Microbiol.* 40, 214–224. doi: 10.1111/j.1365-2958.2004.03988.x

Hendrixson, D. R., and DiRita, V. J. (2004). Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. *Mol. Microbiol.* 52, 471–484. doi: 10.1186/1297-9716-42-82

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., et al. (2011a). Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet. Res.* 42:82. doi: 10.1186/1297-9716-42-82

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., et al. (2011b). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol.* 152, 219–228. doi: 10.1016/j.vetmic.2011.03.010

Hintz, T., Matthews, K. K., and Di, R. (2015). The use of plant antimicrobial compounds for food preservation. *Biomed. Res. Int.* 2015:246264. doi: 10.1155/2015/246264

Hitchcock, P. J. (1983). Aberrant migration of lipopolysaccharide in sodium dodecyl sulfate/polyacrylamide gel electrophoresis. *Eur. J. Biochem.* 133, 685–688. doi: 10.1016/j.fm.2004.08.006

Holley, R. A., and Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol.* 22, 273–292. doi: 10.1016/j.micpath.2017.05.009

Hoseinpour, F., Foroughi, A., Nomanpour, B., and Nasab, R. S. (2017). Identification and differentiation of *Campylobacter* species by high-resolution melting curve analysis. *Microb. Pathog.* 108, 109–113. doi: 10.1016/j.micpath.2017.05.009

Jin, S., Joe, A., Lynett, J., Hani, E. K., Sherman, P., and Chan, V. L. (2001). JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. *Mol. Microbiol.* 39, 1225–1236. doi: 10.3389/fmicb.2017.00487

Johnson, T. J., Shank, J. M., and Johnson, J. G. (2017). Current and potential treatments for reducing *Campylobacter* Colonization in animal hosts and disease in humans. *Front. Microbiol.* 8:487. doi: 10.3389/fmicb.2017.00487

Karlyshev, A. V., and Wren, B. W. (2001). Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using alcian blue dye. *J. Clin. Microbiol.* 39, 279–284. doi: 10.1046/j.1365-2958.1997.4031771.x

Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson, D. E. Jr., and Cieplak, W. Jr. (1997). Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol. Microbiol.* 24, 953–963. doi: 10.1371/journal.pone.0122871

Kovac, J., Simunovic, K., Wu, Z., Klancnik, A., Bucar, F., Zhang, Q., et al. (2015). Antibiotic resistance modulation and modes of action of (-)-alpha-pinene in *Campylobacter jejuni*. *PLoS One* 10:e0122871. doi: 10.1371/journal.pone.0122871

Kummerer, K. (2004). Resistance in the environment. *J. Antimicrob. Chemother.* 54, 311–320. doi: 10.1371/journal.pone.0042842

Lertpiriyapong, K., Gamazon, E. R., Feng, Y., Park, D. S., Pang, J., Botka, G., et al. (2012). *Campylobacter jejuni* type VI secretion system: roles in adaptation to deoxycholic acid, host cell adherence, invasion, and in vivo colonization. *PLoS One* 7:e42842. doi: 10.1371/journal.pone.0042842

Mith, H., Clinquart, A., Zhiri, A., Daube, G., and Delcenserie, V. (2015). The impact of oregano (*Origanum heracleoticum*) essential oil and carvacrol on virulence gene transcription by *Escherichia coli* O157:H7. *FEMS Microbiol. Lett.* 362, 1–7. doi: 10.1093/femsle/fnu021

Monteville, M. R., and Konkel, M. E. (2002). Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infect. Immun.* 70, 6665–6671. doi: 10.1099/00221287-131-8-1973

Morooka, T., Umeda, A., and Amako, K. (1985). Motility as an intestinal colonization factor for *Campylobacter jejuni*. *J. Gen. Microbiol.* 131, 1973–1980.

Nachamkin, I., Yang, X. H., and Stern, N. J. (1993). Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. *Appl. Environ. Microbiol.* 59, 1269–1273. doi: 10.4161/hv.23223

Nanra, J. S., Buitrago, S. M., Crawford, S., Ng, J., Fink, P. S., Hawkins, J., et al. (2013). Capsular polysaccharides are an important immune evasion mechanism for *Staphylococcus aureus*. *Hum. Vaccin. Immunother.* 9, 480–487. doi: 10.1038/ja.2015.26

Oh, E., and Jeon, B. (2015a). Contribution of surface polysaccharides to the resistance of *Campylobacter jejuni* to antimicrobial phenolic compounds. *J. Antibiot.* 68, 591–593. doi: 10.3389/fmicb.2015.01129

Oh, E., and Jeon, B. (2015b). Synergistic anti-*Campylobacter jejuni* activity of fluoroquinolone and macrolide antibiotics with phenolic compounds. *Front. Microbiol.* 6:1129. doi: 10.3389/fmicb.2015.01129

Reuter, M., Mallett, A., Pearson, B. M., and Van Vliet, A. H. (2010). Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76, 2122–2128. doi: 10.1128/AEM.01878-09

Sahin, O., Terhorst, S. A., Burrough, E. R., Shen, Z., Wu, Z., Dai, L., et al. (2017). Key role of capsular polysaccharide in the induction of systemic infection and abortion by hypervirulent *Campylobacter jejuni*. *Infect. Immun.* 85:e00001-17. doi: 10.1128/IAI.00001-17

Salaheen, S., Nguyen, C., Hewes, D., and Biswas, D. (2014). Cheap extraction of antibacterial compounds of berry pomace and their mode of action against the pathogen *Campylobacter jejuni*. *Food Control* 46, 174–181. doi: 10.1038/srep43243

Shi, C., Sun, Y., Liu, Z., Guo, D., Sun, H., Sun, Z., et al. (2017). Inhibition of *Cronobacter sakazakii* virulence factors by citral. *Sci. Rep.* 7:43243. doi: 10.1038/srep43243

Shortt, C., Scanlan, E., Hilliard, A., Cotroneo, C. E., Bourke, B., and Tadhg, O. C. (2016). DNA supercoiling regulates the motility of *Campylobacter jejuni* and is altered by growth in the presence of chicken mucus. *mBio* 7:e01227-16. doi: 10.1128/mBio.01227-16

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., and Hickman, J. J. (2015). TEER measurement techniques for in vitro barrier model systems. *J. Lab. Autom.* 20, 107–126. doi: 10.1177/2211068214561025

Stahl, M., Ries, J., Vermeulen, J., Yang, H., Sham, H. P., Crowley, S. M., et al. (2014). A novel mouse model of *Campylobacter jejuni* gastroenteritis reveals key pro-inflammatory and tissue protective roles for Toll-like receptor signaling during infection. *PLoS Pathog.* 10:e1004264. doi: 10.1371/journal.ppat.1004264

Stratakos, A. C., Sima, F., Ward, P., Linton, M., Kelly, C., Pinkerton, L., et al. (2018). The in vitro effect of carvacrol, a food additive, on the pathogenicity of O157 and non-O157 Shiga-toxin producing *Escherichia coli*. *Food Control* 84, 290–296. doi: 10.1186/s13099-017-0192-y

Stratakos, A. Ch, Sima, F., Ward, P., Linton, M., Kelly, C., Pinkerton, L., et al. (2017). The in vitro and ex vivo effect of Auranta 3001 in preventing *Cryptosporidium hominis* and *Cryptosporidium parvum* infection. *Gut Pathog.* 9:49. doi: 10.1186/s13099-017-0192-y

Teichmann, K., Kuliberda, M., Schatzmayr, G., Pacher, T., Zitterl-Eglseer, K., Joachim, A., et al. (2016). In vitro inhibitory effects of plant-derived by-products against *Cryptosporidium parvum*. *Parasite* 23:41. doi: 10.1051/parasite/2016050

Tsui, W. H., Yim, G., Wang, H. H., Mcclure, J. E., Surette, M. G., and Davies, J. (2004). Dual effects of MLS antibiotics: transcriptional modulation and interactions on the ribosome. *Chem. Biol.* 11, 1307–1316. doi: 10.1111/zph.12176

Ugarte-Ruiz, M., Stabler, R. A., Dominguez, L., Porrero, M. C., Wren, B. W., Dorrell, N., et al. (2015). Prevalence of Type VI secretion system in Spanish *Campylobacter jejuni* isolates. *Zoonoses Public Health* 62, 497–500. doi: 10.1111/zph.12176

Upadhyay, A., Arsi, K., Wagle, B. R., Upadhyaya, I., Shrestha, S., Donoghue, A. M., et al. (2017). Trans-cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes in vitro. *Front. Microbiol.* 8:713. doi: 10.3389/fmicb.2017.00713

van Alphen, L. B., Burt, S. A., Veenendaal, A. K., Bleumink-Pluym, N. M., and Van Putten, J. P. (2012). The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. *PLoS One* 7:e45343. doi: 10.1371/journal.pone.0045343

Wagle, B. R., Upadhyay, A., Arsi, K., Shrestha, S., Venkitanarayanan, K., Donoghue, A. M., et al. (2017). Application of beta-resorcylic acid as potential antimicrobial feed additive to reduce *Campylobacter* colonization in broiler chickens. *Front. Microbiol.* 8:599. doi: 10.3389/fmicb.2017.00599

Yao, R., Burr, D. H., Doig, P., Trust, T. J., Niu, H., and Guerry, P. (1994). Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol. Microbiol.* 14, 883–893. doi: 10.1046/j.1365-2958.1997.2861650.x

Yao, R., Burr, D. H., and Guerry, P. (1997). CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol. Microbiol.* 23, 1021–1031. doi: 10.1038/nrmicro1718

Young, K. T., Davis, L. M., and Dirita, V. J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* 5, 665–679.

Zhu, H., Du, M., Fox, L., and Zhu, M.-J. (2016). Bactericidal effects of *Cinnamon cassia* oil against bovine mastitis bacterial pathogens. *Food Control* 66, 291–299. doi: 10.1016/j.foodcont.2016.02.013

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

METHODS
published: 15 October 2018
doi: 10.3389/fmicb.2018.02401

A Rapid LAMP-Based Method for Screening Poultry Samples for *Campylobacter* Without Enrichment

M. Rosario Romero and Nigel Cook*

Fera Science, Ltd., York, United Kingdom

Campylobacter is the most prominent bacterium associated with foodborne disease and the majority of human infection cases are attributed to chicken. Rapid methods capable of determining the *Campylobacter* status of poultry products in a short time are needed in today's fast-paced food supply chain. In this study, we developed and evaluated an easy to perform, rapid and robust method for direct detection of *Campylobacter* in poultry carcasses based on loop-mediated isothermal DNA AMPlification (LAMP). The method does not require bacterial culture or DNA purification and generates results in just an hour. A total of 171 swabs from chicken and turkey slaughter houses were analyzed in parallel by both LAMP and conventional culture-based enumeration methods to evaluate the performance of the rapid method. *Campylobacter* was detected by LAMP in 100% of swabs with an enumeration result of ≥800 cfu/swab, and 98.6% (69 out of 70) of samples reported as negative by enumeration (≤10 cfu/swab) were also negative by LAMP. The method is also suitable for analysis of boot swabs from poultry houses, and therefore it represents a convenient screening tool that can be implemented on farm, at slaughter houses, processing plants or retail, to help with the control of *Campylobacter* contamination throughout the food supply chain. The inclusion of an internal amplification control prevents any potential false negative results due to DNA amplification inhibitors that might be present in the sample.

Keywords: food safety, poultry, carcasses, *Campylobacter*, detection, LAMP, internal amplification control

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Dario De Medici,
Istituto Superiore di Sanità (ISS), Italy
Annika Flint,
Health Canada, Canada

****Correspondence:***
M. Rosario Romero
rosario.romero@fera.co.uk

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

Received: *19 June 2018*
Accepted: *19 September 2018*
Published: *15 October 2018*

Citation:
Romero MR and Cook N (2018) A Rapid LAMP-Based Method for Screening Poultry Samples for Campylobacter Without Enrichment. Front. Microbiol. 9:2401. doi: 10.3389/fmicb.2018.02401

INTRODUCTION

Campylobacter is the most frequently reported cause of acute bacterial gastroenteritis in humans, with a large proportion of cases implicated with consumption of contaminated poultry products (Efsa Panel On Biological Hazards, 2015). A survey published by the United Kingdom Foods Standard Agency reported that over 60% of fresh chickens from major retailers in the United Kingdom analyzed between July 2015 and March 2016 were positive for *Campylobacter* and 11% showed contamination at high levels (>1,000 colony forming units × g^{-1})[1]. Although these figures represented an improvement with respect to the previous year, the report concluded that

[1]Foods Standards Agency, United Kingdom, 2018. https://www.food.gov.uk/research/foodborne-diseases/a-microbiological-survey-of-campylobacter-contamination-in-fresh-whole-uk-produced-chilled-chickens-at-retail-sale-y234.

the proportion of chicken contaminated with high numbers of *Campylobacter* is still considerable. In order to achieve further reductions in contamination levels, efficient control measures need to be implemented throughout the poultry production chain. Accurate and rapid testing methods are necessary to support *Campylobacter* control strategies.

Current systems to monitor *Campylobacter* incidence on the broiler farm or processing plant involve sending samples to the laboratory for analysis, and then waiting days for the results. Detection of foodborne pathogens by conventional culture methods is a reliable approach, but it requires specialist laboratories and several days to generate results, and therefore there is no real-time information on pathogen presence or absence. Rapid and robust testing systems are needed for implementation on-site to inform rapid action. Other methods for detection and identification of *Campylobacter* have been published in recent years (Velusamy et al., 2010), including antibody-based detection (Wadl et al., 2009), PCR (Josefsen et al., 2004; Leblanc-Maridor et al., 2011; de Boer et al., 2015), DNA microarrays (Quinones et al., 2007; Donatin et al., 2013), and loop-mediated isothermal DNA amplification (LAMP) (Yamazaki et al., 2009; Dong et al., 2014; Sabike et al., 2016). Nevertheless, most procedures are still not fully adequate to be deployed on-site, as they involve specialized skills or facilities to perform certain steps such as DNA extraction or enrichment culture.

A rapid, easy to perform assay should ideally work on crude samples with minimal preparation and sample handling, e.g., no culture-based enrichment or DNA extraction and purification. LAMP-based methods for *Campylobacter* have been produced in recent years. Eiken Chemical, Co., Ltd. (Tokyo, Japan) developed the Loopamp® *Campylobacter* detection kit. This method requires specialized laboratory equipment, as well as pre-enrichment and therefore it is not suitable as a rapid method for on-site application. The Loopamp® *Campylobacter* detection kit has previously been reported as useful for the analysis of chicken meat contaminated with *C. jejuni* and *C. coli* (Yamazaki et al., 2009). The study showed that LAMP achieved 98.5% sensitivity compared with conventional culture tests, and results were obtained in 23.5 to 25.5 h from the start of the enrichment culture, in contrast with the 3–4 days required for the conventional method. However, that approach still involved an enrichment step and a laborious three-step centrifugation protocol. Another research group (Dong et al., 2014) developed a LAMP assay for detection of *C. jejuni* in cattle farm samples with a limit of detection of 400 fg genomic DNA per test tube (approximately 226 genome copies). This assay is rapid, but culture-based sample enrichment was still needed for application to farm samples.

We previously developed a LAMP assay for the detection of themotolerant *Campylobacter* spp. (Romero et al., 2016) in poultry boot swabs. The procedure involved the use of immunomagnetic beads to isolate the bacteria from boot swab samples followed by detection via LAMP. Since then, a new DNA polymerase has become available that is claimed by the manufacturer to possess improved robustness and provide faster amplification rates (GspSSD LF DNA Polymerase, Optigene, Ltd., United Kingdom). In the present work, we have modified our previous assay with this new enzyme to develop a rapid method for the direct detection of *Campylobacter* in poultry carcass swabs. The procedure is performed directly on carcass swab samples without culture enrichment, bacterial isolation, or DNA purification, and results can be generated within 1 h of sample collection.

MATERIALS AND METHODS

Bacterial Strains and Cultures

Campylobacter jejuni NCTC 11168, and *C. coli* NCTC 11350 were used in this study. *Campylobacter* stocks were stored at −20°C and cultured at 41.5 ± 1°C on charcoal cefoperazone desoxycholate agar plates (Oxoid, Basingstoke, United Kingdom) for 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2).

Carcass Swabs

Carcass sponge swab kits (TS/15-BPW, Technical Service Consultants) were used. They are provided pre-wetted in 10 mL of buffer. Poultry carcass swabs were obtained from Faccenda Foods, Ltd., United Kingdom and Bernard Matthews Foods, Ltd., United Kingdom. Duplicate samples were taken from each bird by holding two swabs side by side and swabbing over the whole carcass (chickens) or over the breast (turkey). One of the duplicate swabs was delivered to our laboratory and either stored at −20°C or tested immediately by LAMP, and the other one was tested by the supplier using culture methods. For artificial contamination experiments, the supplier tested for the presence/absence of *Campylobacter* using a culture-based method based on ISO/TS 10272-1:2006 (Anonymous, 2006a). Only swabs from birds testing *Campylobacter*-negative were used for subsequent artificial contamination experiments. For analysis of naturally contaminated carcasses, the supplier tested samples according to an enumeration protocol based on ISO/TS 10272-2:2006 (Anonymous, 2006b).

For comparison of results in samples treated by SonoSteam®[2], carcasses from two different broiler flocks were analyzed, 15 carcasses from each before treatment, just after evisceration, and 15 after the SonoSteam® and air chilling processes. Duplicate swabs from each carcass were analyzed, respectively, by the supplier using bacterial enumeration and by Fera using LAMP.

Boot Swabs

Boot swabs from turkey houses were provided by Bernard Matthews, Ltd., United Kingdom. Samples were collected in duplicate by walking through turkey sheds wearing boot socks. The boot socks were then bagged individually and sent for analysis. One of the duplicate swabs was delivered to our laboratory and either stored at −20°C or tested immediately by LAMP. The second duplicate was tested by the supplier by *Campylobacter* culture methods as above, using 25 g of boot swab plus litter. Enumeration results were provided as cfu × g^{-1}.

[2]https://sonosteam.com/

Extraction of Bacteria From Carcass Swabs

For LAMP testing, the swab was palpitated repeatedly (approximately 10 times) to elute the attached material. A small aliquot of the fluid (around 500 μl) was treated at 85°C for 5 min in a dry hot block. Five μl was then taken and added directly into a LAMP reaction tube.

For the culture methods, each carcass swab was resuspended in 10 mL of buffer and 1 mL was used. Enumeration results were back-calculated to cfu per swab.

Preparation of Boot Swabs for LAMP

Boot swabs were re-suspended in 100 mL of phosphate buffer saline (PBS) + 0.01% Tween 20 (PBST) and shaken for 30 s. An aliquot of the suspension was diluted fourfold in PBST and then 500 μl of this was mixed with an equal volume of 0.3 M KOH and heated at 85°C for 5 min in a dry hot block. Five μl was then added to the LAMP reaction tube.

Detection of *Campylobacter* by LAMP Assay

The LAMP assay was described previously (Romero et al., 2016). It targets a region of the thermotolerant *Campylobacter* spp. 16S RNA gene, and was used with the following modifications: ISO-004 Isothermal Mastermix (OptiGene, Ltd., Horsham, United Kingdom) and a newly designed internal amplification control (IAC) were used in the reaction; this IAC was designed to have a more pronounced annealing temperature difference to the target than that used in the previous assay, and therefore to be more readily distinguishable from the target amplicon (**Figure 1** shows the IAC sequence). Oligo Calc: Oligonucleotide Properties Calculator[3] was used to estimate the annealing temperature of several modifications of the region between the FIP and BIP primers. Four different versions of the IAC were designed, synthesized by Eurofins MWG Operon (Ebersberg, Germany) and tested. The IAC sequence producing the best discrimination from the target during annealing was selected and optimized as published previously (Cook et al., 2013). The optimal concentration for the LAMP assay was 340 IAC copies per reaction.

The master mix (15 μl), primers and IAC were combined in a 20 μl volume per reaction, and 5 μl of sample was added. **Table 1** details primer sequences and concentrations used. LAMP assays were performed in a Genie II (OptiGene, Ltd., Horsham, United Kingdom) using eight-tube strips. Every individual run included a positive control (purified *Campylobacter* DNA) and a negative control (water). The reactions were run at 65°C for 60 min. Amplicon annealing profiling was performed by heating to 98°C then cooling to 80°C at a rate of 0.05°C × sec^{-1}.

For boot swabs, ISO-004LNL Isothermal Mastermix (OptiGene, Ltd., Horsham, United Kingdom) was used, which neutralizes the alkaline solution used to assist bacterial lysis. The rest of the procedure was as described above.

[3]http://biotools.nubic.northwestern.edu/OligoCalc.html

5'-
CTCCTGCTTAACACAAGTTGAGTAGGGAAAGTTTTTCGGTGTAGGATGAGACTATATAG
TATCAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCTATGACGCTTAACTGGTCTGAGA
GGATGATCAGTCACACTGGAACTGAGACACGGTCCGATGGCTCAGCCGATGGCCGAGCC
TCACGCCTAGCGGCGCGTGGCAGCGACGCTCGACGCCAGGCAGTAGCTAGCGAGCGTCG
GGAGGCAGCAGTAGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCG
TGGAGGATGACACTTTTCGGAGCGTAAACTCCTTTTCTTAGGGAAGAATTCTGACGGTAC
CTAAGGAAGCG-3'

FIGURE 1 | DNA sequence of the Internal Amplification Control.

Purification of *Campylobacter* Genomic DNA

Genomic DNA was purified from 100 mL of *Campylobacter* culture using a DNA extraction kit (QIAGEN, Manchester, United Kingdom). A Qubit 4.0 fluorometer (Thermo Fisher Scientific, Wilmington, DE, United States) was used to measure the concentration and purity of the DNA. Purified DNA (1 μl/reaction) was used as a positive control in every LAMP experiment.

Detection Limit of the Method Using Artificial Contamination

Broth cultures of *C. coli* were grown overnight. An aliquot of each culture was used for enumeration and the remainder was kept under hypoxic conditions at room temperature for 2 days, when the number of colony forming units (cfu) was determined. Serial dilutions were then made in maximum recovery diluent (MRD, Oxoid, Basingstoke, United Kingdom). One mL of each serial dilution, containing between 1.0×10^3 and 2.3×10^5 cfu, was added to *Campylobacter*-free turkey carcass swabs, which were then analyzed by the LAMP-based method. A total of 23 swabs were spiked in five independent experiments. Non-spiked swabs were also included as negative control.

RESULTS

Performance of the IAC

The IAC produces an annealing derivative with a peak at 92.5°C, clearly distinguishable from that of the target amplicon (88°C). Negative samples are easily detected by the presence of a single annealing peak at 92.5°C (**Figure 2**).

Limit of Detection of the Method, Using Artificially Contaminated Swabs

The analysis of turkey carcass swabs spiked with different amounts of *C. coli* showed that the method could consistently detect down to 10^4 cfu per swab, and in 2/5 tests down to 10^3 cfu (**Table 2**).

Detection of *Campylobacter* in Naturally Contaminated Chicken and Turkey Carcasses

Table 3 shows the culture enumeration results provided by the supplier's testing laboratories alongside the results obtained with the LAMP-based method. The LAMP based method could

TABLE 1 | *Campylobacter* LAMP primers.

Primer name	Sequence (5′–3′)	Concentration in LAMP (nM)	Reference
OT1559	CTGCTTAACACAAGTTGAGTAGG	200	Uyttendaele et al., 1995
18-1rev	TTCCTTAGGTACCGTCAGAA	200	Lubeck et al., 2003
16SthCampyFIP	GGACCGTGTCTCAGTTCCAGTGTGACGGATGAGACTATATAGTATCAGCTAG	2000	Romero et al., 2016
16SthCampyBIP	CGGGAGGCAGCAGTAGGGAATATTGCTAAGAAAAGGAGTTTACGCTCCG	2000	Romero et al., 2016
16SthCampyF-Loop	GTTAAGCGTCATAGCCTTGGTAA	1000	Romero et al., 2016
16SthCampyB-Loop	GCGTGGAGGATGACACTT	1000	Romero et al., 2016

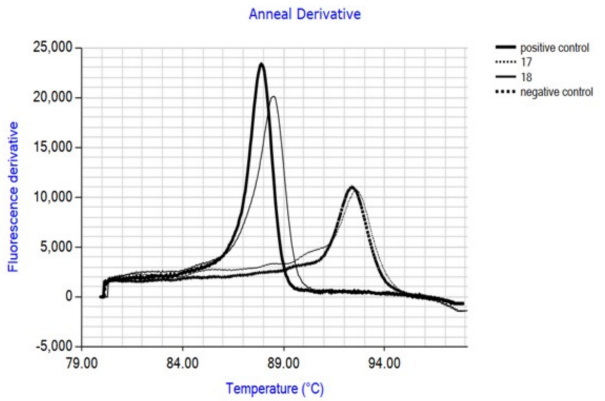

FIGURE 2 | Annealing plots obtained after LAMP amplification showing discrimination of IAC and target. Internal amplification control (IAC) was included in all samples. Positive control: purified *C. coli DNA*; 17: carcass swab not contaminated with *Campylobacter*; 18: carcass swab contaminated with *Campylobacter*; negative control: water.

TABLE 2 | LAMP-based detection of *C. coli* on turkey carcass swabs at various artificial contamination levels.

Contamination level (cfu/swab)	Number of samples that tested *Campylobacter*-positive
10^5	5/5*
10^4	5/5*
10^3	2/5*
10^2	0/4*
10^1	0/4*
Uncontaminated	0/4*

**Number of samples tested at the corresponding contamination level.*

consistently detect contamination at levels of 800 cfu per swab and higher; however, the probability of detection diminished as the counts per swab decreased, resulting in some false negative results in swabs containing 10−760 cfu. Out of 70 samples that tested negative in the enumeration assay, only one generated a positive result by LAMP.

One of the slaughter houses that supplied chicken carcass swabs for this study uses a combination of steam and ultrasound (SonoSteam®) to reduce *Campylobacter* loads on the carcasses. To explore the effectiveness of the LAMP approach in such a setting, samples were collected before and after SonoSteam® treatment from two separate broiler flocks (one positive and one negative for *Campylobacter*, based on farm results). Duplicate swabs from each carcass were analyzed by both enumeration of *Campylobacter* spp. and LAMP, respectively. Results are shown in **Table 4**. All pretreatment samples from the *Campylobacter*-positive flock tested positive by the LAMP-based method, while 14/15 post-treatment samples (ranging from 0 to 410 cfu/swab) were negative for *Campylobacter* by LAMP. All samples from the *Campylobacter*-negative flock tested negative by LAMP, although the enumeration method detected low numbers of cfu/swab in the pre-treatment set.

Detection of *Campylobacter* in Naturally Contaminated Turkey Boot Swabs

Table 5 shows the information obtained when a small set of duplicate turkey boot swabs were tested by the LAMP-based method and by a culture-based method, respectively. LAMP could identify *Campylobacter* contamination at levels of 30 cfu $\times$ g^{-1} and above.

DISCUSSION

Due to the impact of *Campylobacter* on human health, governments and industry have been developing strategies to reduce the levels of *Campylobacter* contamination in poultry, the main vehicle of *Campylobacter* food poisoning for humans. Testing poultry products for the presence of *Campylobacter* is key to monitor the effect of biosecurity measures and to reduce the risk of contaminated products reaching the consumer. In this study, a rapid, robust, and easy to perform method has been optimized and evaluated against standard enumeration methods. The method uses LAMP technology to detect *Campylobacter* spp. directly in crude suspensions of carcass swabs, without pre-enrichment or DNA purification.

The *Campylobacter* LAMP assay has a probability of detection of 95% with 22 genome copies when performed with pure DNA using *Tin* Isothermal Mastermix (OptiGene, Ltd., Horsham, United Kingdom) (Romero et al., 2016). In the method described here, a sponge swab pre-wetted with 10 mL of buffer is used, and after homogenization, the fluid is squeezed out of the sponge and used as template for the reaction. When swabs were artificially contaminated with defined numbers of *Campylobacter* colony forming units, the limit of detection of the method was between 10^3 and 10^4 cfu per swab. Since the total volume of fluid per swab is 10 mL and the volume tested in a single reaction is 5 μl, the *Campylobacter* levels detected are equivalent to 0.5 and 5 cfu

TABLE 3 | Detection of thermophilic *Campylobacter* spp. in naturally contaminated chicken and turkey carcasses, by a culture-based enumeration method and by the LAMP-based method.

	LAMP results			
Enumeration results (CFU/swab)	**Number of swabs**	**Positive swabs**	**Negative swabs**	**Percentage positive samples by LAMP**
≥800	36	36	0	100
400–760	11	5	6	45.4
200–380	14	4	10	28.6
100–190	22	3	19	13.6
10–80	18	0	18	0
0*	70	1	69	1.4
Number of swabs	171	50	121	

**Absence of colonies on the culture plates was reported as <10 cfu/swab, as the limit of detection of the method is 1 cfu × mL^{-1} and samples were diluted 10-fold before plating.*

TABLE 4 | Detection of thermophilic *Campylobacter* spp. in broiler carcasses before and after SonoSteam® treatment by culture-based enumeration and by the LAMP-based method.

***Campylobacter*-positive flock**					
Pre-SonoSteam			**Post-SonoSteam/chill**		
Sample number	**LAMP result**	**Enumeration result (cfu/swab)**	**Sample number**	**LAMP result**	**Enumeration result (cfu/swab)**
1	Positive	1300	16	Negative	30
2	Positive	3600	17	Negative	130
3	Positive	5300	18	Negative	340
4	Positive	1800	19	Negative	90
5	Positive	3100	20	Negative	<10
6	Positive	2600	21	Negative	350
7	Positive	5200	22	Negative	600
8	Positive	3600	23	Positive	120
9	Positive	5100	24	Negative	180
10	Positive	2100	25	Negative	40
11	Positive	2400	26	Negative	90
12	Positive	4700	27	Negative	140
13	Positive	7800	28	Negative	30
14	Positive	1600	29	Negative	480
15	Positive	2100	30	Positive	410
***Campylobacter*-negative flock**					
31	Negative	30	46	Negative	<10
32	Negative	120	47	Negative	<10
33	Negative	220	48	Negative	<10
34	Negative	170	49	Negative	<10
35	Negative	40	50	Negative	<10
36	Negative	250	51	Negative	<10
37	Negative	70	52	Negative	<10
38	Negative	360	53	Negative	<10
39	Negative	80	54	Negative	<10
40	Negative	10	55	Negative	<10
41	Negative	190	56	Negative	<10
42	Negative	100	57	Negative	<10
43	Negative	640	58	Negative	<10
44	Negative	480	59	Negative	<10
45	Negative	60	60	Negative	<10

TABLE 5 | Detection of thermophilic *Campylobacter* spp. in naturally contaminated turkey boot swabs, tested by a culture-based enumeration method and by the LAMP-based method, respectively.

Swab number	Culture enumeration results (cfu/g)	LAMP results
1	<10	Negative
2	<10	Negative
3	<10	Negative
4	1	Negative
5	10	Negative
6	20	Negative
7	30	Positive
8	100	Positive
9	72,000	Positive
10	Spread*	Positive

**Spread: plates were overgrown and individual colonies could not be counted.*

per reaction, respectively. This is lower than the detection levels observed with pure DNA previously suggesting that dead cells were present in the cultures used to spike the swabs. These would not be detected by a culture method, so they would not have been accounted for during enumeration, but would still give a positive signal by LAMP if present at sufficient levels.

The analysis of naturally contaminated samples showed that the LAMP-based method detected *Campylobacter* in all swabs from carcasses with enumeration results of 800 cfu/swab or greater. For samples with enumeration results below 800 cfu/swab, the overall probability of detection of the LAMP assay decreases gradually as the counts per swab decrease, resulting in some false negative results. This is expected, since the LAMP test is performed on a 5 μl aliquot of a 10 mL swab suspension, which on average equates to less than one cfu per reaction at 800 cfu/swab. Despite this, some of the samples with colony counts below 800 tested positive by LAMP. This will be explained by the presence of dead or non-culturable *Campylobacter*, which will only be detected by LAMP.

Out of 70 carcasses for which no colonies were obtained by the culture method, only one was detected as positive by LAMP. In addition, a further set of 60 turkey duplicate swabs were tested in parallel by the LAMP method and by the enrichment culture method that establishes presence/absence of *Campylobacter* (Anonymous, 2006a) (data not shown). All swabs gave a negative result by both methods, confirming that the likelihood of obtaining false positives by the LAMP-based method is very low.

Whilst with artificially contaminated carcass swabs the method consistently produced positive LAMP results with 10^4 cfu/swab, in naturally contaminated samples all swabs with ≥800 cfu/swab tested positive by LAMP. Since the culture-based method detects only viable cells whereas LAMP detects also dead cells, this apparent discrepancy suggests that carcasses that are contaminated with high levels of *Campylobacter* normally contain high numbers of dead bacteria, more so than those that might occur in the pure bacterial cultures used for spiking experiments.

A fundamental difference between DNA amplification methods and culture-based methods is that the latter naturally have higher sensitivity, since the bacteria are incubated for a period of time and allowed to multiply until visible colonies are obtained. However, rapid results at the expense of some sensitivity may be acceptable for certain applications, such as routine screening. The method described here has a limit of detection for poultry carcass of 800 cfu/swab under the conditions applied and with the specific sampling and sample preparation protocols used in this study. However, modifications can be made to these procedures to achieve adequate detection levels, since the LAMP reaction itself is highly sensitive. Should higher sensitivity be required, various adjustments could be explored, such as reducing the volume of buffer the sample is collected in or concentrating the sample, e.g., by centrifugation and resuspension in a smaller volume.

Our original method (Romero et al., 2016) was developed for the analysis of poultry boot swabs, and involved immunoseparation of the bacteria from swab suspensions followed by LAMP using the Tin enzyme (ISO-001 Tin, OptiGene, Ltd., Horsham, United Kingdom). A small set of turkey boot swabs were tested in the current investigation to assess the performance of the new direct LAMP procedure in these samples, which carry fecal and other materials (bedding, feathers) and might pose a bigger analytical challenge. Although the number of samples is small, these preliminary results show a similar trend to those from carcass swabs, suggesting that the method is robust enough to be applicable to different types of samples, including very complex materials that would inhibit other molecular reactions such as PCR.

A recent publication (Sabike et al., 2016) reported the use of *Campylobacter* LAMP assay for direct detection in chicken fecal samples. They achieved detection of 3.89 and 3.60 log cfu × g^{-1} of fecal sample for *C. jejuni* and *C. coli*, respectively, in artificially contaminated fecal samples, and a three-step DNA precipitation protocol was used to improve sensitivity. In contrast, the approach presented here does not require DNA precipitation, making it faster and simpler to perform. The only equipment needed is a hot block and a small and portable battery powered instrument to perform the reaction and read-out of results. Thus, our method is suitable for rapid screening of *Campylobacter* in different settings including processing plants and poultry farms. The method has also been successfully tested in cattle carcass swabs, with and without artificial contamination (data not shown), suggesting that as expected, it can be applied in settings other than poultry production.

Sabike et al. (2016) reported that their analysis of naturally contaminated specimens from one particular farm showed a high frequency of false negative results for *Campylobacter* loadings ranging from <3 to 5.81 log cfu × g^{-1}. The authors discussed that this high rate of false negatives may be explained by inhibitors of DNA amplification, possibly derived from substances in the poultry feeding stuffs. Indeed, in our preliminary investigation with boot swabs (fecal material) a difference in method performance between different poultry farms was also observed. To deal with potential inhibitors frequently present in complex matrices such as food and fecal samples, our assay includes a

competitive IAC (Hoorfar et al., 2004). The presence of inhibitors of DNA amplification in a sample may result in false negative results. However, in our assay, if DNA amplification has taken place, a signal will always be produced, and the temperature of the annealing peak will indicate if it is a *Campylobacter*-positive or a true negative result. The IAC is also a useful tool for assay optimization; in the absence of target amplification, the IAC fails to amplify it is an indication that the reaction has not proceeded and changes to the sample preparation can be tested. This was applied in our trials with boot swabs and it helped to establish that a fourfold dilution of the swab suspension produced optimal results.

The direct LAMP-based method was applied to carcasses that had been treated with a combination of steam with ultrasound (SonoSteam®) at slaughter to reduce *Campylobacter* loads. As observed previously (Musavian et al., 2014), treatment with SonoSteam® significantly reduced viable *Campylobacter* levels on chicken carcasses (average reduction of 1.4 $\log_{10}$ cfu in the sample set used in this study). The results obtained with LAMP are similar to those obtained with the culture-based method in that in most post-treatment samples a reduction of *Campylobacter* load is indicated. Therefore, the direct LAMP method is suitable for monitoring *Campylobacter* levels in plants where poultry carcasses have been treated by SonoSteam® or similar decontamination procedures, and to ensure that high level contamination is eliminated. Nevertheless, the results obtained with samples post-treatment indicate that low residual levels of *Campylobacter* that would be detected by culture methods may result in false negatives by LAMP due to the limit of sensitivity of the method. As an additional observation, the absence of a LAMP signal in most post-treatment samples suggests that the SonoSteam®/air chilling procedure is not simply killing *Campylobacter*, in which case dead cells on the carcass surface would be detected by LAMP and more positive results would be expected. Instead, the treatment might be removing the bacteria from the carcass surface or damaging their DNA so that it can no longer be amplified by LAMP.

The United Kingdom government/industry set a target to reduce the prevalence of chickens containing greater than 1000 cfu per g skin, considered the most contaminated, to below 10% at the end of the slaughter process[4]; it was considered that achieving this target should consequently reduce the prevalence of *Campylobacter* in the food chain. A survey to determine progress toward this target was carried out in 2016, analyzing chickens sold at retail using a culture-based method[5]. The LAMP-based method presented here is suitable for deployment not just at point of sale, but also before and immediately after the slaughter process, and thus closer to the stages where the *Campylobacter* colonization status of a bird is determined. The colonization status of a chicken is recognized as the most important factor affecting numbers of *Campylobacter* cfu on a carcass (European Food Safety Authority [EFSA], 2010), and therefore the ability to assess this as soon as conveniently possible will be highly useful to allow meat inspectors/slaughter house/processing plant personnel rapid logistic decisions. It could also help to monitor the effectiveness of biosecurity measures implemented to control *Campylobacter* contamination. The LAMP-based system described furthermore offers scope for automation and indeed preliminary work is being carried out in this respect (unpublished data).

This study reports validation data on the performance of a novel rapid method for *Campylobacter* detection on poultry carcasses as compared with conventional ISO-based culture methods. Further validation work will be conducted to provide more information about the equivalence of the method to ISO standards for different purposes and to establish how the rapid method could be integrated into surveillance and enforcement activities and thus contribute to food safety. The method in its present state of development has the acknowledged limitation of a lack of sensitivity below 800 cfu per swab, which will result in some analyses producing negative results if the contamination level is below that figure. However, it must be stressed that the sampling protocol used in this study for both LAMP and culture (half or whole carcass swabbing) is different to that used in the ISO culture method on which current United Kingdom and EU recommendations are based (cutting out a small piece of skin). Some of the carcasses from this study that resulted in less than 800 cfu per swab (whole chicken carcass swabbed) may have also produced a negative result by culture following the standard method that analyses only 10–25 g of skin. Therefore, the sensitivity of the method is sufficient for detection of the *Campylobacter* contamination level (1000 cfu $\times$ g^{-1} skin) which is a process hygiene criterion recommended in European regulation[6]. It has been estimated (Efsa Panel On Biological Hazards, 2011) that full compliance with this criterion by the poultry production industry would lead to a public health risk reduction of over 50%. The LAMP-based method can therefore be a useful tool for rapid screening of poultry carcasses at primary production, assisting in the management of foodborne *Campylobacter* transmission.

AUTHOR CONTRIBUTIONS

MR contributed design and coordination of the study, testing by LAMP and data analysis and interpretation. NC contributed to the LAMP assay design and data interpretation. Both authors contributed to manuscript writing.

FUNDING

This work was funded by the United Kingdom Technology Strategy Board (Grant No. 101736) (An Integrated Platform for Rapid Testing of Pathogens in the Meat/Poultry Supply Chain).

[4]Foods Standards Agency, United Kingdom, 2013. https://acmsf.food.gov.uk/sites/default/files/multimedia/pdfs/board/board-papers-2013/fsa-130904.pdf.

[5]Foods Standards Agency, United Kingdom, 2018. https://www.food.gov.uk/research/foodborne-diseases/a-microbiological-survey-of-campylobacter-contamination-in-fresh-whole-uk-produced-chilled-chickens-at-retail-sale-y234.

[6]https://publications.europa.eu/en/publication-detail/-/publication/218f322f-8890-11e7-b5c6-01aa75ed71a1

ACKNOWLEDGMENTS

We would like to express their gratitude to the project consortium members, especially Terry Motley (Faccenda Foods, Ltd.) for his advice and support, and to Cassandra Denton and Quin Leyden (Faccenda Foods, Ltd.), and Sherrilee Bekker (Bernards Matthews, Ltd.) for providing swabs and test results. We are grateful to the Food Microbiology team at Fera Science, Ltd., especially Lucy Vickers-Smith and Deb Jones for their technical assistance with bacterial cultures, to Martin D'Agostino for assistance with IAC design and to James Rainford for his contribution to data discussion.

REFERENCES

Anonymous (2006a). *ISO/TS 10272-1:2006. Microbiology of food and Animal Feeding Stuffs - Horizontal Method for Detection and Enumeration of Campylobacter spp. - Part 1: C. Detection Method*. Geneva: International Standards Organisation.

Anonymous (2006b). *ISO/TS 10272-2:2006. Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for Detection and Enumeration of Campylobacter spp. - Part 2: Colony-count Technique*. Geneva: International Standards Organisation.

Cook, N., De Ridder, G. A., D′agostino, M., and And Taylor, M. B. (2013). "Internal amplification controls in real-time polymerase chain reaction-based methods for pathogen detection," in *Real-Time PCR in Food Science: Current Technology and Applications*, ed. D. E. Rodriguez-Lazaro (Poole: Caister Academic Press), 35–41.

de Boer, P., Rahaoui, H., Leer, R. J., Montijn, R. C., and Van Der Vossen, J. M. (2015). Real-time PCR detection of *Campylobacter* spp.: a comparison to classic culturing and enrichment. *Food Microbiol.* 51, 96–100. doi: 10.1016/j.fm.2015.05.006

Donatin, E., Buffet, S., Leroy, Q., Raoult, D., and Drancourt, M. (2013). A DNA microarray for the versatile diagnosis of infectious diarrhea. *APMIS* 121, 634–642. doi: 10.1111/apm.12081

Dong, H. J., Cho, A. R., Hahn, T. W., and Cho, S. (2014). Development of a loop-mediated isothermal amplification assay for rapid, sensitive detection of *Campylobacter jejuni* in cattle farm samples. *J. Food Prot.* 77, 1593–1598. doi: 10.4315/0362-028X.JFP-14-056

Efsa Panel On Biological Hazards (2011). Scientific Opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA J.* 9:2105. doi: 10.2903/j.efsa.2011.2105

Efsa Panel on Biological Hazards (2015). Scientific opinion on quantification of the risk posed by broiler meat to human Campylobacteriosis in the EU. *EFSA J.* 8:1437.

European Food Safety Authority [EFSA]. (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008; Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA J.* 8:1503.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., and Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J. Clin. Microbiol.* 42, 1863–1868. doi: 10.1128/JCM.42.5.1863-1868.2004

Josefsen, M. H., Jacobsen, N. R., and Hoorfar, J. (2004). Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of food-borne thermotolerant *Campylobacter*. *Appl. Environ. Microbiol.* 70, 3588–3592. doi: 10.1128/AEM.70.6.3588-3592.2004

Leblanc-Maridor, M., Garenaux, A., Beaudeau, F., Chidaine, B., Seegers, H., Denis, M., et al. (2011). Quantification of *Campylobacter* spp. in pig feces by direct real-time PCR with an internal control of extraction and amplification. *J. Microbiol. Methods* 85, 53–61. doi: 10.1016/j.mimet.2011.01.013

Lubeck, P. S., Cook, N., Wagner, M., Fach, P., and Hoorfar, J. (2003). Toward an international standard for PCR-based detection of food-borne thermotolerant Campylobacters: validation in a multicenter collaborative trial. *Appl. Environ. Microbiol.* 69, 5670–5672. doi: 10.1128/AEM.69.9.5670-5672.2003

Musavian, H. S., Krebs, N. H., Nonboe, U., Corry, J. E., and Purnell, G. (2014). Combined steam and ultrasound treatment of broilers at slaughter: a promising intervention to significantly reduce numbers of naturally occurring Campylobacters on carcasses. *Int. J. Food Microbiol.* 176, 23–28. doi: 10.1016/j.ijfoodmicro.2014.02.001

Quinones, B., Parker, C. T., Janda, J. M. Jr., Miller, W. G., and Mandrell, R. E. (2007). Detection and genotyping of *Arcobacter* and *Campylobacter* isolates from retail chicken samples by use of DNA oligonucleotide arrays. *Appl. Environ. Microbiol.* 73, 3645–3655. doi: 10.1128/AEM.02984-06

Romero, M. R., D′agostino, M., Arias, A. P., Robles, S., Casado, C. F., Iturbe, L. O., et al. (2016). An immunomagnetic separation/loop-mediated isothermal amplification method for rapid direct detection of thermotolerant *Campylobacter* spp. during poultry production. *J. Appl. Microbiol.* 120, 469–477. doi: 10.1111/jam.13008

Sabike, I. I., Uemura, R., Kirino, Y., Mekata, H., Sekiguchi, S., Okabayashi, T., et al. (2016). Use of direct LAMP screening of broiler fecal samples for *Campylobacter jejuni* and *Campylobacter coli* in the positive flock identification strategy. *Front. Microbiol.* 7:1582. doi: 10.3389/fmicb.2016.01582

Uyttendaele, M., Schukkink, R., Van Gemen, B., and Debevere, J. (1995). Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). *Appl. Environ. Microbiol.* 61, 1341–1347.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., and Adley, C. (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

Wadl, M., Polzler, T., Flekna, G., Thompson, L., Slaghuis, J., Kofer, J., et al. (2009). Easy-to-use rapid test for direct detection of *Campylobacter* spp. in chicken feces. *J. Food Prot.* 72, 2483–2488. doi: 10.4315/0362-028X-72.12.2483

Yamazaki, W., Taguchi, M., Kawai, T., Kawatsu, K., Sakata, J., Inoue, K., et al. (2009). Comparison of loop-mediated isothermal amplification assay and conventional culture methods for detection of *Campylobacter jejuni* and *Campylobacter coli* in naturally contaminated chicken meat samples. *Appl. Environ. Microbiol.* 75, 1597–1603. doi: 10.1128/AEM.02004-08

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 October 2018
doi: 10.3389/fmicb.2018.02452

Comprehensive Longitudinal Microbiome Analysis of the Chicken Cecum Reveals a Shift From Competitive to Environmental Drivers and a Window of Opportunity for *Campylobacter*

Umer Zeeshan Ijaz[1†], *Lojika Sivaloganathan*[2†], *Aaron McKenna*[3†], *Anne Richmond*[3], *Carmel Kelly*[4], *Mark Linton*[4], *Alexandros Ch. Stratakos*[4], *Ursula Lavery*[3], *Abdi Elmi*[2], *Brendan W. Wren*[2], *Nick Dorrell*[2*], *Nicolae Corcionivoschi*[4*] and *Ozan Gundogdu*[2*]

[1] School of Engineering, University of Glasgow, Glasgow, United Kingdom, [2] Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, [3] Moy Park, Armagh, United Kingdom, [4] Agri-Food and Biosciences Institute, Food Microbiology, Newforge Lane, Belfast, United Kingdom

Chickens are a key food source for humans yet their microbiome contains bacteria that can be pathogenic to humans, and indeed potentially to chickens themselves. *Campylobacter* is present within the chicken gut and is the leading cause of bacterial foodborne gastroenteritis within humans worldwide. Infection can lead to secondary sequelae such as Guillain-Barré syndrome and stunted growth in children from low-resource areas. Despite the global health impact and economic burden of *Campylobacter*, how and when *Campylobacter* appears within chickens remains unclear. The lack of day to day microbiome data with replicates, relevant metadata, and a lack of natural infection studies have delayed our understanding of the chicken gut microbiome and *Campylobacter*. Here, we performed a comprehensive day to day microbiome analysis of the chicken cecum from day 3 to 35 (12 replicates each day; final $n = 379$). We combined metadata such as chicken weight and feed conversion rates to investigate what the driving forces are for the microbial changes within the chicken gut over time, and how this relates to *Campylobacter* appearance within a natural habitat setting. We found a rapidly increasing microbial diversity up to day 12 with variation observed both in terms of genera and abundance, before a stabilization of the microbial diversity after day 20. In particular, we identified a shift from competitive to environmental drivers of microbial community from days 12 to 20 creating a window of opportunity whereby *Campylobacter* can appear. *Campylobacter* was identified at day 16 which was 1 day after the most substantial changes in metabolic profiles observed. In addition, microbial variation over time is most likely influenced by the diet of the chickens whereby

OPEN ACCESS

Edited by:
Michael J. Rothrock, United States Department of Agriculture, United States

Reviewed by:
Heriberto Fernandez, Universidad Austral de Chile, Chile
Kristina Marie Feye, University of Arkansas, United States

***Correspondence:**
Nick Dorrell
nick.dorrell@lshtm.ac.uk
Nicolae Corcionivoschi
Nicolae.Corcionivoschi@afbini.gov.uk
Ozan Gundogdu
ozan.gundogdu@lshtm.ac.uk

[†]*These authors share first authorship*

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

Received: *21 July 2018*
Accepted: *25 September 2018*
Published: *15 October 2018*

Citation:
Ijaz UZ, Sivaloganathan L, McKenna A, Richmond A, Kelly C, Linton M, Stratakos AC, Lavery U, Elmi A, Wren BW, Dorrell N, Corcionivoschi N and Gundogdu O (2018) Comprehensive Longitudinal Microbiome Analysis of the Chicken Cecum Reveals a Shift From Competitive to Environmental Drivers and a Window of Opportunity for Campylobacter. Front. Microbiol. 9:2452. doi: 10.3389/fmicb.2018.02452

significant shifts in OTU abundances and beta dispersion of samples often corresponded with changes in feed. This study is unique in comparison to the most recent studies as neither sampling was sporadic nor *Campylobacter* was artificially introduced, thus the experiments were performed in a natural setting. We believe that our findings can be useful for future intervention strategies and help reduce the burden of *Campylobacter* within the food chain.

Keywords: chicken, microbiome, *Campylobacter,* environmental filtering, phylogenetic signal, competitive exclusion, diversity

INTRODUCTION

Chickens (*Gallus gallus domesticus*) are an important food source for humans with over 50 billion reared annually for meat and eggs (Part et al., 2016). Feed conversion and the health of chickens is heavily dependent on the largely unexplored complex gut microbial community which plays a role in nutrient assimilation, vitamin and amino acid production and prevention of pathogen colonization (Józefiak et al., 2004; Apajalahti, 2005; Mcnab, 2007; Sergeant et al., 2014). In chickens, the organ with the highest number and variety of bacteria is the cecum (10^{10}-10^{11} cells/g) which plays an essential role in the digestion of non-starch polysaccharides (NSPs) found in chicken feed (Barnes et al., 1972; Józefiak et al., 2004; Bjerrum et al., 2006). The importance of this organ is demonstrated when up to 10% of energy needs can be recovered from a well-functioning cecum (Hegde et al., 1982; Józefiak et al., 2004). The cecum remains a source of bacterial human infection and a reservoir of antibiotic resistance determinants.

The chicken cecum contracts several times a day releasing contents toward the ileum and the cloaca (Pauwels et al., 2015). Notably the cecal drop contains *Campylobacter*, a Gram-negative spiral shaped bacterium which causes an estimated 400 million human infections each year (Friedman et al., 2000; Walker, 2005). *Campylobacter* causes bloody diarrhea, fever and abdominal pains in humans and can also cause post infectious sequelae such as Guillain-Barré syndrome which is a potentially fatal paralytic autoimmune illness. In low-resource areas, asymptomatic and occasionally persistent *Campylobacter* infections are common in children younger than 1 year and correlate with stunted growth and therefore life-long physical and cognitive deficits (Amour et al., 2016). Approximately 80–90% of these infections are attributed to *Campylobacter jejuni*, with poultry as the most important source of human campylobacteriosis within industrialized countries (Humphrey et al., 2007; Mullner et al., 2009; Sheppard et al., 2009). *C. jejuni* colonizes the chicken cecum with relatively high numbers (10^9 CFU per gram) and whereas traditionally was considered a commensal of the chicken gut, more recently has been demonstrated to be pathogenic to the chicken, with this dependent on the genetics of the host and the strain of infection (Van Deun et al., 2008; Hermans et al., 2012; Humphrey et al., 2014, 2015; Wigley, 2015). Natural colonization of chickens is reported to be at approximately day 14 of the chicken life cycle, although we do not know how and why this occurs, and what the impact of *Campylobacter* is on the microbiome (Neill et al., 1984; Kalupahana et al., 2013; Thibodeau et al., 2015).

The microbiome of chickens develop rapidly from days 1–3 where *Enterobacteriaceae* dominate, with *Firmicutes* increasing in abundance and taxonomic diversity from approximately day 7 onwards (Danzeisen et al., 2011; Ballou et al., 2016; Mancabelli et al., 2016). Bacterial populations within the chicken gut are subsequently driven by the rearing environment and from the bacteria present in food and water (Connerton et al., 2018). How and when *Campylobacter* appears and the impact on the chicken gut microbiome remains unanswered. The presence of *Campylobacter* has been noted to prompt an increase in *Bifidobacterium* and modify abundances of *Clostridia* and *Mollicutes* (Thibodeau et al., 2015). The identification of a number of hydrogenases within the ceca may lead to a potential hydrogen sink and provide an explanation as to the high abundance of genera such as *Campylobacter* (Sergeant et al., 2014). Comparison of broilers not exposed and exposed to *C. jejuni* at day 6 or day 20 revealed reductions in the relative abundance of operational taxonomic units (OTUs). These were within the taxonomic families *Lactobacillaceae* and the *Clostridium* cluster XIVa, with specific members of the *Lachnospiraceae* and *Ruminococcaceae* families exhibiting transient shifts in microbial community populations dependent upon the age at which the birds become colonized by *C. jejuni* (Connerton et al., 2018). These studies have enhanced our understanding of the chicken cecal microbiome, however the lack of day to day microbiome data, suitable replicate numbers, relevant metadata, and lack of natural infectivity studies have not allowed us to fully appreciate what is occurring in a natural habitat in relation to how and when *Campylobacter* appears within the chicken gut. To answer these questions, in this study we have performed a comprehensive analysis of the chicken cecal microbiome from days 3 to 35, with 12 replicates per day (final $n = 379$), correlating additional metadata such as chicken weight and feed conversion rates with *Campylobacter* detection in a natural environmental setting.

MATERIALS AND METHODS

Ethics Statement

Approval to conduct the experiments were granted by Agri-Food and Biosciences Institute (AFBI) Establishment License 5002 for AFBI Veterinary Science Division. Euthanasia of birds were

carried out by methods laid out in Animal Scientific Protection Act (ASPA) schedule 1.

Experimental Design, Broilers and Sample Collection

This study was performed using a total of 396 Ross-308 male broiler chickens provided by Moy Park (39 Seagoe Industrial Estate, Portadown, Craigavon, Co. Armagh, BT63 5QE, UK). The birds were divided into 12 pens; each pen contained 33 chickens (**Supplementary Figure 1**). Birds were raised on three phase diets from day 0 to day 35. Starter diets were offered to the birds from days 0 to 10, grower diets from days 11 to 25 and finisher diets from days 26 to 35. Every 24 h, a single chicken from each of the 12 pens was removed at random, and euthanized according to ASPA schedule 1 guidelines. Briefly, birds under 250 g were euthanized by dislocation of the neck whereas those over 250 g and up to 1 kg were euthanized by dislocation of the neck following anesthesia using isoflurane. Birds over 1 kg were euthanized by an overdose of anesthetic (isoflurane) followed by dislocation of the neck. Anesthesia was carried out using an anesthetic mask fitted over the bird's head to deliver the vapourised isoflurane with oxygen with death confirmed in all birds by the onset of rigor mortis. Following this, genomic DNA (gDNA) was extracted from the chicken cecum. Out of 396 samples, a total of 17 were removed from the final analysis due to poor gDNA quality giving a final $n = 379$.

Poultry Growth and Performance Measurements

The performance parameters investigated were mean body weight (BW_mean), body weight gain (Gain), feed intake (FI) and feed conversion ratio (FCR). Measurements were taken at time points 3–7 days, 8–14 days, 15–24 days, and 25–35 days. These variables were then correlated with the microbial community's composition in various statistical analyses.

DNA Extraction, 16S rRNA Amplification and Sequencing

Cecal gDNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions and stored at −20°C. 16S metagenomic sequencing library construction was performed using Illumina guidelines (Illumina, U.S.A). The 16S ribosomal primers used were V3 (tcgtcggcagcgtcagatgtgtataagag acagcctacgggnggcwgcag) and V4 (gtctcgtgggctcggagatgtgtataaga gacaggactachvgggtatctaatcc) (Klindworth et al., 2013; D'Amore et al., 2016). A second PCR step was performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit. Sequencing was performed on the Illumina MiSeq at LSHTM using a v3 300 bp paired-end kit.

Bioinformatics

Abundance tables were obtained by constructing OTUs (a proxy for species) as follows. Paired-end reads were trimmed and filtered using Sickle v1.200 (Joshi and Fass, 2011) by applying a sliding window approach and trimming regions where the average base quality drops below 20. Following this we applied a 10 bp length threshold to discard reads that fall below this length. We then used BayesHammer (Nikolenko et al., 2013) from the Spades v2.5.0 assembler to error correct the paired-end reads followed by pandaseq (v2.4) with a minimum overlap of 20 bp to assemble the forward and reverse reads into a single sequence spanning the entire V3-V4 region. The above choice of software was as a result of author's recent work (Schirmer et al., 2015; D'Amore et al., 2016) where it was shown that the above strategy reduces the substitution rates (main form of error) significantly. After having obtained the consensus sequences from each sample, we used the VSEARCH (v2.3.4) pipeline (all these steps are documented in https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline) for OTU construction. The approach is as follows: we pool the reads from different samples together and add barcodes to keep an account of the samples these reads originate from. We then dereplicate the reads and sort them by decreasing abundance and discard singletons. In the next step, the reads are clustered based on 97% similarity, followed by removing clusters that have chimeric models built from more abundant reads (–uchime_denovo option in vsearch). A few chimeras may be missed, especially if they have parents that are absent from the reads or are present with very low abundance. Therefore, in the next step, we use a reference-based chimera filtering step (–uchime_ref option in vsearch) using a gold database (https://www.mothur.org/w/images/f/f1/Silva.gold.bacteria.zip). The original barcoded reads were matched against clean OTUs with 97% similarity (a proxy for species level separation) to generate OTU table (a total of 18,588 unique sequences) for $n = 379$ samples.

The representative OTUs were then taxonomically classified against the SILVA SSU Ref NR database release v123 database with assign_taxonomy.py script from the Qiime (Caporaso et al., 2010) workflow. To find the phylogenetic distances between OTUs, we first multisequence aligned the OTUs against each other using Kalign v2.0.4 (Lassmann and Sonnhammer, 2005) (using the options -gpo 11 -gpe 0.85) and then used FastTree v2.1.7 (Price et al., 2010) to generate the phylogenetic tree in NEWICK format. Finally make_otu_table.py from Qiime workflow was employed to combine abundance table with taxonomy information to generate biome file for OTUs. Tax4Fun (Aßhauer et al., 2015) was used to predict the functional capabilities of microbial communities based on 16S rRNA datasets (all prokaryotic KEGG organisms are available in Tax4Fun for SILVA v123 and KEGG database release 64.0) and then utilizing ultrafast protein classification (UProC) tool (Meinicke, 2015) to generate metabolic functional profiles after normalizing the data for 16S rRNA gene copy numbers. In Tax4Fun, we used MoP-Pro approach (Asshauer and Meinicke, 2013) to give pre-computed 274 KEGG Pathway reference profiles. Although Tax4Fun based metabolic prediction is constrained by the taxa available in the reference database, it gives a statistic called fraction-of-taxonomic-units-unexplained (FTU) which reflects the amount of sequences assigned to a taxonomic unit and not transferable to KEGG reference organisms. This can be used as a measure of confidence in

trusting the predictions. Summary statistics of FTUs returned in this study are as follows: 1st Quantile:0.09129; Median:0.13995; Mean:0.14902; and 3rd Quantile:0.19800 (**Figure 1H**). Thus, on average metabolic profiles of ∼86% of the taxa were present and therefore with this high representation, we used the pathways in the statistical analysis.

Statistical Analysis

Statistical analyses were performed in R using the tables and data generated as above as well as the meta data associated with the study. For community analysis (including alpha and beta diversity analyses) we used the vegan package (Oksanen et al., 2015). For alpha diversity measures, we calculated: *Richness*, estimated number of species/features per sample; and *Shannon* entropy: a commonly used index to measure the balance of a community within a sample. Exponentiating Shannon entropy gives the richness profile. These alpha diversity measures are calculated after rarefying the abundance table to minimum library size, as is the norm. To calculate Unifrac distances (that account for phylogenetic closeness), we used the phyloseq (McMurdie and Holmes, 2013) package. Nonmetric Distance Scaling (NMDS) plot of community data (OTUs) used different distance measures (Vegan's metamds() function): *Bray-Curtis*, considers the species abundance count; *Unweighted Unifrac,* considers the phylogenetic distance between the branch lengths of OTUs observed in different samples without taking into account the abundances; and *Weighted Unifrac,* unweighted unifrac distance weighted by the abundances of OTUs. The samples are grouped for different treatments as well as the mean ordination value and spread of points (ellipses were drawn using Vegan's ordiellipse() function that represent the 95% confidence interval of the standard errors of the groups).

To understand multivariate homogeneity of groups dispersion (variances) between multiple conditions, we used Vegan's betadisper() function in which the distances between objects and group centroids are handled by reducing the original distances (BrayCurtis, Unweighted Unifrac, or Weighted Unifrac) to principal coordinates and then performing ANOVA on them. We used Vegan's adonis() for analysis of variance using distance matrices (BrayCurtis/Unweighted Unifrac/Weighted Unifrac) i.e., partitioning distance matrices among sources of variation (Grouping type i.e., weeks, body weight, feed intake, feed conversion ratio etc.). This function, henceforth referred to as PERMANOVA, fits linear models to distance matrices and uses a permutation test with pseudo-F ratios.

To find OTUs that are significantly different between multiple conditions (days/weeks), we used DESeqDataSetFromMatrix() function from DESeq2 (Love et al., 2014) package with the adjusted *p*-value significance cut-off of 0.05 and log2 fold change cut-off of 2. This function uses negative binomial GLM to obtain maximum likelihood estimates for OTUs log fold change between two conditions. Then Bayesian shrinkage is applied to obtain shrunken log fold changes subsequently employing the Wald test for obtaining significances. To find KEGG pathways significantly up/down-regulated between multiple conditions (days/weeks), the Kruskal-Wallis test was used with *p*-values adjusted for multiple comparisons using the fdrtool package (Klaus and Strimmer, 2013, 2015).

We performed Local Contribution to Beta Diversity (LCBD) analysis (Legendre and De Cáceres, 2013) by using LCBD.comp() from adespatial package (Dray et al., 2018). We used the Hellinger distance (abundances), unweighted (phylogenetic distance) and weighted Unifrac (phylogenetic distance weighted by abundance) dissimilarities. LCBD gives the sample-wise local contributions to beta diversity that could be derived as a proportion of the total beta diversity. In the context of this longitudinal study, it provides a mean to show how markedly different the microbial community structure of a single sample is from the average (with higher LCBD values representing outliers), and also provides a mean to show when the community structure has stabilized in a temporal setting.

To characterize the phylogenetic community composition within each sample whether the microbial community structure is stochastic (driven by competition among taxa) or deterministic (driven by strong environmental pressure i.e. host environment), we quantified: mean-nearest-taxon-distance (MNTD) and the nearest-taxon-index (NTI) using mntd(), and ses.mntd(); and mean-phylogenetic-diversity (MPD) and nearest-relative-index (NRI) using mpd() and ses.mpd() function from the picante (Kembel et al., 2010) package. NTI and NRI represent the negative of the output from ses.mntd() and ses.mpd(), respectively. They also quantify the number of standard deviations that the observed MNTD/MPD is from the mean of the null distribution (999 randomization by using null.model = "richness" in the ses.mntd() and ses.mpd() functions and only considering the taxa as present/absent without taking their abundances). We used the top 1,000 most abundant OTUs for calculation of these measures based on the recommendations given in (Stegen et al., 2012).

We used the "BVSTEP" routine (Clarke and Ainsworth, 1993), an algorithm that searches for highest correlation (Mantel test) between dissimilarities of a fixed and variable multivariate datasets using bvStep() from sinkr package (Taylor, 2014) by permuting through 2^n-1 possible combinations of features in the variable dataset. Testing all feature combinations is unrealistic and computationally intractable when the feature space is high (18,588 OTUs in our case). Thus, we used the abundance table with 1000 most abundant OTUs (with the premise that the most abundant species that may have a significant role to play) to best correlate with the overall similarities given all the OTUs (18,588 in our case). This analysis is complimentary to the differential analysis and identified the OTUs that were causing the major shifts in beta diversity.

The phylogenetic tree and annotations summarizing the findings of this study were drawn using Evolview (http://www.evolgenius.info/evolview/).

We considered analyses on two different groupings of the sample data, comparison of microbial profiles on a daily basis to reveal temporal patterns, and on a weekly basis (4 weeks), primarily because the poultry growth and performance parameters were recorded on a weekly basis. The statistical scripts and workflows for all above can be found at http://userweb.eng.gla.ac.uk/umer.ijaz#bioinformatics.

RESULTS

Daily Diversity Patterns Converge to a Stable Community as We Go Forward in Time

Although alpha diversity (Shannon) on microbial counts (**Figure 1A**) shows a rapid increase over the first ten days, it follows a plateauing effect where the microbiome normalizes at approximately day 12. This is in line with previous reports whereby the gastrointestinal (GI) tract of poultry comes into contact with exogenous microorganisms immediately after hatch and as the host grows, this microbiome becomes highly diverse until it reaches a relatively stable yet dynamic state (Pan and Yu, 2014). The same temporal phenomenon can be observed when considering local contributions to beta diversity based on abundance count (Hellinger distance; **Figure 1B**). When considering phylogenetic distances only (Unweighted Unifrac; **Figure 1C**), although the decrease in beta diversity contributions is marginally slower than the abundance counts counterpart, there is a sudden increase around day 20. Using both abundances and phylogenetic distances this seems to disappear (Weighted Unifrac; **Figure 1D**). It should be noted that a higher LCBD value suggests the diversity patterns of a sample is markedly different from the rest of the samples in an average sense. In contrast, the level of microbial diversity between the different pens was relatively stable (results not significant and thus not shown) suggesting less or no variability amongst pens. *Campylobacter* was detected in three chickens from the 12 pens at day 16 (**Figure 1A**). This is in line with previous reports where natural colonization of chickens has been reported at approximately day 14 of the chicken life cycle (Neill et al., 1984; Hermans et al., 2011; Kalupahana et al., 2013; Thibodeau et al., 2015). *Campylobacter* was also identified in one of the chickens at day 3 and previously it has also been reported that chickens between 0 and 3 days of age can become infected with *Campylobacter* (Cawthraw et al., 1996).

Window of Opportunity for *Campylobacter* Between Day 12 and Day 20

Next, we explored ecological drivers of microbial community to determine whether there is any environmental pressure (host environment) responsible for assemblage of microbial community or if it is driven purely by competition. Using NTI and NRI (**Figures 1E,F**), one can observe a step function response around day 12. For a single community, NTI/NRI greater than $+2$ indicates strong phylogenetic clustering (driven by environmental filtering) and less than -2 indicates phylogenetic overdispersion (environment has little or no role to play). Since chicken ceca are already a constrained environment to begin with (as opposed to real environmental datasets), the lower bound of -2 may not be feasible and hence the values should be taken relatively with an increasing value implying increasing host environmental pressure. It should be noted that NRI reflects the phylogenetic clustering in a broad sense (whole phylogenetic tree) with the negative values representing evenly spread community. On the other hand, NTI focuses more on the tips of the tree with positive values of NTI indicating that species co-occur with more closely related species than expected, and negative values indicating that closely related species do not co-occur. We have chosen presence/absence of species while calculating these measures without taking into account the abundances as they mask the phenomenon similar to LCBD profiles (**Figures 1C,D**). When we consider differential analysis of OTUs (**Supplementary Table 1**), we can notice that between days 9 and 11 there is a high proportion of OTUs that were log2 fold different. After day 20, we also observe the same between days 26 and 28 with the changes in phylogenetic structure responsible for peaks in NTI/NRI. Interestingly, chickens were raised on three phase diets; starter diets (days 0–10), grower diets (days 11–25) and finisher diets (days 26–35). The high proportion of OTUs that were log2 fold different between days 26 and 28 may be attributed to the change in feed from grower to finisher feed. Since the NTI/NRI are already significantly higher than 2, we do not consider this as an upper bound and revert back to day 20 as an upper bound for the window. Based on beta dispersion analysis (**Table 1**), we observe days 11–13 and days 19–21 when the dispersions of the microbial communities are changing significantly. The alteration in the chicken feed from starter diet (days 0–10) to grower diets (days 11–25) may also play a role in the significant beta dispersion between days 11 and 13, although the feed change does not seem a likely explanation for days 19–21. For completeness we also generated differential analysis of genus level where *Campylobacter* was identified as being significantly down-regulated between day 16 and day 17 (**Supplementary Table 2**).

If we consider the richness of metabolic pathways (**Figure 1G**), we notice that they achieve stability before the microbial community at around day 6 with no obvious patterns to suggest anything apparent between day 12 and day 20 other than a marginal decrease to day 16 and increasing again onwards. However, if we consider the differential expression analysis of pathways (**Supplementary Table 3**), we can notice a large proportion of these pathways changing between day 14 and 15, a day before *Campylobacter* was first observed. We identified a reduction in lysine degradation (ko00310) from day 14 to day 15, and an increase in D-Alanine metabolism (ko00473) from day 14 to day 15. *C. jejuni* typically cannot utilize sugars as a carbon source as it lacks the glycolytic enzyme phosphofructokinase and so depends on the availability of free amino and keto acids scavenged from the host or from the intestinal microbiome (Parkhill et al., 2000; Velayudhan and Kelly, 2002; Lee and Newell, 2006). *C. jejuni* utilizes serine, aspartate, glutamate and proline preferentially as nutritional substrates *in vitro* with serine catabolism required for colonization of the intestinal tract (Elharrif and Mégraud, 1986; Leach et al., 1997; Hendrixson and DiRita, 2004; Velayudhan et al., 2004). Amino acids can also potentially be deaminated to a small number of intermediates that can directly feed into the central metabolism, including pyruvate (from serine and alanine), oxaloacetate (from aspartate), and 2-oxoglutarate (from glutamate) (Velayudhan et al., 2004). The variation of such metabolic pathways may give an indication as to the appearance of *Campylobacter* at this time point. We also identified a reduction from days 14 to day

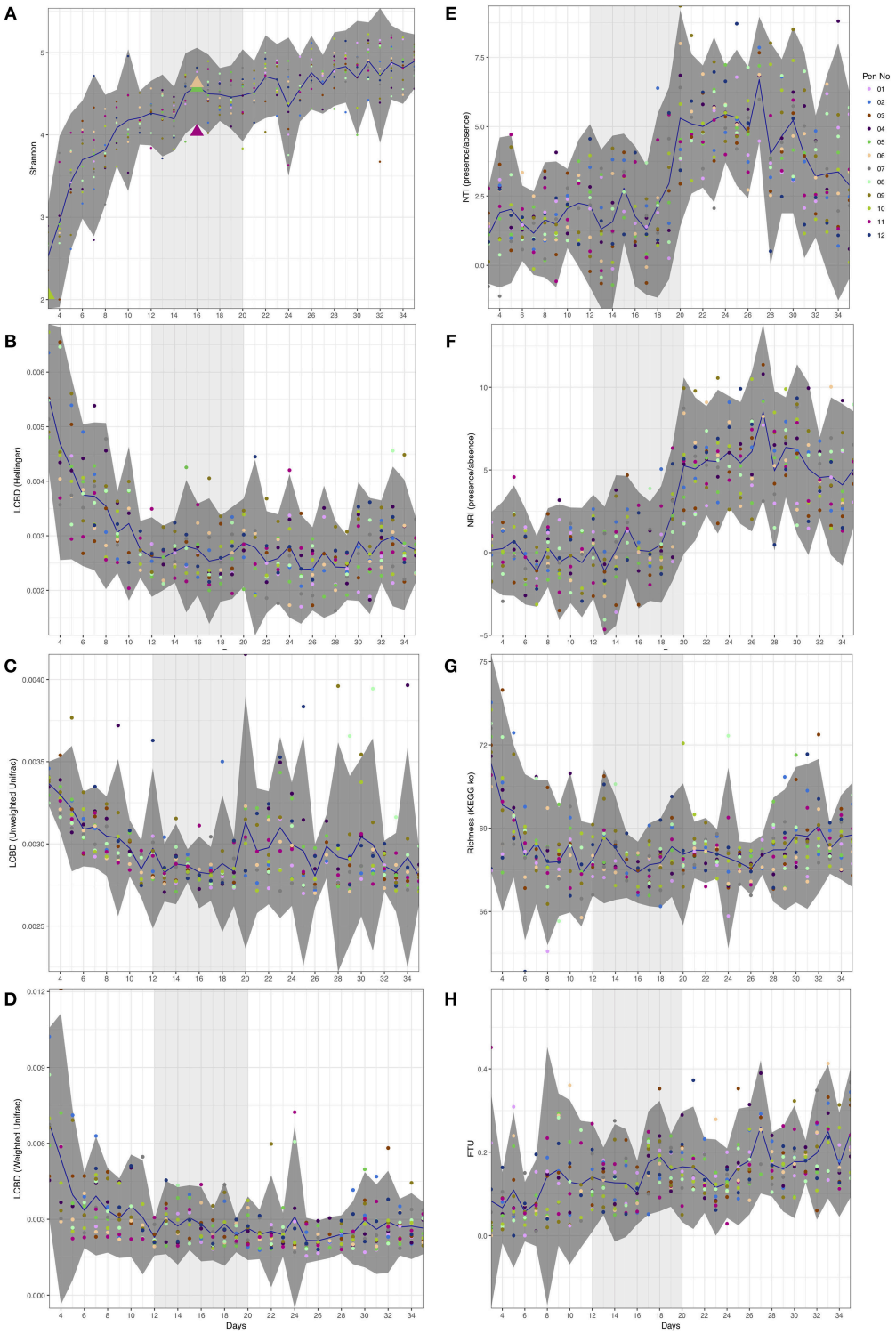

FIGURE 1 | Day-wise statistical measures calculated on the microbiome data. **(A)** Shannon entropy with first appearance of *Campylobacter* (≥5 sequences) highlighted as triangles. **(B–D)** Local contribution to beta diversity (LCBD) calculated by using Hellinger transform on the microbial counts, Unweighted Unifrac dissimilarity (phylogenetic distances only), and Weighted Unifrac dissimilarity (phylogenetic distances weighted with abundance counts) respectively **(E,F)** Nearest-Taxon-Index (NTI) and nearest-relative-index (NRI) considering presence/absence of OTUs in samples **(G)** Richness calculated as exponentiation of Shannon entropy on the proportional representation of KEGG pathways on samples, and **(H)** fraction-of-taxonomic-units-unexplained (FTU) calculated on each sample. In all subfigures, the mean value is represented by solid blue line with 95% confidence interval of standard deviation given as dark shaded region around the mean. The samples are colored with respect to the pens they originate from. Based on the analysis given in this study, we have identified days 12–20 of importance and are thus highlighted as lighter shaded regions.

TABLE 1 | Statistics for beta dispersion comparison on daily microbiome data.

Day comparisons	Bray-curtis	Unweighted unifrac	Weighted unifrac
3–4	$p = 0.018071$ (*)	$p = 0.18436$	$p = 0.085112$
4–5	$p = 0.85255$	$p = 0.18547$	$p = 0.25546$
5–6	$p = 0.60961$	$p = 0.1225$	$p = 0.73468$
6–7	$p = 0.82972$	$p = 0.94104$	$p = 0.21369$
7–8	$p = 0.71257$	$p = 0.88392$	$p = 0.47401$
8–9	$p = 0.060007$	$p = 0.94453$	$p = 0.36231$
9–10	$p = 0.9966$	$p = 0.11357$	$p = 0.53314$
10–11	$p = 0.20247$	$p = 0.20845$	$p = 0.13289$
11–12	$p = 0.38794$	$p = 0.014818$ (*)	$p = 0.62198$
12–13	$p = 0.88847$	$p = 0.064143$	$p = 0.013623$ (*)
13–14	$p = 0.63766$	$p = 0.16696$	$p = 0.41304$
14–15	$p = 0.9467$	$p = 0.64383$	$p = 0.46855$
15–16	$p = 0.89972$	$p = 0.055618$	$p = 0.79989$
16–17	$p = 0.59807$	$p = 0.37379$	$p = 0.41167$
17–18	$p = 0.70773$	$p = 0.66013$	$p = 0.30413$
18–19	$p = 0.40112$	$p = 0.92525$	$p = 0.5994$
19–20	$p = 0.020548$ (*)	$p = 0.087076$	$p = 0.56858$
20–21	$p = 0.033097$ (*)	$p = 0.12251$	$p = 0.52086$
21–22	$p = 0.29506$	$p = 0.055585$	$p = 0.90226$
22–23	$p = 0.24688$	$p = 0.90221$	$p = 0.99695$
23–24	$p = 0.79886$	$p = 0.71275$	$p = 0.34913$
24–25	$p = 0.21019$	$p = 0.67687$	$p = 0.11096$
25–26	$p = 0.14334$	$p = 0.20716$	$p = 0.97116$
26–27	$p = 0.96286$	$p = 0.044866$ (*)	$p = 0.80425$
27–28	$p = 0.50377$	$p = 0.096107$	$p = 0.1382$
28–29	$p = 0.91052$	$p = 0.87339$	$p = 0.69398$
29–30	$p = 0.34265$	$p = 0.60245$	$p = 0.11773$
30–31	$p = 0.61843$	$p = 0.55324$	$p = 0.20403$
31–32	$p = 0.24674$	$p = 0.082761$	$p = 0.50328$
32–33	$p = 0.73392$	$p = 0.53114$	$p = 0.62586$
33–34	$p = 0.7431$	$p = 0.36694$	$p = 0.57642$
34–35	$p = 0.16111$	$p = 0.20181$	$p = 0.77382$

Asterisks denote a statistically significant difference ($p < 0.05$).*

15 of a number of pathways relating to specific bacteria; *Vibrio cholerae* pathogenic cycle (ko05111; Biofilm formation - *Vibrio cholerae*), *Escherichia coli* (ko05130; Pathogenic *Escherichia coli* infection), *Salmonella* species (ko05132; *Salmonella* infection). In addition, we identified a reduction from day 14 to 15 of Bacterial secretion systems (ko03070). Future studies are needed to elucidate and confirm the predicted pathways. In view of these findings, *Camplyobacter* appears at day 16 within this window of opportunity (**Figure 1**) where there exists a shift from competitive to environmental drivers of microbial community, with day 16 lying immediately after the most substantial changes in metabolic profiles observed over the whole period.

Analysis of Dominant Bacterial Taxa Over Time

Analysis of the 50 most abundant genera (**Supplementary Figure 2**) have identified trends that were reported previously in the literature i.e., chicken microbiome contains *Enterobacteriaceae* at early days of development, and that *Firmicutes* increase in abundance and taxonomic diversity over time (Danzeisen et al., 2011; Ballou et al., 2016; Mancabelli et al., 2016). *Escherichia.Shigella* (Phylum *Proteobacteria*; Family *Enterobacteriaceae*) was identified as being highly abundant at day 3 and showed a general reduction up to approximately day 7. *Escherichia.Shigella* was also noted to be present after day 28. This pattern was observed for *Eisenbergiella* (Phylum *Firmicutes*; Family *Lachnospiraceae*) which displayed a decrease from early time points, but remained present throughout. This pattern was also observed for *Ruminiclostridium* (Phylum *Firmicutes*; Family *Ruminococcaceae*) which however was not in the abundant genera after day 23. *Flavonifractor* (Phylum *Firmicutes*; Family -) was identified consistently at early time points, but was rarely abundant after day 19. *Enterobacter* (Phylum *Proteobacteria*; Family *Enterobacteriaceae)* was only observed at days 3 and 4 and was not abundant at any other time points. Here we identified that *Ruminiclostridium.5* and *Ruminiclostridium.9* (Phylum *Firmicutes*; Family *Ruminococcaceae*) which were consistently present throughout at a relatively significant level of abundance. This was also the case for *Anaerotruncus* (Phylum *Firmicutes*; Family *Clostridiaceae*), but at a lower level of abundance, especially before day 7. *Faecalibacterium* (Phylum *Firmicutes*; Family *Clostridiaceae*) was rarely abundant at early time points, however was observed consistently at a relative high abundance after day 14. *Lachnoclostridium* (Phylum *Firmicutes*; Family *Lachnospiraceae*) was found to be present throughout with a relatively high level of fluctuation. Certain genera such as *Ruminococcaceae.UCG.005* and *Ruminococcaceae.UCG.014* (Phylum *Firmicutes*; Family *Ruminococcaceae*) were not abundant at high levels at early time points however increased significantly at approximately days 16-19. Finally, *Megamonas* (Phylum *Firmicutes*; Family *Veillonellaceae*) and *Intestinimonas* (Phylum *Firmicutes*; Family -) were not abundant throughout most time points, before appearing post day 22-25 onwards.

Weekly Microbial Profiles and Analysis of Poultry Performance Metadata

The metadata collected here included Bird Weight (BW_Mean; grams), Body Weight Gain (Gain; g/bird), Feed Intake (FI), Feed Conversion Ratio (FCR), and was recorded on a weekly basis where we have considered grouping the microbiome samples accordingly; days 03–07 (week 1), days 08–14 (week 2), days 15–24 (week 3), and days 25–35 (week 4). As is the case with the daily microbiome profile, alpha diversity (rarefied richness and Shannon; **Figure 2A**) increases over time, however, due to the nature of this grouping, we lose the plateauing effect over time. In accordance with daily analysis, we can see a major shift in the parameters as we transition from days 08–14 to days 15–24 (**Figure 2B**). FCR in particular increases substantially in this period remaining stable for week 4 (days 25–35). Gain is also significantly elevated in this transition period (days 08–14 to days 15–24) when compared to other periods. In terms of beta diversity (**Figure 2C**), we observe the samples more sparsely spread in the first week (days 03–07)

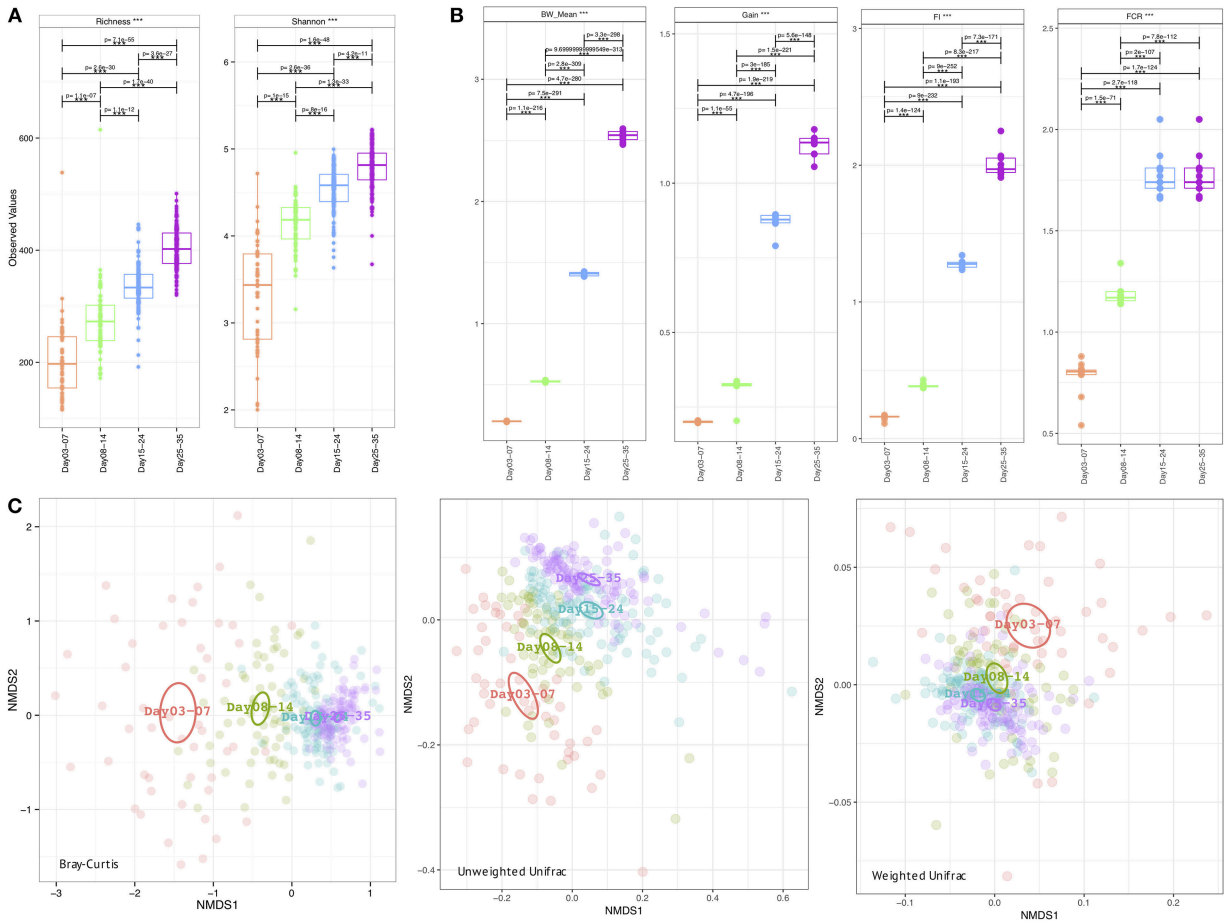

FIGURE 2 | Week-wise measures calculated on the microbiome data **(A)** Alpha diversity measures: richness (after rarefying the samples to minimum library size) and Shannon entropy **(B)** Extrinsic parameters calculated on weekly basis were mean body weight (BW_mean), body weight gain (Gain), feed intake (FI), feed conversion ratio (FCR), and **(C)** Beta diversity measures using Bray-Curtis (counts), Unweighted Unifrac (phylogenetic distance), and Weighted Unifrac (phylogenetic distance weighted by abundance counts). In **(A,B)** we have performed pair-wise ANOVA and where significant the pairs were connected with *p*-values drawn on top. In **(C)** the ellipses represent the 95% confidence interval of the standard error of the ordination points of a given grouping with labels drawn at the center (mean) of the ordination points.

TABLE 2 | Statistics for pairwise beta dispersion and PERMANOVA when using different dissimilarity measures on weekly microbiome data.

Beta dispersion		Bray-curtis	Unweighted unifrac	Weighted unifrac
Day 03–07	Day08–14	$p = 0.0061142$ (**)	$p = 0.00014712$ (***)	$p = 9.6914e{-}05$ (***)
	Day15–24	n.s.	$p = 0.010418$ (*)	$p = 2.5203e{-}09$ (***)
	Day25–35	$p = 0.042066$ (*)	$p = 0.00015112$ (***)	$p = 3.5789e{-}12$ (***)
Day08–14	Day15–24	$p = 0.00077017$ (***)	n.s.	$p = 0.019953$ (*)
	Day25–35	n.s.	n.s.	$p = 0.0011717$ (**)
Day15–24	Day25–35	$p = 0.0075651$ (**)	$p = 0.020128$ *	n.s.
PERMANOVA				
Groups		$R^2 = 0.16763$ ($p = 0.001$) (***)	$R^2 = 0.06048$ ($p = 0.001$) (***)	$R^2 = 0.17577$ ($p = 0.001$) (***)
BW_Mean		$R^2 = 0.11721$ ($p = 0.001$) (***)	$R^2 = 0.03964$ ($p = 0.001$) (***)	$R^2 = 0.08723$ ($p = 0.001$) (***)
FI		$R^2 = 0.11856$ ($p = 0.001$) (***)	$R^2 = 0.04069$ ($p = 0.001$) (***)	$R^2 = 0.09301$ ($p = 0.001$) (***)
FCR		$R^2 = 0.1086$ ($p = 0.001$) (***)	$R^2 = 0.03842$ ($p = 0.001$) (***)	$R^2 = 0.11787$ ($p = 0.001$) (***)
Gain		$R^2 = 0.11886$ ($p = 0.001$) (***)	$R^2 = 0.04146$ ($p = 0.001$) (***)	$R^2 = 0.0998$ ($p = 0.001$) (***)

Asterisks denote a statistically significant difference ($p < 0.05$, **$p < 0.01$, ***$p < 0.001$).*
In beta dispersion analysis, the pair-wise differences in distances from group center/mean were subjected to ANOVA after performing Principle Coordinate Analysis, and if significant ($p \leq 0.05$) the values are shown. In PERMANOVA analysis, R^2 represents the proportion of variability explained, for example, using "Groups" and "Bray-Curtis" dissimilarity, the weeks explain 16.8% variability in microbial community structure.

TABLE 3 | Subset analysis from BVSTEP routine listing top 18 subsets with highest correlation with the full OTU table considering Bray-Curtis distance done on weekly basis.

	Subsets of top 1000 most abundant OTUs	Correlation with full OTU table (R)	PERMANOVA (full OTU table)				
			Groups	BW_Mean	FI	FCR	Gain
			R^2 = 0.16763 (p = 0.001) (***)	**R^2 = 0.11721 (p = 0.001) (***)**	**R^2 = 0.11856 (p = 0.001) (***)**	**R^2 = 0.1086 (p = 0.001) (***)**	**R^2 = 0.11886 (p = 0.001) (***)**
			PERMANOVA (subsets)				
			Groups	BW_Mean	FI	FCR	Gain
S1	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_157 + OTU_15 + OTU_24 + OTU_3028 + OTU_2496 + OTU_1024 + OTU_10 + OTU_3 + OTU_2555	0.833	R^2 = 0.14768 (p = 0.001) (***)	R^2 = 0.10732 (p = 0.001) (***)	R^2 = 0.10784 (p = 0.001) (***)	R^2 = 0.10117 (p = 0.001) (***)	R^2 = 0.11143 (p = 0.001) (***)
S2	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_157 + OTU_15 + OTU_24 + OTU_3028 + OTU_2496 + OTU_1024 + OTU_3 + OTU_2555	0.83	R^2 = 0.13977 (p = 0.001) (***)	R^2 = 0.10028 (p = 0.001) (***)	R^2 = 0.09987 (p = 0.001) (***)	R^2 = 0.09203 (p = 0.001) (***)	R^2 = 0.1025 (p = 0.001) (***)
S3	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_157 + OTU_15 + OTU_24 + OTU_3028 + OTU_2496 + OTU_1024 + OTU_3	0.827	R^2 = 0.14186 (p = 0.001) (***)	R^2 = 0.10205 (p = 0.001) (***)	R^2 = 0.10165 (p = 0.001) (***)	R^2 = 0.09344 (p = 0.001) (***)	R^2 = 0.10435 (p = 0.001) (***)
S4	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_157 + OTU_15 + OTU_24 + OTU_2496 + OTU_1024 + OTU_3	0.823	R^2 = 0.14241 (p = 0.001) (***)	R^2 = 0.10262 (p = 0.001) (***)	R^2 = 0.10228 (p = 0.001) (***)	R^2 = 0.0939 (p = 0.001) (***)	R^2 = 0.10509 (p = 0.001) (***)
S5	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_15 + OTU_24 + OTU_2496 + OTU_1024 + OTU_3	0.816	R^2 = 0.14289 (p = 0.001) (***)	R^2 = 0.10313 (p = 0.001) (***)	R^2 = 0.10279 (p = 0.001) (***)	R^2 = 0.09436 (p = 0.001) (***)	R^2 = 0.1056 (p = 0.001) (***)
S6	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_23 + OTU_2474 + OTU_6 + OTU_28 + OTU_15 + OTU_24 + OTU_2496 + OTU_1024	0.809	R^2 = 0.14742 (p = 0.001) (***)	R^2 = 0.10587 (p = 0.001) (***)	R^2 = 0.10556 (p = 0.001) (***)	R^2 = 0.098 (p = 0.001) (***)	R^2 = 0.1084 (p = 0.001) (***)
S7	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_6 + OTU_28 + OTU_15 + OTU_24 + OTU_2496 + OTU_1024	0.799	R^2 = 0.17779 (p = 0.001) (***)	R^2 = 0.12583 (p = 0.001) (***)	R^2 = 0.12598 (p = 0.001) (***)	R^2 = 0.11968 (p = 0.001) (***)	R^2 = 0.12936 (p = 0.001) (***)
S8	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_6 + OTU_28 + OTU_15 + OTU_24 + OTU_2496	0.789	R^2 = 0.14605 (p = 0.001) (***)	R^2 = 0.0875 (p = 0.001) (***)	R^2 = 0.08646 (p = 0.001) (***)	R^2 = 0.08927 (p = 0.001) (***)	R^2 = 0.08866 (p = 0.001) (***)

(Continued)

TABLE 3 | Continued

Subsets of top 1000 most abundant OTUs		Correlation with full OTU table (R)	PERMANOVA (full OTU table)				
			Groups	**BW_Mean**	**FI**	**FCR**	**Gain**
			R^2 = 0.16763 (p = 0.001) (***)	**R^2 = 0.11721 (p = 0.001) (***)**	**R^2 = 0.11856 (p = 0.001) (***)**	**R^2 = 0.1086 (p = 0.001) (***)**	**R^2 = 0.11886 (p = 0.001) (***)**
			PERMANOVA (subsets)				
			Groups	**BW_Mean**	**FI**	**FCR**	**Gain**
S9	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_28 + OTU_15 + OTU_24 + OTU_2496	0.777	R^2 = 0.14132 (p = 0.001) (***)	R^2 = 0.10216 (p = 0.001) (***)	R^2 = 0.10313 (p = 0.001) (***)	R^2 = 0.0989 (p = 0.001) (***)	R^2 = 0.10736 (p = 0.001) (***)
S10	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_28 + OTU_15 + OTU_24	0.763	R^2 = 0.13922 (p = 0.001) (***)	R^2 = 0.10051 (p = 0.001) (***)	R^2 = 0.10121 (p = 0.001) (***)	R^2 = 0.09676 (p = 0.001) (***)	R^2 = 0.10532 (p = 0.001) (***)
S11	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_28 + OTU_15	0.746	R^2 = 0.13173 (p = 0.001) (***)	R^2 = 0.09081 (p = 0.001) (***)	R^2 = 0.09291 (p = 0.001) (***)	R^2 = 0.09023 (p = 0.001) (***)	R^2 = 0.09828 (p = 0.001) (***)
S12	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474 + OTU_28	0.723	R^2 = 0.09574 (p = 0.001) (***)	R^2 = 0.04819 (p = 0.001) (***)	R^2 = 0.04936 (p = 0.001) (***)	R^2 = 0.05663 (p = 0.001) (***)	R^2 = 0.05163 (p = 0.001) (***)
S13	OTU_2165 + OTU_2448 + OTU_33 + OTU_1121 + OTU_2474	0.696	R^2 = 0.0952 (p = 0.001) (***)	R^2 = 0.04875 (p = 0.001) (***)	R^2 = 0.05019 (p = 0.001) (***)	R^2 = 0.05606 (p = 0.001) (***)	R^2 = 0.05246 (p = 0.001) (***)
S14	OTU_2165 + OTU_2448 + OTU_1121 + OTU_2474	0.661	R^2 = 0.10232 (p = 0.001) (***)	R^2 = 0.05483 (p = 0.001) (***)	R^2 = 0.05623 (p = 0.001) (***)	R^2 = 0.06108 (p = 0.001) (***)	R^2 = 0.05869 (p = 0.001) (***)
S15	OTU_2448 + OTU_33 + OTU_1121 + OTU_2474	0.655	R^2 = 0.06994 (p = 0.001) (***)	R^2 = 0.02489 (p = 0.001) (***)	R^2 = 0.02416 (p = 0.001) (***)	R^2 = 0.03155 (p = 0.001) (***)	R^2 = 0.02375 (p = 0.001) (***)
S16	OTU_2448 + OTU_33 + OTU_2474	0.604	R^2 = 0.03489 (p = 0.001) (***)	R^2 = 0.01348 (p = 0.003) (**)	R^2 = 0.01238 (p = 0.005) (**)	R^2 = 0.01154 (p = 0.006) (**)	R^2 = 0.01053 (p = 0.012) (*)
S17	OTU_33 + OTU_1121 + OTU_2474	0.599	R^2 = 0.06662 (p = 0.001) (***)	R^2 = 0.01995 (p = 0.001) (***)	R^2 = 0.02047 (p = 0.001) (***)	R^2 = 0.03201 (p = 0.001) (***)	R^2 = 0.02183 (p = 0.001) (***)
S18	OTU_1121 + OTU_2474	0.538	R^2 = 0.07 (p = 0.001) (***)	R^2 = 0.02571 (p = 0.001) (***)	R^2 = 0.02628 (p = 0.001) (***)	R^2 = 0.03796 (p = 0.001) (***)	R^2 = 0.02793 (p = 0.001) (***)

*Asterisks denote a statistically significant difference (***$p < 0.001$).*
For each subset, PERMANOVA was performed against different sources of variations.
OTU_2165,Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae.
OTU_2448:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium.
OTU_33:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium 5.
OTU_1121:Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Eisenbergiella.
OTU_23:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium 9.
OTU_2474:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium 5.
OTU_6:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae.
OTU_28:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae.
OTU_157:Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus.
OTU_15:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae.
OTU_24:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium.
OTU_3028:Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae.
OTU_2496:Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Tyzzerella.
OTU_1024:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Faecalibacterium.
OTU_10:Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae.
OTU_3:Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminiclostridium 5.
OTU_2555:Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae.

as compared to other weeks on abundance (Bray-Curtis) alone. The phylogenetic dispersion (Unweighted Unifrac) on the other hand is more preserved. We can also notice a gradient forming with later weeks more or less close to suggest convergence as we established in the case of daily profiles. Based on beta dispersion analysis (**Table 2**), we can notice that the dispersion in week 1 is significantly different to other weeks with 16, 6, and 17% variability in microbial community explained by

PERMANOVA using counts alone (Bray-Curtis), phylogenetic distance alone (Unweighted Unifrac), and combination of the two (Weighted Unifrac), respectively. With this grouping, main sources of variation are then the distribution of species rather than their phylogenetic relatedness. The metadata explains 10–12% variability (all significant) in terms of counts alone (Bray-Curtis) with 3–6% in terms of phylogeny (Unweighted Unifrac). For the sake of completeness, we also performed differential analysis of OTUs and pathways on a consecutive weekly basis (lower halves of **Supplementary Tables 1**–**3**); however, these should be interpreted with great care as main source of variability are the daily changes and grouping samples on weekly basis will always return more significant OTUs and pathways.

Key Species Representing Majority of the Shift in Community Dynamics

In addition to differential analysis on OTUs (**Supplementary Table 1**) which returned OTUs that were log2 fold different between consecutive days, we also considered the subset analysis where we imploded the abundance table to the minimum set of OTUs, the resulting reduced-order abundance table correlated highly with the full table by

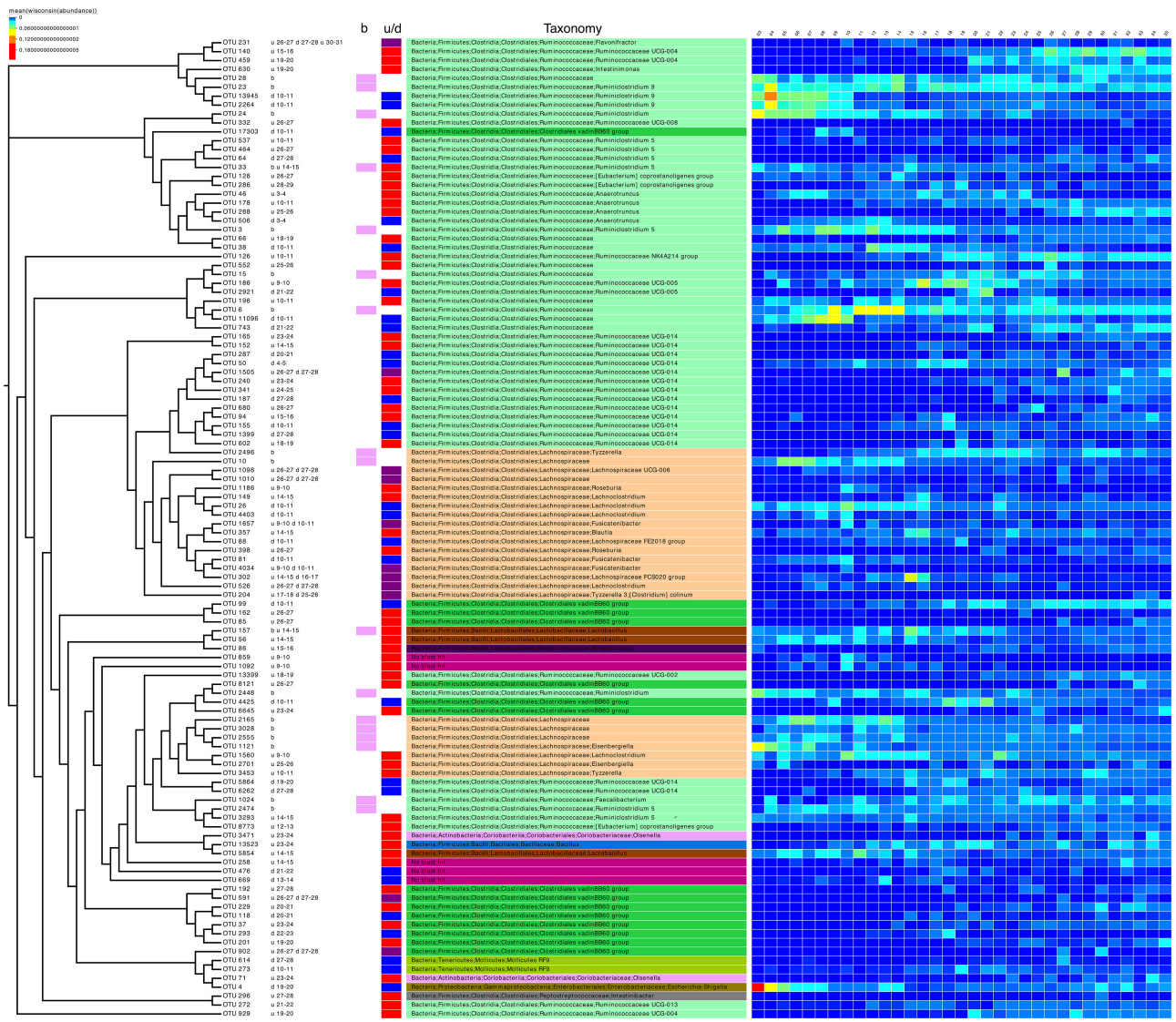

FIGURE 3 | Phylogenetic tree of the subset of OTUs selected as significant on differential analysis (based on **Table 3** and **Supplementary Table 1**). Next to the OTU labels are descriptive text representing where the OTUs were found to be significant, for example, the first entry for OTU 231, "u 26-27 d 27-28 u 30-31," can be read as upregulated going from day 26 to 27 and then from day 30 to 31 and downregulated going from day 27 to 28. "b" represents the OTUs selected in the subset analysis. The next two columns are a pictorial representation of the above-mentioned descriptive text with pink color representing OTUs selected in subset analysis, red color for upregulated OTUs, blue for downregulated OTUs, and purple for OTUs which show the both trends (up/down regulation). The next column shows the taxonomy of the OTUs according to SILVA v123 with coloring at unique family level. The heatmap was drawn by collating the mean values of OTUs for samples from the same day after performing proportional standardization on the full OTU table using wisconsin() function.

preserving the beta diversity between the samples (**Table 3**). To see how much variability is lost, the PERMANOVA with full OTU table (18,588 OTUs) is provided as a reference. The 17 OTUs listed represent only ∼2% (Subset S1 in **Table 3**) loss in variability and thus represent the main OTUs that are driving the community dynamics. In terms of metadata, the loss in variability is ∼1% (Subset S1 in **Table 3**). The subset of the phylogenetic tree of these OTUs, in addition to those selected in the differential analysis (daily comparisons), a total of 110 OTUs were then extracted and annotated in **Figure 3** along with taxonomy information. It can be seen that majority of these (>50%) belong to *Firmicutes* (*Bacillaceae*, *Ruminococcaceae*, *Lachnospiracaeae*, *Lactobacillaceae*, *Peptostreptococcaceae*, and *Clostridiales vadin BB60 group*), with a small proportion belonging to *Actinobacteria* (*Coriobacteriacaea*), *Tenericutes* (*Mollicutes RF9*), and *Proteobacteria* (*Enterobacteriaceae* including *Escherichia.Shigella* as mentioned before).

DISCUSSION

Comprehensive investigation of the chicken cecal microbiome at a day to day level revealed a rapid increase in diversity up to day 12, with microbial variation observed both in terms of genera and abundance. We suspect this early variation is due to competitive factors determined by space and available food resources. Post day 20 there exists a considerable stabilization of the chicken cecal microbiome where the relative microbial diversity and abundances are standardized, with environmental factors (in this case the host chicken) exerting a greater influence on any change in the microbial diversity. Between days 12 and 20 we observe a shift from competitive to environmental drivers of microbial community creating a window of opportunity whereby *Campylobacter* appears. We identified *Campylobacter* at day 16 with this day lying immediately after the most substantial changes in metabolic profiles observed over the whole period. Whilst we identified *Campylobacter* within 25% of the pens on day 16, we would naturally expect *Campylobacter* to spread to other chickens and pens and also be identified on subsequent days. We suspect that the experimental set-up here was such that following random selection of birds from each pen on each day, sacrificing the bird (to perform gDNA extraction from the ceca) did not allow for an opportunity for *Campylobacter* to spread to other chickens or pens. Clearly in a typical farm set-up this would not be the case and *Campylobacter* would spread naturally.

Microbial variation over time is most likely influenced by diet of the chickens whereby significant shifts in OTU abundances and beta dispersion of the samples often corresponded with changes in feed. Notably, the relatively high proportion of OTUs that were log2 fold different between days 9 and 11, and days 26 and 28, and beta dispersion for days 11–13 corresponded with changes in feed from grower to finisher. Further studies investigating different feed content is required to ascertain the complete impact on chicken cecal microbiome.

Previous microbiome studies of chicken ceca have often lacked the day to day sampling points, replicate numbers, relevant metadata and have often provided external *Campylobacter* infection that may potentially perturb the natural habitat. These have not allowed us to fully appreciate what is occurring in a natural environment in relation to how and when *Campylobacter* appears within the chicken gut. Thus, we believe the major strength of this study is that we have filled these gaps by performed the most comprehensive analysis of the chicken cecal microbiome to date. This was made possible by sampling from days 3 to 35, with 12 replicates per day (final $n = 379$), correlating additional metadata such as chicken weight and feed conversion rates and with *Campylobacter* detection in a natural environmental setting giving the most comparable experimental design to a farm set-up. As we were not able to sample the same chicken for all time points, future studies should investigate this further with added dietary information than what we have considered here, with experimental designs also to investigate and confirm the predicted pathways.

CONCLUSIONS

Industry has endeavored to reduce the burden of *Campylobacter* within chicken production lines with supplements often administered with the aim of performance enhancing and/or reducing bacteria such as *Campylobacter*, typically post day 25. The relative stability of the chicken cecal microbiota at this time point may explain the efficacy of such products, however the identification of a window of opportunity for bacteria such as *Campylobacter* may call for intervention strategies between days 12 and 20, or even earlier. This study can act as a baseline for future intervention strategies and help reduce the burden of *Campylobacter* within chickens.

AVAILABILITY OF SUPPORTING DATA

The raw sequence files supporting the results of this article are available in the European Nucleotide Archive under the project accession number PRJEB25776.

AUTHOR CONTRIBUTIONS

AM, AR, UL, BW, ND, NC, and OG contributed to the study design. ND, NC, and OG managed the study. CK, AM, AS, and ML performed the sample collection and DNA extraction. LS, AM, AE, and OG performed the library preparation and Illumina MiSeq sequencing at the LSHTM. UZI wrote the analysis scripts to generate the figures and tables in this paper. UZI and OG performed the bioinformatics and statistical analysis. UZI, ND, NC, and OG drafted the initial version of the manuscript with all authors contributed to redrafting.

FUNDING

The authors acknowledge research funding from Moy Park. UZI is funded by NERC Independent Research Fellowship (NE/L011956/1).

ACKNOWLEDGMENTS

This manuscript is available as a preprint on bioRxiv (Ijaz et al., 2018). We thank Cadi Davies (LSHTM), Janie Liaw (LSHTM), and Gianna Di Sario (UCL School of Pharmacy) for helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02452/full#supplementary-material

Supplementary Figure 1 | Spatial arrangement of pens. Triangles indicate pens with *Campylobacter*.

Supplementary Figure 2 | Relative abundance of 50 most abundant genera in this study.

Supplementary Table 1 | Differential analysis of OTUs that are up/down-regulated between different groups (Adjusted *P* values $\leq$ 0.05) where positive log2 fold change represent OTUs becoming abundant as we go forward in time. Here only the significant OTUs are shown for both daily and weekly comparisons.

Supplementary Table 2 | Differential analysis of genera that are up/down regulated between different groups (Adjusted *P* values $\leq$ 0.05) where positive log2 fold change represent genera becoming abundant as we go forward in time. Here only the significant genera are shown for daily comparisons.

Supplementary Table 3 | Differential analysis of pathways becoming significant based on Kruskal-Wallis test (Adjusted *P* values $\leq$ 0.05). Here results are shown for both daily and weekly comparisons.

REFERENCES

Amour, C., Gratz, J., Mduma, E., Svensen, E., Rogawski, E. T., Mcgrath, M., et al. (2016). Epidemiology and impact of campylobacter infection in children in 8 low-resource settings: results from the MAL-ED study. *Clin. Infect. Dis.* 63, 1171–1179. doi: 10.1093/cid/ciw542

Apajalahti, J. (2005). Comparative gut microflora, metabolic challenges, and potential opportunities. *J. Appl. Poult. Res.* 14, 444–453. doi: 10.1093/japr/14.2.444

Asshauer, K. P., and Meinicke, P. (2013). "On the estimation of metabolic profiles in metagenomics," in *German Conference on Bioinformatics 2013*, eds T. Beissbarth, M. Kollmar, A. Leha, B. Morgenstern, A. Schultz, S. Waack and E. Wingender (Dagstuhl: Schloss Dagstuhl–Leibniz-Zentrum fuer Informatik), 1–13.

Aßhauer, K. P., Wemheuer, B., Daniel, R., and Meinicke, P. (2015). Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* 31, 2882–2884. doi: 10.1093/bioinformatics/btv287

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., et al. (2016). Development of the chick microbiome: how early exposure influences future microbial diversity. *Front. Vet. Sci.* 3:2. doi: 10.3389/fvets.2016.00002

Barnes, E. M., Mead, G. C., Barnum, D. A., and Harry, E. G. (1972). The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *Br. Poult. Sci.* 13, 311–326. doi: 10.1080/00071667208415953

Bjerrum, L., Engberg, R. M., Leser, T. D., Jensen, B. B., Finster, K., and Pedersen, K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poult. Sci.* 85, 1151–1164. doi: 10.1093/ps/85.7.1151

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cawthraw, S. A., Wassenaar, T. M., Ayling, R., and Newell, D. G. (1996). Increased colonization potential of Campylobacter jejuni strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol. Infect.* 117, 213–215. doi: 10.1017/S0950268800001333

Clarke, K. R., and Ainsworth, M. (1993). A method of linking multivariate community structure to environmental variables. *Mar. Ecol. Prog. Ser.* 92, 205–219. doi: 10.3354/meps092205

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of the timing of exposure to Campylobacter jejuni on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome* 6:88. doi: 10.1186/s40168-018-0477-5

D'Amore, R., Ijaz, U. Z., Schirmer, M., Kenny, J. G., Gregory, R., Darby, A. C., et al. (2016). A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. *BMC Genomics* 17:55. doi: 10.1186/s12864-015-2194-9

Danzeisen, J. L., Kim, H. B., Isaacson, R. E., Tu, Z. J., and Johnson, T. J. (2011). Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS ONE* 6:e27949. doi: 10.1371/journal.pone.0027949

Dray, S., Blanchet, G., Borcard, D., Clappe, S., Guenard, G., Jombart, T., et al. (2018). *"adespatial: Multivariate Multiscale Spatial Analysis". 0.0–9 ed*. Available online at: https://CRAN.R-project.org/package=adespatial

Elharrif, Z., and Mégraud, F. (1986). Characterization of thermophilicCampylobacter: I. Carbon-substrate utilization tests. *Curr. Microbiol.* 13, 117–122. doi: 10.1007/BF01568505

Friedman, C. R., Neimann, J., Wegener, H. C., and Tauxe, R. V. (2000). "Epidemioogy of *Camylobacter jejuni* infections in the United States and other industrialized nations," in *Campylobacter, 2nd Edn,* eds I. Nachamkin, and M. J. Blaser (Washington, DC: American Society for Microbiology Press), 121–138.

Hegde, S., Rolls, B., and Coates, M. E. (1982). The effects of the gut microflora and dietary fibre on energy utilization by the chick. *Br. J. Nutr.* 48, 73–80. doi: 10.1079/BJN19820089

Hendrixson, D. R., and DiRita, V. J. (2004). Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract. *Mol. Microbiol.* 52, 471–484. doi: 10.1111/j.1365-2958.2004.03988.x

Hermans, D., Pasmans, F., Heyndrickx, M., Van Immerseel, F., Martel, A., Van Deun, K., et al. (2012). A tolerogenic mucosal immune response leads to persistent Campylobacter jejuni colonization in the chicken gut. *Crit. Rev. Microbiol.* 38, 17–29. doi: 10.3109/1040841X.2011.615298

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., et al. (2011). Colonization factors of Campylobacter jejuni in the chicken gut. *Vet. Res.* 42:82. doi: 10.1186/1297-9716-42-82

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., et al. (2014). *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. *MBio* 5:e01364–e01314. doi: 10.1128/mBio.01364-14

Humphrey, S., Lacharme-Lora, L., Chaloner, G., Gibbs, K., Humphrey, T., Williams, N., et al. (2015). Heterogeneity in the infection biology of *Campylobacter jejuni* isolates in three infection models reveals an invasive and virulent phenotype in a ST21 isolate from poultry. *PLoS ONE* 10:e0141182. doi: 10.1371/journal.pone.0141182

Humphrey, T., O'Brien, S., and Madsen, M. (2007). Campylobacters as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol.* 117, 237–257. doi: 10.1016/j.ijfoodmicro.2007.01.006

Ijaz, U. Z., Sivaloganathan, L., Mckenna, A., Richmond, A., Kelly, C., Linton, M., et al. (2018). Comprehensive longitudinal microbiome analysis of the chicken cecum reveals a shift from competitive to environmental drivers and a window of opportunity for Campylobacter. *bioRxiv*. doi: 10.1101/341842

Joshi, N., and Fass, J. (2011). *Sickle: A Sliding-Window, Adaptive, Quality-Based Trimming Tool for Fastq Files (Version 1.33). 1.33 ed.* San Francisco, CA. Available online at: https://github.com/najoshi/sickle

Józefiak, D., Rutkowski, A., and Martin, S. A. (2004). Carbohydrate fermentation in the avian ceca: a review. *Anim. Feed Sci. Technol.* 113, 1–15. doi: 10.1016/j.anifeedsci.2003.09.007

Kalupahana, R. S., Kottawatta, K. S., Kanankege, K. S., Van Bergen, M. A., Abeynayake, P., and Wagenaar, J. A. (2013). Colonization of *Campylobacter* spp. in broiler chickens and laying hens reared in tropical climates with low-biosecurity housing. *Appl. Environ. Microbiol.* 79, 393–395. doi: 10.1128/AEM.02269-12

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Klaus, B., and Strimmer, K. (2013). Signal identification for rare and weak features: higher criticism or false discovery rates? *Biostatistics* 14, 129–143. doi: 10.1093/biostatistics/kxs030

Klaus, B., and Strimmer, K. (2015). "*fdrtool: Estimation of (Local) False Discovery Rates and Higher Criticism." 3.0 ed*. Available online at: https://CRAN.R-project.org/package=fdrtool.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Lassmann, T., and Sonnhammer, E. L. (2005). Kalign–an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* 6:298. doi: 10.1186/1471-2105-6-298

Leach, S., Harvey, P., and Wali, R. (1997). Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. *J. Appl. Microbiol.* 82, 631–640. doi: 10.1111/j.1365-2672.1997.tb02873.x

Lee, M. D., and Newell, D. G. (2006). Campylobacter in poultry: filling an ecological niche. *Avian Dis.* 50, 1–9. doi: 10.1637/7474-111605R.1

Legendre, P., and De Cáceres, M. (2013). Beta diversity as the variance of community data: dissimilarity coefficients and partitioning. *Ecol. Lett.* 16, 951–963. doi: 10.1111/ele.12141

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Mancabelli, L., Ferrario, C., Milani, C., Mangifesta, M., Turroni, F., Duranti, S., et al. (2016). Insights into the biodiversity of the gut microbiota of broiler chickens. *Environ. Microbiol.* 18, 4727–4738. doi: 10.1111/1462-2920.13363

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Mcnab, J. M. (2007). The avian caeca: a Review. *Worlds Poultry Sci. J.* 29, 251–263. doi: 10.1079/WPS19730014

Meinicke, P. (2015). UProC: tools for ultra-fast protein domain classification. *Bioinformatics* 31, 1382–1388. doi: 10.1093/bioinformatics/btu843

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

Neill, S. D., Campbell, J. N., and Greene, J. A. (1984). Campylobacter species in broiler chickens. *Avian Pathol.* 13, 777–785. doi: 10.1080/03079458408418574

Nikolenko, S. I., Korobeynikov, A. I., and Alekseyev, M. A. (2013). BayesHammer: bayesian clustering for error correction in single-cell sequencing. *BMC Genomics* 14(Suppl. 1):S7. doi: 10.1186/1471-2164-14-S1-S7

Oksanen, J., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'hara, R., et al. (2015). *"Vegan: Community Ecology Package, R Package version 2.2-1." version 2.2-1 ed.)*. San Francisco, CA.

Pan, D., and Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* 5, 108–119. doi: 10.4161/gmic.26945

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Part, C. E., Edwards, P., Hajat, S., and Collins, L. M. (2016). Prevalence rates of health and welfare conditions in broiler chickens change with weather in a temperate climate. *R. Soc. Open Sci.* 3:160197. doi: 10.1098/rsos.160197

Pauwels, J., Taminiau, B., Janssens, G. P., De Beenhouwer, M., Delhalle, L., Daube, G., et al. (2015). Cecal drop reflects the chickens' cecal microbiome, fecal drop does not. *J. Microbiol. Methods* 117, 164–170. doi: 10.1016/j.mimet.2015.08.006

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

Schirmer, M., Ijaz, U. Z., D'amore, R., Hall, N., Sloan, W. T., and Quince, C. (2015). Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nucleic Acids Res.* 43:e37. doi: 10.1093/nar/gku1341

Sergeant, M. J., Constantinidou, C., Cogan, T. A., Bedford, M. R., Penn, C. W., and Pallen, M. J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. *PLoS ONE* 9:e91941. doi: 10.1371/journal.pone.0091941

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

Stegen, J. C., Lin, X., Konopka, A. E., and Fredrickson, J. K. (2012). Stochastic and deterministic assembly processes in subsurface microbial communities. *ISME J.* 6, 1653–1664. doi: 10.1038/ismej.2012.22

Taylor, M. (2014). *sinkr: A Collection of Functions Featured on the Blog 'me nugget'*. Available online at: https://github.com/menugget/sinkr [Accessed].

Thibodeau, A., Fravalo, P., Yergeau, E., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS ONE* 10:e0131978. doi: 10.1371/journal.pone.0131978

Van Deun, K., Pasmans, F., Ducatelle, R., Flahou, B., Vissenberg, K., Martel, A., et al. (2008). Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut. *Vet. Microbiol.* 130, 285–297. doi: 10.1016/j.vetmic.2007.11.027

Velayudhan, J., Jones, M. A., Barrow, P. A., and Kelly, D. J. (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect. Immun.* 72, 260–268. doi: 10.1128/IAI.72.1.260-268.2004

Velayudhan, J., and Kelly, D. J. (2002). Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology* 148, 685–694. doi: 10.1099/00221287-148-3-685

Walker, R. I. (2005). Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries. *Vaccine* 23, 3369–3385. doi: 10.1016/j.vaccine.2004.12.029

Wigley, P. (2015). Blurred lines: pathogens, commensals, and the healthy gut. *Front. Vet. Sci.* 2:40. doi: 10.3389/fvets.2015.00040

Conflict of Interest Statement: AM, AR and UL are employed by company Moy Park. AM is also a PhD student between Moy Park and AFBI.

The remaining 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: 02 November 2018
doi: 10.3389/fmicb.2018.02454

Semi-Quantification of Total *Campylobacter* and *Salmonella* During Egg Incubations Using a Combination of 16S rDNA and Specific Pathogen Primers for qPCR

*Michael J. Rothrock Jr.[1], Kristina M. Feye[2], Sun Ae Kim[3], Si Hong Park[4], Aude Locatelli[1], Kelli L. Hiett[5†], John Gamble[6], Holly Sellers[7] and Steven C. Ricke[2]**

[1] Egg Safety and Quality Research Unit, U.S. National Poultry Research Center, United States Department of Agriculture – Agricultural Research Service, Athens, GA, United States, [2] Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [3] Department of Food Science and Engineering, Ewha Womans University, Seoul, South Korea, [4] Department of Food Science and Technology, Oregon State University, Corvallis, OR, United States, [5] Poultry Management Safety and Processing Unit, U.S. National Poultry Research Center, United States Department of Agriculture – Agricultural Research Services, Athens, GA, United States, [6] Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, United States, [7] Department of Veterinary Medicine, Poultry Diagnostic and Research Center, University of Georgia, Athens, GA, United States

Rapid molecular techniques that evaluate eggs for the presence of foodborne pathogens is an essential component to poultry food safety monitoring. Interestingly, it is not just table eggs that contribute to outbreaks of foodborne disease. Broiler layer production actively contributes to sustaining of foodborne pathogens within a flock. The surface contamination of production eggs with invasive pathogens such as *Salmonella enterica*, *Campylobacter jejuni*, and *Listeria monocytogenes* during embryogenesis results in gastrointestinal tract (GIT) colonization. Pathogens that secure a niche within the GIT during embryonic development are nearly impossible to eradicate from the food chain. Therefore, current monitoring paradigms are not comprehensive because they fail to capture the presence of invasive pathogens within the embryonic GIT rapidly. By developing tools to recognize the pathogens' presence in the GIT during embryogenesis, producers are then able to spot evaluate broiler eggs for their potential risk as carriers of foodborne pathogens. In this study a novel qPCR assay was developed to semi-quantify pathogen load relative to total bacterial burden. Eggs sampled from three independent production broiler flocks of different ages were assayed for *S. enterica (invA)*, *C. jejuni (HipO)*, and *L. monocytogenes (HlyA)* against total microbial load (*16s*). The eggs were sampled at 1-day post-set within each flock, 2 weeks post-set, after vaccination (at 2.5 weeks) and 1-day post-hatch. The eggs were washed, and the yolk and embryonic chick GIT were collected. The DNA was extracted and subjected to a qPCR assay. The results confirm a novel technique for pathogen monitoring relative to total bacterial load and a unique method for monitoring the dynamics of foodborne pathogen invasion throughout broiler egg production.

Keywords: *Campylobacter*, *Salmonella*, poultry, eggs, qPCR, semi-quantification

OPEN ACCESS

Edited by:
Juan Aguirre, Universidad de Chile, Chile

Reviewed by:
Divya Ammalia Jaroni, Oklahoma State University, United States
Julio Parra-Flores, University of the Bío Bío, Chile
Beatrix Stessl, Veterinärmedizinische Universität Wien, Austria

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

***Present address:**
Kelli L. Hiett, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD, United States

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

Received: *28 June 2018*
Accepted: *25 September 2018*
Published: *02 November 2018*

Citation:
Rothrock MJ Jr, Feye KM, Kim SA, Park SH, Locatelli A, Hiett KL, Gamble J, Sellers H and Ricke SC (2018) Semi-Quantification of Total Campylobacter and Salmonella During Egg Incubations Using a Combination of 16S rDNA and Specific Pathogen Primers for qPCR. Front. Microbiol. 9:2454. doi: 10.3389/fmicb.2018.02454

INTRODUCTION

Substantial data indicates that the pathogen load innately carries a certain level of risk; therefore, the absolute presence of any pathogen is substantial enough to remove a carcass from the processing line (Rajan et al., 2017). Broiler eggs are unique reservoirs for foodborne disease, with low levels of foodborne pathogens making the detection and recovery of bacterial cells difficult. The egg contamination occurs in one of two routes—through surface contamination during oviposition and via the invasion of eggs by foodborne pathogens (Cox et al., 2000; European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control, 2012). Once on the egg, invasive pathogens are capable of entering the embryonic chickens' GIT (Cox et al., 2002; Heyndrickx et al., 2002). The early exposure of the chicken GIT to foodborne pathogens results in the direct colonization of the pre-immune chick, which makes the eradication of these pathogens from flocks extraordinarily difficult, if not impossible (Claud and Walker, 2001).

Therefore, production layer facilities rely on significant monitoring strategies designed for identifying sources of contamination and contamination events. Efforts directed toward reducing the threat of foodborne pathogens in production layer facilities include the monitoring of eggs via surface swabbing, egg washes, egg carton swabbing, and production hen sampling (United States Department of Agriculture-Animal and Plant Health Inspection Services [USDA-APHIS], 2007). The samples are subsequently assayed via microbiological and molecular analyses to determine pathogen load and prevalence. Unfortunately, these methods fail to detect pathogens from the surface that have successfully invaded the embryonic chicken GIT. Being unable to evaluate the embryonic chick and the yolk for pathogen penetrance fails to truly indicate the risk that individual layer flocks may pose to the contamination risk of hatcheries and broiler facilities.

The data presented herein uses a relative 16s rDNA semi-quantitative qPCR assay to quantitate indigenous *S. enterica*, *L. monocytogenes*, and *C. jejuni* against the total bacterial load of eggs. This study uses this technology to investigate the risk of GIT penetrance associated with three ages of broiler hen flocks: new (25 weeks), mid-life (40 weeks), and old (65 weeks). Egg washes (EW) were compared to yolk and embryonic chick GIT pathogen load. The chicks were sampled 1-week post-set, 2 weeks post-set, after *in ovo* vaccination (at 2.5 weeks), and 1-day post-hatch. Evaluating the penetrance of foodborne pathogens at the post-vaccination time-point in eggs can potentially help identify weak-points in egg hatching that make eggs and developing chicks vulnerable to pathogen invasion. Furthermore, this assay uses rDNA, which provides a relative quantitation of the viable bacterial load against the specific pathogen load. While RNA is an attractive target, field applications can be less user friendly. Therefore, by employing a semi-quantitative, DNA based qPCR assay provides data that comprehensively evaluates broiler eggs for pathogen load and provides a rapid method to track the spread of *Campylobacter* and *Salmonella* throughout egg production.

MATERIALS AND METHODS

Broiler Hatchery

A commercial broiler hatchery in the southeastern U.S. provided all of the eggs for this study. Two independent trials were conducted at two different ages of the broiler breeder flocks. **Figure 1** shows a visual depiction of the sampling strategy used for this study. In the first trial, eggs from broiler breeder flocks from three separate broiler breeder farms were sampled. The hens were individually caged. These broiler breeder farms were chosen based on the age of the breeders: (1) Young flock (F1) just entering egg production (flock age = 25 weeks), (2) peak egg production flock (F2; flock age = 40 weeks), and (3) older flock producing its last set of eggs prior to culling (F3; flock age = 60 weeks). Throughout the second trial, a single broiler breeder flock (F1-Y; flock F1 from trial 1, representing the young flock age) was followed and was sampled. The F1-Y flock had eggs sampled at the peak (F1-P) and old (F1-O) ages as defined by trial 1. The young and peak flocks from both trials were similarly managed based on integrator's guidelines. However, the old flocks did experience a proprietary feed formulation change for the old flock at approximately 45 weeks of age. Throughout both studies, the sample collection and processing methods were the same. Once the eggs were set in the commercial hatchery, eggs were collected at four independent time points: (1) 1 week after set (T1: day 8), (2) 2 weeks after set (T2: day 15), (3) after *in ovo* immunization (T3: day 20), and (4) one-day post hatch (T4) (**Figure 1**).

Gastrointestinal Tract (GIT) and Egg Yolk Collection

All of the necropsies for this study were performed at the University of Georgia Poultry Diagnostic and Research Center, Athens, GA, United States. All the animal work conducted throughout the study was in accordance with the approved policies and procedures of the University of Georgia Institutional Animal Care and Use Committee (IACUC) number A2010 11-568-Y1-A0. At each sampling event, 60 eggs per flock were collected (F1, F2, F3 or F1-Y, F1-P, F1-O for trials 1 and 2, respectively, **Figure 1**). Researchers aseptically removed the GIT and yolks from each egg during necropsy. For T1 and T2, eggs from one flock at a time were removed from the 37°C incubator, placed in the Type II biosafety cabinet, sprayed with 0.4% Bioguard (Neogen Corp, Lansing, MI, United States) and allowed to dry prior to sampling. Once the embryos were dry, sterile forceps were used to crack the air cell end of the egg. The embryos were removed from the shell with sterile forceps, the shell was discarded, and the birds were subsequently euthanized by cervical dislocation. The embryos were pooled in groups of seven into a sterile 110 mm^3 petri dish for sampling. Sterile scissors were used to open the abdominal cavity of the embryos and the intestines were removed with sterile forceps per group. The GIT samples from each group of seven embryos was placed into a small filtered stomacher bag (Seward Laboratory Systems, Inc., Davie, FL, United States). The egg yolks from the eggs of the seven embryos were pooled into large filtered stomacher bags

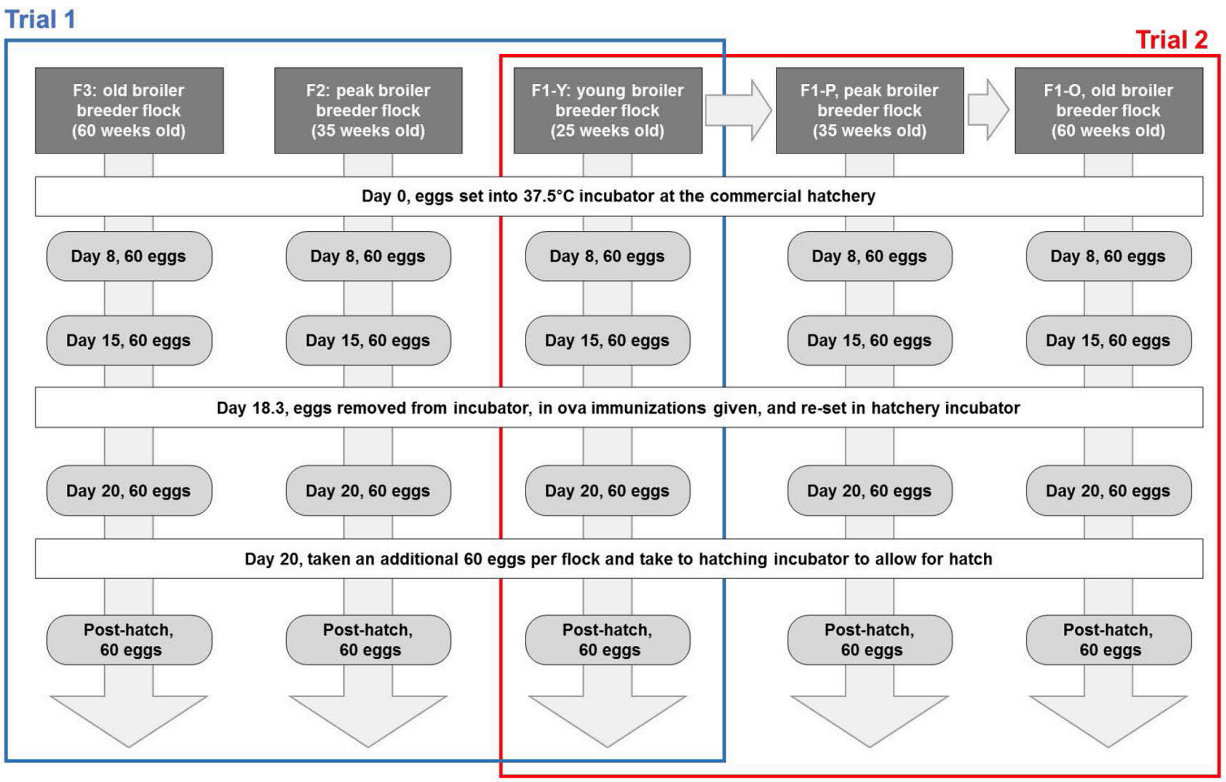

FIGURE 1 | Sampling scheme for this study. Eggs were collected from the commercial hatchery from broiler breeder flocks at three different ages (young, peak, old) for trial 1 (F1, F2, and F3, respectively) and trial 2 (F1-Y, F1-P, and F1-O, respectively). These eggs were collected at four embryonic ages [1 week post set (PS) (T1), 2 weeks PS (T2), 2.5 weeks PS, after *in ovo* immunization (T3), and 1 day post hatch (PH) (T4)]. At every sampling event, triplicate pooled gastrointestinal tract (GIT) and yolk samples were created (n = 7 eggs per pool) for embryonic ages. The triplicate pooled egg shell washes (EW) also created for the T3 sampling time only.

(Seward Laboratory Systems, Inc.) and the remaining amniotic fluid and shell was discarded.

Each of the pooled GIT and yolk samples were weighed and sterile 1x phosphate-buffered saline (PBS) was added to pooled (3:1; 1x PBS volume: GIT mass) to ensure that there was enough homogenate available for all sampling needs. Pooled GIT samples were homogenized via stomaching (Seward Laboratory Systems, Inc.) on maximum speed for 60 s, while the pooled yolk samples were homogenized manually for 60 s.

Collection of Egg Shell Washes

Egg shell washes (EW) were performed at sampling time T3 to see if the puncturing of the egg shell during *in ovo* immunization introduced egg shell microbial populations into the internal egg environment. Eggs from each breeder flock at T3 were washed in brain heart infusion (BHI) broth by placing each egg, the same ones selected for the selected for embryo harvest in a sampling bag containing 10 mL of BHI broth. The bag was rotated to coat the entire surface of the egg. Then, the eggs were removed, placed on a clean fiberboard flat, placed in the biosafety cabinet and allowed to dry. The eggs were subsequently sprayed with 0.4% Bioguard and allowed to dry. The embryo sampling was conducted as previously described with the following adjustments for the egg washes. For the T4 samples, an extra set of eggs were collected from the commercial hatchery at T3. Those specific eggs were placed into hatching baskets that were arranged by breeder flock, then set into a single stage Natureform Hatcher (NatureForm Hatchery Technologies, Jacksonville, FL, United States), and hatched out at the University of Georgia Hatchery. The chicks were removed from the hatcher by flock, placed in ventilated transport containers and transported to the lab. The chicks were euthanized by group via cervical dislocation, and then placed into sterile 110 mm^3 petri dishes contained within the biosafety cabinet. The pooled EW samples were centrifuged at 5000 g for 10 min, the supernatant was discarded and the pellet was suspended in 1x PBS. The EW samples were stored at −20 °C until DNA extraction.

DNA Extraction

Two 0.5 mL aliquots of each pooled sample of either the GIT, yolk, or EW were placed into separate FastPrep Lysing Matrix A tubes (MP Biomedicals, Solon, OH, United States). After lysis, all of the tubes were frozen at −20°C until DNA extraction. The genomic DNA was extracted from the GIT, yolk and EW samples using a hybrid extraction method optimized for poultry samples (Rothrock et al., 2014). Exactly 1 mL of Qiagen ASL buffer (Qiagen, Valencia, CA, United States) was added to each sample tube and vortexed at the maximum setting for 1 min,

followed by a more thorough homogenization using the FastPrep 24 (MP Biomedicals) at 6.0 m/s for 45 s. After centrifugation (14,000 × g for 10 min), supernatant was removed, added to a sterile 2 mL tube and incubated at 25°C (yolk) or 95°C in a water bath (GIT, EW) for 5 min. From thereon out, all of the samples were processed using the QIAamp DNA Stool Mini Kit (Qiagen) using the standard stool pathogen detection protocol on the QIAcube robotic workstation (Qiagen). After the automated extraction and purification steps, the two extracted aliquots for each pooled sample were combined in 100 mL sterile molecular grade water using Vacufuge™ Plus (Eppendorf, Hauppage NY, United States), and the DNA concentration in each sample was determined spectrophotometrically using the Take3® plate in conjunction with the Synergy H4 multimode plate reader (BioTek, Winooski, VT, United States).

qPCR for Bacterial Absolute Enumeration

Total bacteria, *Salmonella* spp. and *C. jejuni*, were targeted using the 16S rDNA (Harms et al., 2003), *invA* (Fey et al., 2004), *hipO* (He et al., 2010), and *hlyA* (Suo et al., 2010) genes, respectively. All DNA extractions analyzed with qPCR were performed on Mastercycler® ep Realplex s2 and s4 thermocycling machines (Eppendorf) in 20 μL reaction mixture was prepared using 10 μL of 2x PerfeCTa qPCR ToughMix, ROX (Quanta BioSciences, Gaithersburg, MD, United States) and 5 μL template of 1:10 diluted sample (containing 10 to 15 ng genomic DNA). Previously published thermocycling protocols were followed and the salient information for all qPCR reactions can be found in **Table 1**. The PCR amplification efficiency and detection sensitivity were determined by using a series of 10-fold dilutions of standards (10^8 to 10^1 copies per reaction) created from purified plasmids for the target gene. The target gene copy number was determined using Mastercycler ep Realplex software (Eppendorf).

Statistical Analyses

All qPCR data was $\log_{10}$-transformed prior to statistical analyses. Two-way ANOVAs evaluated the effect of breeder flock age or embryonic age, as well as their interaction were analyzed via using Prism 6.0 (GraphPad Software Inc., La Jolla, CA, United States). To determine the changes in the pathogen target gene copy numbers relative to the total bacterial population, the $\log_{10}$-transformed *invA*, *hipO*, or the *hlyA* values were divided by $\log_{10}$-transformmed 16S rDNA values for each pooled samples. The result provided a measure of the relative abundance of each pathogen.

RESULTS

Bacterial Population in Trial 1

Time and the flock age dictated pathogen and microbial load. Bacterial loads were low in the GIT samples during the first two weeks post-set for all three broiler breeder flocks (T1 and T2; 4.50–4.54, 4.84–4.52, and 5.61–5.52 log copies/g for F1, F2, and F3, respectively). All three broiler breeder flocks exhibited approximately a 2-$\log_{10}$ increase in GIT total bacterial load by 1-day post hatch (T4; 6.07, 7.30, 7.50 $\log_{10}$ copies/g for F1, F2, and F3, respectively) (**Table 2**). The impact of the age of the broiler layer flock was detectable. The youngest flock (F1) exhibited significantly lower GIT total bacterial load as compared to the oldest flock (F3), with the peak age flock (F2) matching the F1 flock early during embryonic development (T1, T2) and the F3 flock later during development (T3, T4). Two of the broiler breeder flocks (F1, F3) yielded low total bacterial loads within the yolk regardless of embryonic age (4.09–4.59 and 4.22–4.58 $\log_{10}$ copies/g, respectively), although total bacterial concentrations in the yolks of the F1 and F2 flock both significantly changed by T4 (**Table 2**). While there were differences in directions of the total bacterial concentration

TABLE 1 | qPCR primer, probes, and protocols used for this study.

Target Group	Gene	Name	Primer/Probe Sequence (5′–3′)	Final concentration (nM)	Tm (°C)	Reference
Total Bacteria	16S	1055F	ATG GCT GTC GTC AGC T	600	58	Harms et al., 2003
		1392R	ACG GGC GGT GTG TAC	600		
		16STaqIII5-BHQ	FAM -CAA CGA GCG –ZEN -CAA CCC –3IABkFQ	200		
Salmonella spp.[1]	*invA*	invA2-F	ATT CTG GTA CTA ATG GTG ATG ATC	400	60	Fey et al., 2004
		invA2-R	GCC AGG CTA TCG CCA ATA AC	400		
Campylobacter jejuni	*hipO*	hipO-Cj-F	TCC AAA ATC CTC ACT TGC CAT T	500	60	He et al., 2010
		hipO-Cj-R	TGC ACC AGT GAC TAT GAA TAA CGA	500		
		hipO-Cj-P	FAM- TTG CAA CCT CAC TAG CAA AAT CCA CAGCT-BHQ-1	250		
Listeria monocytogenes	*hlyA*	hlyA-LisM-F	ACT GAA GCA AAG GAT GCA TCT G	600	60	Suo et al., 2010
		hlyA-LisM-R	TTT TCG ATT GGC GTC TTA GGA	600		
		hlyA-LisM-P	FAM-CAC CAC CAG CAT CTC CGC CTG C -BHQ-1	200		

[1] *Superscript letter indicates that Salmonella spp. was evaluated using a SYBR kit and not TaqMan.*

TABLE 2 | GIT, Yolk, and EW log transformed qPCR data for three commercial broiler breeder flocks at four times during embryonic development (trial I)[1,2].

Target		F1				F2				F3			
		T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
GIT[3]	Total Bacteria (16S)	$4.50^{B,Y}$ (0.10)	$4.54^{B,Y}$ (0.66)	$4.25^{B,Y}$ (0.12)	$6.07^{A,Y}$ (1.33)	$4.84,^{B,C,Y}$ (0.32)	$4.52^{C,Y}$ (0.13)	$5.39^{B,X}$ (0.09)	$7.30^{A,X}$ (0.48)	$5.61^{B,X}$ (0.17)	$5.52^{B,X}$ (0.12)	$6.01^{B,X}$ (0.03)	$7.50^{A,X}$ (0.40)
	Salmonella (invA)[6]	0.00^{B} (0.00)	0.00^{B} (0.00)	$0.46^{A,X}$ (0.72)	0.00^{B} (0.00)	0.00 (0.00)	0.00 (0.00)	0.00^{Y} (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00^{Y} (0.00)	0.00 (0.00)
	C. jejuni (hipO)	0.48 (1.18)	0.00 (0.00)	0.00 (0.00)	0.49 (1.20)	0.00 (0.00)	0.41 (1.00)	0.67 (1.65)	0.66 (1.61)	0.54 (1.33)	1.38 (1.52)	1.11 (1.73)	0.00 (0.00)
Yolk[3]	Total Bacteria (16S)	$4.09^{B,Y}$ (0.19)	4.30^{B} (0.14)	4.59^{A} (0.06)	$4.58^{A,Y}$ (0.03)	$6.99^{a,X}$ (0.06)	4.40^{C} (0.08)	4.32^{C} (0.61)	$5.83^{B,X}$ (1.92)	4.22^{Y} (0.20)	4.63 (0.29)	4.63 (0.17)	4.56^{Y} (0.08)
	Salmonella (invA)[6]	0.00 (0.00)	0.23 (0.57)	0.20 (0.48)	0.30 (0.72)	0.25 (0.61)	0.50 (0.78)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.26 (0.63)	0.25 (0.61)	0.20 (0.50)
	C. jejuni (hipO)	$0.61^{B,Y}$ (0.95)	2.97^{A} (0.15)	$0.00^{B,Y}$ (0.00)	$2.89^{A,X}$ (0.24)	$2.88^{A,X}$ (0.15)	2.85^{A} (0.33)	$0.29^{B,X,Y}$ (0.70)	$2.83^{A,X}$ (0.19)	$2.95^{A,X}$ (0.13)	2.83^{A} (0.13)	$0.69^{B,X}$ (1.07)	$0.00^{B,Y}$ (0.00)
EW[4,5]	Total Bacteria (16S)	ND	ND	6.56^{B} (0.08)	ND	ND	ND	6.63^{B} (0.03)	ND	ND	ND	6.91^{A} (0.14)	ND
	Salmonella (invA)[6]	ND	ND	0.00^{B} (0.00)	ND	ND	ND	0.00^{B} (0.00)	ND	ND	ND	1.78^{A} (1.40)	ND
	C. jejuni (hipO)	ND	ND	1.56 (1.24)	ND	ND	ND	2.51 (0.23)	ND	ND	ND	1.95 (1.05)	ND

[1]F1, Young Flock; F2, Peak Flock; F3, Old Flock; T1, 1 week after set; T2, 2 weeks after set; T3, After in ovo immunization (2.5 weeks after set); T4, 1 day after hatch; GIT, Gastrointestinal Tract; EW, Egg Wash.

[2]Values represent the Mean (Standard Deviation) of duplicate qPCR runs for triplicate pooled homogenate (GIT, Yolk) or rinse (EW) samples. Samples from 7 eggs constitute a pooled sample. Values represent copies g^{-1} (GIT, Yolk) or copes mL^{-1} (EW).

[3]Superscript letters A, B, C indicate significant differences ($p < 0.05$) of qPCR data between embryonic ages (T1, T2, T3, T4) for a given broiler breeder flock (F1, F2, F3). Superscript letters X, Y, Z indicate significant differences ($p < 0.05$) of qPCR data between broiler breeder flocks (F1, F2, F3) at different embryonic ages (T1, T2, T3, T4).

[4]Superscript letters indicate significant differences ($p < 0.05$) of qPCR data between broiler breeder flocks (F1, F2, F3).

[5]ND, not determined.

[6]Superscript letter indicates that Salmonella spp. was evaluated using a SYBR kit and not TaqMan.

changes (e.g., increase, decrease, no change) between the GIT and yolk samples, two-way ANOVA analyses revealed that breeder flock age, embryonic age, and the interaction of these two ages were considered highly significant for both (**Table 3**). The age of the broiler breeder flock significantly affected the total bacterial concentrations recovered after T3 for the EW samples (**Table 3**). The old flock (F3) exhibited greater total bacterial concentrations (6.91 $\log_{10}$ copies/mL) compared to the other two flocks (6.56 and 6.63 $\log_{10}$ copies/mL for F1 and F2, respectively) (**Table 2**).

While the total bacterial load is important, the load of the three main bacterial foodborne pathogens (*Salmonella*, *C. jejuni, L. monocytogenes*) were also targeted by this study. Of these pathogens, only *Salmonella* and *C. jejuni* were detected in the GIT, yolk, and EW samples. *L. monocytogenes* was neither recovered nor quantified in the samples or trials. Across time, in all of the GIT and yolk samples demonstrated that *Salmonella* was only found once in the GIT (F1, T3) (**Table 2**). *Salmonella* was only detectable in EW from one flock (F3; 1.79 $\log_{10}$ copies/mL). Statistically, the age of the broiler breeder flock influences the *Salmonella* and microbial load in the GIT and the EW, but not the yolk (**Table 3**). *C. jejuni* was the most consistently detected foodborne pathogen in this trial (**Table 2**). The concentrations of the *C. jejuni* were much higher in many of the yolk samples, with each breeder flock having approximately 2.8 to 2.9-$\log_{10}$ copies/g yolk at two or more embryonic ages (T2, T4 for F1; T1, T3, T4 for F2; T1, T2 for F3). Unlike *Salmonella*, there was no significant impact of the embryonic age of the egg nor the age of the production flock alone on *C. jejuni* load. However, a significant interaction occurred between the age of the broiler breeder flock and the egg's embryonic age on *C. jejuni* load in the yolk (**Table 3**).

Bacterial Populations in Trial 2

The effects of breeder flock age on microbial populations within the developing embryo are an important nuance because data gathered can affect flock management. Throughout both studies, temporal effects of age and development were impacted total microbial load. The eggs from a single broiler breeder flock from the first trial (F1) were sampled once that flock reached the peak production (F1-P) and old (F1-O) ages. Low bacterial loads were quantified during the beginning of embryonic develop and were closely followed by approximately a 2-$\log_{10}$ increase in total bacterial load by T4 in the GIT samples for the young and old flocks (6.07 and 8.16 log copies/g GIT, respectively) (**Table 4**). Unlike the first trial, no significant GIT differences were observed in the F1-P flock load. The youngest flock (F1-Y) exhibited the lowest bacterial load; however, the peak flock (F1-P) consistently yielded the highest GIT total bacterial except for one case (T4, F1-O). The total bacterial loads quantified in the yolks were low in the young and old flocks, with the highest microbial load occurring in the peak-age samples (F1-P). The load of microbial populations in the yolk significantly increased between T1 and T4 for both the F1-Y and F1-P flocks. Yet, interestingly, the peak-age yolk concentrations significantly decreased in trial 1 (**Table 4**). Likewise, the age of the broiler breeding flock and the embryonic age of the egg independently and together significantly affect the total

TABLE 3 | Two-way (GIT, Yolk) and One-way (EW) ANOVA results for three commercial broiler breeder flocks at four times during embryonic development (trial I)[1,2].

Type		GIT					Yolk					EW				
		SS	DF	MS	F	*P*-value	SS	DF	MS	F	*P* value	SS	DF	MS	F	*P*-value
Total Bacteria (16S)	Breeder Flock Age	20.77	2	10.38	28.73	**<0.0001**	14.11	2	7.05	29.66	**<0.0001**	0.41	2	0.21	22.55	**<0.0001**
	Embryonic Age	51.85	3	17.28	91.76	**<0.0001**	5.93	3	1.98	4.98	**0.0046**					
	Interaction	3.68	6	0.61	3.26	**0.0096**	25.12	6	4.19	10.55	<0.0001					
	Subjects (matching)	5.4	15	0.36	1.92	0.06	3.57	15	0.24	0.60	0.86					
	Residual	8.48	45	0.19			17.86	45	0.40			0.14	15	0.01		
Salmonella (invA)[3]	Breeder Flock Age	0.21	2	0.11	2.46	0.12	0.0015	2	0.0008	0.0032	1.00	12.72	2	6.34	9.77	**0.0019**
	Embryonic Age	0.32	3	0.11	2.46	0.08	0.60	3	0.20	0.76	0.52					
	Interaction	0.64	6	0.11	2.46	**0.04**	0.10	6	0.17	0.63	0.71					
	Subjects (matching)	0.65	15	0.04	1.00	0.47	3.51	15	0.23	0.88	0.59					
	Residual	1.95	45	0.04			11.96	45	0.27			9.76	15	0.65		
C. jejuni (hipO)	Breeder Flock Age	3.27	2	1.64	1.14	0.35	5.66	2	2.83	11.02	**0.0011**	2.69	2	1.35	1.50	0.26
	Embryonic Age	1.00	3	0.33	0.25	0.86	62.56	3	20.85	91.1	**<0.0001**					
	Interaction	9.00	6	1.50	1.13	0.36	49.90	6	8.32	36.33	**<0.0001**					
	Subjects (matching)	21.49	15	1.43	1.08	0.40	3.85	15	0.26	1.12	0.37					
	Residual	59.55	45	1.32			10.30	45	0.23			13.5	15	0.9		

[1]GIT, Gastrointestinal Tract; EW, Egg Wash; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean of Squares. [2]Bolded P-values indicate that ANOVA analyses found those variables to be significant ($p \leq 0.05$). [3]Superscript letter indicates that Salmonella spp. was evaluated using a SYBR kit and not TaqMan.

TABLE 4 | GIT, Yolk, and EW log transformed qPCR data at three different flock ages for one commercial broiler breeder flock at four times during embryonic development (trial 2)[1,2].

Type		F1-Y				F1-P				F1-O			
		T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
GIT[3]	Total Bacteria (16S)	$4.42^{B,Y}$ (0.16)	$4.54^{B,Y}$ (0.66)	$4.25^{B,Y}$ (0.12)	$6.07^{A,Z}$ (1.33)	6.83^{X} (0.25)	6.76^{X} (0.08)	6.74^{X} (0.04)	6.99^{Y} (0.22)	$4.51^{B,Y}$ (0.16)	$4.47^{B,Y}$ (0.09)	$4.47^{B,Y}$ (0.19)	$8.16^{A,X}$ (0.35)
	Salmonella (invA)[6]	0.00^{Y} (0.00)	0.00^{Y} (0.00)	0.46^{Y} (0.72)	0.00^{Y} (0.00)	0.00^{Y} (0.00)	0.00^{Y} (0.00)	0.38^{Y} (0.92)	0.00^{Y} (0.00)	3.19^{X} (0.23)	2.60^{X} (1.28)	3.05^{X} (0.25)	3.00^{X} (0.23)
	C. jejuni (hipO)	0.47^{Z} (1.15)	0.00^{Z} (0.00)	0.00^{Z} (0.00)	0.49^{Y} (1.20)	$5.61^{A,X}$ (0.71)	$5.59^{A,X}$ (0.19)	$5.51^{A,X}$ (0.02)	$4.61^{B,X}$ (0.04)	4.52^{Y} (0.12)	4.48^{Y} (0.17)	4.37^{Y} (0.27)	4.45^{X} (0.48)
Yolk[3]	Total Bacteria (16S)	$4.09^{B,Y}$ (0.19)	$4.30^{B,Y}$ (0.14)	$4.59^{A,Y}$ (0.06)	$4.58^{A,Y}$ (0.04)	$6.47^{C,X}$ (0.42)	$7.23^{A,X}$ (0.02)	$7.26^{A,X}$ (0.01)	$6.84^{B,X}$ (0.04)	4.18^{Y} (0.20)	4.10^{Y} (0.05)	4.02^{Z} (0.12)	4.04^{Z} (0.14)
	Salmonella (invA)[6]	0.00^{Y} (0.00)	0.23^{Y} (0.57)	0.20^{Y} (0.48)	0.30^{Y} (0.72)	0.00^{Y} (0.00)	0.00^{Y} (0.00)	0.28^{Y} (0.69)	0.00^{Y} (0.00)	3.14^{X} (0.21)	2.88^{X} (0.17)	2.95^{X} (0.07)	3.22^{X} (0.13)
	C. jejuni (hipO)	$0.61^{B,Z}$ (0.95)	$2.97^{A,Y}$ (0.15)	$0.00^{B,Z}$ (0.00)	$2.89^{A,Y}$ (0.24)	$5.60^{B,X}$ (0.79)	$6.70^{A,X}$ (0.05)	$6.75^{A,X}$ (0.09)	$6.36^{A,X}$ (0.19)	3.05^{Y} (0.24)	3.03^{Y} (0.24)	3.05^{Y} (0.24)	3.13^{Y} (0.08)
EW[4,5]	Total Bacteria (16S)	ND	ND	6.56^{B} (0.08)	ND	ND	ND	6.85^{A} (0.08)	ND	ND	ND	5.41^{C} (0.31)	ND
	Salmonella (invA)[6]	ND	ND	0.00^{B} (0.00)	ND	ND	ND	0.00^{B} (0.00)	ND	ND	ND	2.74^{A} (0.21)	ND
	C. jejuni (hipO)	ND	ND	1.56^{C} (1.24)	ND	ND	ND	6.23^{A} (0.06)	ND	ND	ND	2.96^{B} (0.35)	ND

[1]F1-Y, Young Age; F1-P, Peak Age; F1-0, Old Age; T1, 1 week after set; T2, 2 weeks after set; T3, After in ovo immunization (2.5 weeks after set); T4, 1 day after hatch; GIT, Gastrointestinal Tract; EW, Egg Wash. [2]Values represent the Mean (Standard Deviation) of duplicate qPCR runs for triplicate pooled homogenate (GIT, yolk) or rinse (EW) samples. Samples from 7 eggs constitute a pooled sample. Values represent copies g^{-1} (GIT, yolk) or copes mL^{-1} (EW). [3]Superscript letters A, B, C indicate significant differences ($p < 0.05$) of qPCR data between embryonic ages (T1, T2, T3, T4) for a given broiler breeder flock (F1, F2, F3). Superscript letters X, Y, Z indicate significant differences ($p < 0.05$) of qPCR data between broiler breeder flocks (F1, F2, F3) at different embryonic ages (T1, T2, T3, T4). [4]Superscript letters indicate significant differences ($p < 0.05$) of qPCR data between broiler breeder flocks (F1-Y, F1-P, F1-O). [5]ND, not determined. [6]Superscript letter indicates that Salmonella spp. was evaluated using a SYBR kit and not TaqMan.

microbial concentrations of the GIT and yolk samples (**Table 5**). The total microbial load recovered from the surface of the egg (EW) was also significantly impacted by the age of the flock producing the egg, with the F1-Y and F1-P exhibiting greater total bacterial load (6.562 and 6.853 $\log_{10}$ copies mL-^{1}, respectively) compared to the F1-O flock (5.41 $\log_{10}$ copies/mL) (**Table 4**). This was a complete reversal of what was observed in the first trial.

While *L. monocytogenes* was not detected in any of the samples from this trial, both *Salmonella* and *C. jejuni* were more prevalent in both the GIT and yolk samples from the F1-P (*C. jejuni* only) and F1-O (*Salmonella* and *C. jejuni*) flocks compared to trial 1 (**Table 4**). *Salmonella* GIT and yolk concentrations were not affected by the embryonic age. Interestingly, the highest GIT, yolk, and EW pathogen loads were found in the oldest broiler breeder flock. Compared to the first trial, the embryonic age affected *C. jejuni* concentrations from both GIT and yolk samples from the F1-P flock. Furthermore, *C. jejuni* significantly decreased by T4 in the GIT samples, whereas they significantly increased in the T4 yolk samples, as compared to the concentrations found at T1. Overall, *C. jejuni* GIT, yolk, and EW concentrations were 1 to 3 $\log_{10}$ and 4 to 6 $\log_{10}$ higher in the F1-P flock compared to the F1-O and F1-Y (**Table 4**). Overall, embryonic age was only found to significantly affect *C. jejuni* yolk concentrations, whereas broiler breeder flock age had a highly significant ($p < 0.0001$) effect on *Salmonella* and *C. jejuni* concentrations for all three sample types (**Table 5**).

DISCUSSION

Foodborne pathogens that are able to contaminate eggs through vertical integration are a significant concern to the broiler industry. The rise in antibiotic resistant foodborne pathogens is exceptionally well documented, increasing the scrutiny of poultry production from egg to fork (Economou and Gousia, 2015; McCrackin et al., 2015). However, two facts need to be considered concerning eggs and food safety: (1) other foodborne pathogens that cause significant concern, such as *C. jejuni*, are transmitted to the GIT of embryonic broiler chicks, and (2) broiler eggs can be a potential contributor to foodborne outbreaks (Cox et al., 2002; Gole et al., 2014; Mughini-Gras et al., 2014).

Perhaps the most historically common pathogen that reduces production quality and decreases food safety is *Salmonella*. Table eggs are considered to be the other primary source of foodborne *Salmonella* besides poultry meat (Finstad et al., 2012; Howard et al., 2012; Ricke, 2017). *Salmonella*'s ability to infect eggs by invading and colonizing the reproductive tract of hens is a documented route of transmission in poultry with significant consequences to the industry (Gantois et al., 2009; Ricke, 2017). The early establishment of *Salmonella* increases the pervasive threat that the pathogen poses throughout poultry reproduction, rearing, and processing; it can actively disseminate to peripheral organs and contaminate meat. Interestingly, the internal environment of the egg became a more prominent concern after increasing the number of *S. enterica* Enteritidis

TABLE 5 | Two-way (GIT, Yolk) and One-way (EW) ANOVA results at three different flock ages for one commercial broiler breeder flock at four times during embryonic development (trial 2)[1,2].

Type		GIT					Yolk					EW				
		SS	DF	MS	F	*P*-value	SS	DF	MS	F	*P*-value	SS	DF	MS	F	*P*-value
Total Bacteria (16S)	Breeder Flock Age	51.34	2	25.67	91.57	**<0.0001**	118.90	2	59.46	1971.0	**<0.0001**	6.97	2	3.49	98.01	**<0.0001**
	Embryonic Age	46.52	3	15.51	81.72	**<0.0001**	1.41	3	0.47	18.65	**<0.0001**					
	Interaction	27.33	6	4.56	24.01	**<0.0001**	2.20	6	0.37	14.52	**<0.0001**					
	Subjects (matching)	4.21	15	0.28	1.48	0.15	0.45	15	0.03	1.19	0.31					
	Residual	8.54	45	0.19			1.14	45	0.03			0.53	15	0.04		
Salmonella (invA)[3]	Breeder Flock Age	130.30	2	65.17	278.20	**<0.0001**	136.90	2	68.44	411.7	**<0.0001**	30.07	2	15.03	1059	**<0.0001**
	Embryonic Age	1.76	3	0.59	2.14	0.11	0.24	3	0.08	0.64	0.60					
	Interaction	1.00	6	0.17	0.61	0.72	0.85	6	0.14	1.11	0.37					
	Subjects (matching)	3.51	15	0.23	0.86	0.61	2.49	15	0.17	1.30	0.24					
	Residual	12.32	45	0.27			5.76	45	0.13			0.21	15	0.014		
C.jejuni (hipO)	Breeder Flock Age	355.60	2	177.80	656.20	<0.0001	282.20	2	141.10	12490.00	**<0.0001**	68.89	2	34.44	62.16	**<0.0001**
	Embryonic Age	1.14	3	0.38	1.20	0.32	18.54	3	6.18	37.42	**<0.0001**					
	Interaction	4.48	6	0.75	2.36	**0.05**	29.14	6	4.86	29.41	**<0.0001**					
	Subjects (matching)	4.06	15	0.27	0.85	0.62	1.69	15	0.11	0.68	0.79					
	Residual	14.28	45	0.32			7.43	45	0.17			8.31	15	0.55		

[1]GIT, Gastrointestinal Tract; EW, Egg Wash; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean of Squares. [2]Bolded P-values indicate that ANTOVA analyses found those variables to be significant ($p \leq 0$).
[3]Reflects that the assay was performed using SYBR not TaqMan.

outbreaks occurred with some of these originating from internal contamination of table shell eggs (St Louis et al., 1988; Henzler, et al., 1994; Cox et al., 2000; Gantois et al., 2009; Howard et al., 2012; Ricke, 2017). In this instance, *S.* Enteritidis was identified by USDA-APHIS from multiple farms and houses on a farm (Henzler et al., 1994). Epidemiologists were able to link *Salmonella* contamination with three independent outbreaks of salmonellosis in the U.S. to those barns, and specifically to the broiler eggs. Environmental sampling of a layer facility is consistently correlated with egg contamination by pathogens (Gole et al., 2014).

Controversy remains as to the ability of other pathogens use their invasion apparatus and colonize the embryonic chicken. Previous studies demonstrate the ability to isolate naturally occurring *C. jejuni* from the circulating blood of broilers as well as the internal organs such as the spleen, liver, adrenal glands, and gall bladder. While this isolation of the pathogen in the periphery can be indicative of the breakdown of stability of the gut barrier, additional evidence has emerged to implicate the vertical transmission of *C. jejuni.* Studies documented the pathogen's presence in the immature and mature follicles of breeders and the egg shells in commercial laying hens (Cox et al., 2005, 2006, 2007; Richardson et al., 2011; Jones et al., 2016). This is a concern because the establishment of *C. jejuni* in the embryonic gut may have significant consequences for production. The experimental inoculation of chickens by Salmonella and C. jejuni both stimulate the immune and intestinal inflammatory responses (Humphrey et al., 2014; Han et al., 2016). The resulting inflammatory response negatively affects poultry health and production. However, despite the evidence to the contrary and presented herein, the vertical transmission and immune stimulating potential of *Campylobacter* in poultry remains controversial because data challenging the establishment of the vertical route of transmission paradigm continues to emerge (Sahin et al., 2002, 2003; Herman et al., 2003; Newell and Fearnley, 2003; Snelling et al., 2005; Humphrey et al., 2007; Silva et al., 2011).

Current qPCR strategies are also highly correlated to microbial plating techniques to detect total microbial load (Gole et al., 2014) and have been used to quantitate expression of individual genes in *Salmonella* and *C. jejuni* as well as quantitation of both organisms (Gharst et al., 2013; Park et al., 2014; Papić et al., 2017; Neal-McKinney et al., 2018; Ricke et al., 2018). This study directly challenges the sampling paradigm because EW were largely negative throughout the study, with the exception of post-vaccination time point during each trial. Egg washes do not appear to be a good predictor of pathogen invasion within the yolk. Furthermore, this study successfully quantified microbial and pathogen load in an egg. With the exception of *L. monocytogenes*, the pathogens *S. enterica* and *C. jejuni* were identified in the GIT and yolk of the embryonic chick.

The improved detection of pathogens has two goals: (1) improving poultry health which safeguards the food supply; and (2) reducing the transmission of antibiotic resistant pathogens and elements. There is a rise in foodborne pathogens containing antibiotic resistance elements that are capable of disseminating to the naïve microbiota (Nguyen et al., 2016; Kassem et al., 2017; Vita et al., 2018). Therefore, the uncontrolled spread of antibiotic resistant foodborne pathogens increases the risk to the food supply and potentially result in resistant clinical infections (Economou and Gousia, 2015; McCrackin et al., 2015; Battersby et al., 2017; Kassem et al., 2017). Therefore, the detection of these pathogens and the antibiotic resistance profile exhibited is essential to safeguard the food supply. To address these concerns, future studies should include the development of a qPCR system to detect various serovars of *Salmonella* (Mughini-Gras et al., 2014). By expanding on these ideas, the facilitation of the assignment of responsibility and improvement of food safety is innovated.

The implementation of the qPCR strategy delineated in this paper could actively mitigate the risk of the vertical transmission of foodborne pathogens. The sampling of hens occurs monthly or during outbreaks. However, it is well documented that cloacal swabs are not direct indicators of colonization (Langkabel et al., 2014; Battersby et al., 2017). Therefore, by using a "spot check" strategy that looks directly at the egg and the GIT of embryonic chicks is valuable. By doing so, hens with a high rate of transmission of invasive pathogens can be readily identified and culled.

AUTHOR CONTRIBUTIONS

MR, AL, KH, JG, SP, and HS designed the experiments, executed the experiments. MR, KF, SK, and SR wrote the manuscript. MR, KF, and SR provided the final analysis, provided ample edits, and finalized the manuscript.

REFERENCES

Battersby, T., Walsh, D., Whyte, P., and Bolton, D. (2017). Evaluating and improving terminal hygiene practices on broiler farms to prevent Campylobacter cross-contamination between flocks. *Food Microbiol.* 64, 1–6. doi: 10.1016/j.fm.2016.11.018

Claud, E. C., and Walker, W. A. (2001). Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *FASEB* 15, 1398–1403. doi: 10.1096/fj.00-0833hyp

Cox, N. A., Bailey, J. S., Richardson, L. J., Buhr, R. J., Cosby, D. E., Wilson, J. L., et al. (2005). Presence of naturally occurring Campylobacter and *Salmonella* in mature and immature ovarian follicles of late-life broiler breeding hens. *Avian Dis.* 49, 285–287. doi: 10.1637/7324-011005

Cox, N. A., Berrang, M. E., and Cason, J. A. (2000). *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs—a review. *Poult. Sci.* 79, 1571–1574. doi: 10.1093/ps/79.11.1571

Cox, N. A., Richardson, L. J., Buhr, R. J., Fedorka-Cray, P. J., Bailey, J. S., Wilson, J. L., et al. (2006). Natural presence of *Campylobacter* spp. in various internal organs of commercial breeder hens. *Avian Dis.* 50, 450–453. doi: 10.1637/7481-120205R.1

Cox, N. A., Richardson, L. J., Buhr, R. J., Northcutt, J. K., Bailey, J. S., Cray, P. F., et al. (2007). Recovery of *Campylobacter* and *Salmonella* serovars from spleen, liver, gallbladder, and ceca of six- and eight-week-old commercial broilers. *Poult. Sci.* 16, 477–480.

Cox, N. A., Stern, N. J., Hiett, K. L., and Berrang, M. E. (2002). Identification of a new source of *Campylobacter* contamination in poultry: transmission from breeder hens to broiler chickens. *Avian Dis.* 46, 535–541.

Economou, V., and Gousia, P. (2015). Agriculture as food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 8, 49–61. doi: 10.2147/IDR.S55778

European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control. (2012). The European Union summary report on trends and sources of zoonoses, zoonotic agents, and foodborne outbreaks in 2010. *EFSA J.* 10:2597. doi: 10.2903/j.efsa.2012.2597

Fey, A., Eichler, S., Flavier, S., Christen, R., Hofle, M. G., and Guzman, C. A. (2004). Establishment of a real-time PCR-based approach for the accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. *Appl. Environ. Microbiol.* 70, 3618–3623. doi: 10.1128/AEM.70.6.3618-3623.2004

Finstad, S., O-Bryan, C. A., Marcy, J. A., Crandall, P. G., and Ricke, S. C. (2012). *Salmonella* and other broiler processing in the United States: relationship to foodborne salmonellosis. *Food Res. Int.* 45, 789–794. doi: 10.1016/j.foodres.2011.03.057

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., et al. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiol. Rev.* 33, 718–738. doi: 10.1111/j.1574-6976.2008.00161.x

Gharst, G., Oyarzabal, O. A., and Hussain, S. K. (2013). Review of current methodologies to isolate and identify *Campylobacter* spp. from foods. *J. Microbiol. Methods* 95, 84–92. doi: 10.1016/j.mimet.2013.07.014

Gole, V. C., Torok, V., Sexton, M., Caraguel, C. G. B., and Chousalkar, K. K. (2014). Association between indoor environmental contamination by *Salmonella enterica* and contamination of eggs on layer farms. *JCM* 57, 3250–3258. doi: 10.1128/JCM.00816-14

Han, Z., Willer, T., Reisticker, C., Gerzova, L., Rychlik, I., and Rautenschlein, S. (2016). Differences in host breed and diet influence colonization by Campylobacter jejuni and induction of local immune responses in chickens. *Gut Pathog.* 8, 1–14. doi: 10.1186/s13099-016-1

Harms, G., Layton, A. C., Dionisi, H. M., Gregory, I. R., Garrett, V. M., Hawkins, S. A., et al. (2003). Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ. Sci. Technol.* 37, 343–351. doi: 10.1021/es0257164

He, Y., Yao, X., Gunther, N. W., Xie, Y., Tu, S. I., and Shi, X. (2010). Simultaneous detection and differentiation of *Campylobacter jejuni*, *C. coli*, and *C. lari* in chickens using a multiplex real-time PCR assay. *Food Anal. Methods* 3, 321–329. doi: 10.1007/s12161-010-9136-6

Henzler, D. J., Ebel, E., Sanders, J., Krandel, D., and Mason, J. (1994). *Salmonella* Enteritidis in eggs from commercial chicken layer flocks implicated in human outbreaks. *Avian Dis.* 38, 37–43. doi: 10.2307/1591834

Herman, L., Heyndrickx, M., Grijspeerdt, K., Vandekerchove, D., Roller, I., and DeZutter, L. (2003). Routes for *Campylobacter* contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiol. Infect.* 131, 1169–1180. doi: 10.1017/S0950268803001183

Heyndrickx, M., Vandekerchove, D., Herman, L., Rollier, I., Grijspeerdt, K., and De Zutter, L. (2002). Routes for Salmonella contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiol. Infect.* 129, 253–265. doi: 10.1017/S0950268802007380

Howard, Z. R., O-Bryan, C. A., Crandall, P. G., and Ricke, S. C. (2012). *Salmonella* Enteritidis in shell eggs: current issues and prospects for control. *Food Res. Int.* 45, 755–764. doi: 10.1016/j.foodres.2011.04.030

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., et al. (2014). Campylobacter jejuni is not merely a commensal in commercial broiler chickens and affects bird welfare. *mBio* 5:e1364-14. doi: 10.1128/mBio.01364-14

Humphrey, T., O'Brien, S., and Madsen, M. (2007). Campylobacters as zoonotic pathogens: a food production study. *Int. J. Food Microbiol.* 117, 237–257. doi: 10.1016/j.ijfoodmicro.2007.01.006

Jones, D. R., Guard, J., Gast, R. K., Buhr, R. J., Gedorka-Cray, P. J., Abdo, Z., et al. (2016). Influence of commercial laying hens housing systems on the incidence and identification of *Salmonella* and *Campylobacter*. *Poult. Sci.* 95, 1116–1124. doi: 10.3382/ps/pew036

Kassem, I. I., Kehinde, O., Kumar, A., and Rajashekra, G. (2017). Antimicrobial-resistant Campylobacter in organically and conventionally raised layer chickens. *Foodborne Pathog. Dis.* 14, 29–34. doi: 10.1089/fpd.2016.2161

Langkabel, N., Klose, A., Irsigler, H., Jaeger, D., Bräutigam, L., Hafez, H. M., et al. (2014). Comparison of methods for the detection of *Salmonella* in poultry. *JAPR* 23, 403–408. doi: 10.3382/jarp.2013-0085

McCrackin, M. A., Kelke, K. L., Galloway, A. M., Poole, A. Z., Salgado, C. D., and Marriott, B. P. (2015). Effect of antimicrobial use in agriculture animals on drug-resistant foodborne Campylobacteriosis in humans: a systemic literature review. *Crit. Rev. Food Sci. Nutr.* 56, 2115–2132. doi: 10.1080/10408398.2015.1119798

Mughini-Gras, L., Enserink, R., Friesma, I., Heck, M., van Duynhoven, Y., and van Pelt, W. (2014). Risk factors for human Salmonellosis originating from pigs, cattle, broiler chickens, and egg laying hens: a combined case-control and source attribution analysis. *PLoS One* 9:e87933. doi: 10.137/journal.pone.0087933

Neal-McKinney, J. M., Liu, K. C., Jinneman, K. C., Wu, W. H., and Rice, D. H. (2018). Whole genome sequencing and multiplex of qPCR methods to identify *Campylobacter jejuni* encoding cst-11 or cst-III sialyltransferase. *Front. Microbiol.* 9:408. doi: 10.3389/fmicb.2018.00408

Newell, D. G., and Fearnley, C. (2003). Sources of Campylobacter colonization in broiler chickens. *Appl. Exp. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Nguyen, T. N. M., Hotzel, H., Njeru, J., Mwituria, J., B-Adaway, H., Tomaso, H., et al. (2016). Antimicrobial resistance of *Campylobacter* isolates from small scale and backyard chickens in Kenya. *Gut Pathog.* 8:39. doi: 10.1186/sf13099-016-0121-5

Papić, B., Pate, M., Henigman, U., Zajc, U., Gruntar, I., Biasizzo, M., et al., (2017). New approaches on Quantification of *Campylobacter jejuni* in poultry samples: the use of digital PCR and real-time PCR against the ISO standard plate count method. *Front. Microbiol.* 8:331. doi: 10.3389/fmicb.2017.00331

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., et al. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food Microbiol.* 38, 250–262. doi: 10.1016/j.fm.2013.10.002

Rajan, K., Shi, Z., and Ricke, S. C. (2017). Current aspects of *Salmonella* contamination in the US poultry production chain and the potential application of risk strategies in understanding emerging hazards. *Crit. Rev. Microbiol.* 43, 370–392. doi: 10.1080/1040841X.2016.1223600

Richardson, J. L., Cox, N. A., Buhr, R. J., and Harrison, M. A. (2011). Isolation of *Campylobacter* from circulating blood of commercial broilers. *Avian Dis.* 55, 375–378. doi: 10.1637/9613-121310-Reg.1

Ricke, S. C. (2017). Insights and challenges of *Salmonella* infection of laying hens. *Curr. Opin. Food Sci.* 18, 43–49. doi: 10.1016/j.cofs.2017.10.012

Ricke, S. C., Kim, S. A., Shi, Z., and Park, S. H. (2018). Molecular-based identification and detection of *Salmonella* in food production systems: current prospectives. *J. Appl. Microbiol.* 125, 313–327. doi: 10.1111/jam.13888

Rothrock, M. J. Jr., Hiett, K. L., Gamble, J., Caudill, A. C., Cicconi-Hogan, K. M., and Caporaso, J. G. (2014). A hybrid DNA extraction method for the qualitative and quantitative assessment of bacterial communities from poultry production samples. *J. Vis. Exp.* 94:52161. doi: 10.3791/52161

Sahin, O., Kobalka, P., and Zhang, Q. (2003). Detection and survival of Campylobacter in chicken eggs. *J. Appl. Microbiol.* 95, 1070–1079. doi: 10.1046/j.1365-2672.2003.02083.x

Sahin, O., Morishita, T. Y., and Zhang, Q. (2002). Campylobacter colonization in poultry: sources of infection and modes of transmission. *CAB Int.* 3, 95–105. doi: 10.1079/AHRR200244

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeria, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Snelling, W. J., Moore, J. E., and Dooley, J. S. G. (2005). The colonization of broilers with *Campylobacter*. *Worlds Poult. Sci. J.* 61, 655–662. doi: 10.1079/WPS200577

St Louis, M. E., Morse, D. L., Potter, M. E., DeMelfi, T. M., Guzewich, J. J., Tauxe, R. V., et al. (1988). The emergence of grade A eggs as a major source of *Salmonella* Enteritidis infections: new implications for the control of salmonellosis. *JAMA* 259, 2103–2107. doi: 10.1001/jama.1988.03720140023028

Suo, B., He, Y., Tu, S., and Shi, X. (2010). A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* 157, and *Listeria monocytogenes* in meat products. *Foodborne Pathog. Dis.* 7, 619–628. doi: 10.1089/fpd.2009.0430

United States Department of Agriculture-Animal and Plant Health Inspection Services [USDA-APHIS] (2007). *Prevention, Detection and Control of Salmonella in Poultry. CAC/RCP 15/1976.* Available at: https://www.aphis.usda.gov/import_export/animals/oie/downloads/tahc_sep09/tahc_pre_detec_cont_salm_poul_78_sep09.pdf

Vita, S., Devros, A., Perkaska, K., Dace, R., Elvira, L., Piekuse, L., et al. (2018). Gut colonization with extended-spectrum β lactamase-producing *Enterobacteriaceae* may increase disease activity in biological-naïve outpatients with ulcerative colitis: an interm analysis. *Eur. J. Gastroenterol. Hepatol.* 30, 92–100.

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 November 2018
doi: 10.3389/fmicb.2018.02674

Transducer-Like Protein in *Campylobacter jejuni* With a Role in Mediating Chemotaxis to Iron and Phosphate

*Kshipra Chandrashekhar[1], Vishal Srivastava[1], Sunyoung Hwang[2†], Byeonghwa Jeon[3], Sangryeol Ryu[2] and Gireesh Rajashekara[1]**

[1] Food Animal Health Research Program, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH, United States, [2] Department of Food and Animal Biotechnology – Department of Agricultural Biotechnology, Center for Agricultural Biomaterials, Seoul National University, Seoul, South Korea, [3] School of Public Health, University of Alberta, Edmonton, AB, Canada

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Young Min Kwon,
University of Arkansas, United States
Ximin Zeng,
University of Tennessee, Knoxville, United States

***Correspondence:**
Gireesh Rajashekara
rajashekara.2@osu.edu

†Present address:
Sunyoung Hwang,
National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong, South Korea

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

Received: 07 August 2018
Accepted: 19 October 2018
Published: 16 November 2018

Citation:
Chandrashekhar K, Srivastava V, Hwang S, Jeon B, Ryu S and Rajashekara G (2018) Transducer-Like Protein in Campylobacter jejuni With a Role in Mediating Chemotaxis to Iron and Phosphate. Front. Microbiol. 9:2674. doi: 10.3389/fmicb.2018.02674

Chemotaxis-mediated motility enables *Campylobacter jejuni* to navigate through complex environmental gradients and colonize diverse niches. *C. jejuni* is known to possess several methyl accepting chemotaxis proteins (MCPs), also called transducer-like proteins (Tlps). While the role of some of the Tlps in chemotaxis has been identified, their regulation and role in virulence is still not very clear. Here, we investigated the contribution of Tlp2 to *C. jejuni* chemotaxis, stress survival and colonization of the chicken gastrointestinal tract. The Δ*tlp2* deletion mutant showed decreased chemotaxis toward aspartate, pyruvate, inorganic phosphate (Pi), and iron ($FeSO_4$). Transcriptional analysis of *tlp2* with a promoter fusion reporter assay revealed that the *tlp2* promoter (P_{tlp2}) was induced by Pi and iron, both in the ferrous (Fe^{2+}) and ferric form (Fe^{3+}). RT-PCR analysis using overlapping primers indicated that the *phoX* gene, located immediately downstream of *tlp2*, is co-transcribed with *tlp2*. A transcription start site was identified at 53 bp upstream of the *tlp2* start codon. The Δ*tlp2* mutant showed decreased colonization of the chicken gastrointestinal tract. Collectively, our findings revealed that the *tlp2* plays a role in *C. jejuni* pathogenesis and colonization in the chicken host and its expression is regulated by iron.

Keywords: transducer like protein, chemotaxis, iron, regulation, promoter

INTRODUCTION

Foodborne gastrointestinal illness caused by a gram negative bacterium, *Campylobacter jejuni*, has seen a surge in incidence in the recent years (CDC, 2013). In the United States, Food and Drug Administration (FDA) has placed *Campylobacter* species in the list of "qualifying pathogens" capable of posing a serious public health risk (Food and Drug Administration and HHS, 2014). The prevalence and transmission of *Campylobacter* can be attributed to its widespread colonization in the gastrointestinal tract of farm animals, especially chickens (Hermans et al., 2012). It is well established that *C. jejuni* employs motility and chemotaxis to colonize the avian and mammalian gastrointestinal tract (Yao et al., 1994; Hendrixson and DiRita, 2004; Young et al., 2007; Hermans et al., 2011; Chandrashekhar et al., 2015, 2017). Directional motility in *C. jejuni* is mediated by

the chemotaxis system, composed of chemoreceptors and other core signal transduction proteins (Lertsethtakarn et al., 2011).

Transducer like proteins (Tlps) are the key components involved in sensing environmental signals through chemotaxis or energy taxis in *C. jejuni*. (Marchant et al., 2002; Vegge et al., 2009; Korolik, 2010; Tareen et al., 2010; Reuter and van Vliet, 2013; Rahman et al., 2014). Amino acids (aspartate, glutamate and serine), organic acid salts (succinate, isocitrate, and formate), bile and mucin are chemoattractants for *C. jejuni* (Hugdahl et al., 1988; Hartley-Tassell et al., 2010; Tareen et al., 2010). *C. jejuni* Tlps have been classified into three groups (A-C), based on sequence analysis and structural homology (Marchant et al., 2002; Chandrashekhar et al., 2017). The *C. jejuni* Tlp2 (CJJ81176_0180) is a group A transducer-like protein (Marchant et al., 2002) with transmembrane domains, a periplasmic ligand binding domain and a cytoplasmic signaling domain. BLAST analysis of the predicted amino acid sequence of Tlp2 shows greatest homology to *C. jejuni* Tlp3 and Tlp4 (60% identity). The cytoplasmic signaling domain is identical to Tlp3 but the periplasmic domain shows only 38% identity with Tlp3 (Rahman et al., 2014). An earlier study in *C. jejuni* NCTC11168 strain revealed that *tlp2* deletion mutant exhibited no chemotaxis and invasion defects (Vegge et al., 2009). However, recent evidence indicates that *tlp2* is one of the most abundantly expressed *tlps* in mice infected with *C. jejuni* NCTC 11168-O (Day et al., 2012), thus emphasizing the significance of understanding the role of Tlp2 in *C. jejuni* pathophysiology. This warranted us to further investigate the role of *C. jejuni* Tlp2 in chemotaxis, virulence, and host colonization.

Iron is an essential nutrient and a cofactor for proteins involved in cellular metabolism, enzyme catalysis, and sensing extracellular and intracellular signals (Lill, 2009). The bioavailability of iron in the host and environment (10^{-18}–10^{-24}M) being lower than the minimum requirement for bacterial growth (10^{-7}M), makes iron a key player in the host-pathogen interaction (Braun and Hantke, 2003). Chemotaxis toward iron has been studied in *Shewanella oneidensis* and the magnetotactic bacteria *Geobacter metallireducens* (Childers et al., 2002; Bencharit and Ward, 2005). In these bacteria, the chemotactic response to iron is due to the fact that it serves as an insoluble electron acceptor (Childers et al., 2002; Bencharit and Ward, 2005; Harris et al., 2010). Knowledge about the role of iron as an electron acceptor in *C. jejuni,* chemotaxis toward iron and/or regulation of Tlp genes by iron in *C. jejuni* is still scarce. However, a study in *Helicobacter pylori* indicated that *tlpB,* chemoreceptor for sensing bicarbonate and arginine, is induced by iron through a *fur*-independent mechanism (Ernst et al., 2005). Interestingly, a recent study in *C. jejuni* has identified that *tlp* genes (Cj0262c and Cj1110c) are regulated by iron and/or Ferric uptake regulator (Fur) protein (Butcher et al., 2012). The study also revealed that *cj0145* (*phoX),* a gene located immediately downstream of *tlp2,* is induced in the presence of iron although the specific mechanism of regulation is still unexplored (Butcher et al., 2012).

Here we investigated the role *tlp2* in *C. jejuni* chemotaxis, *in-vitro* virulence and colonization of the chicken gastrointestinal tract. We provide evidence that iron regulates chemotaxis in *C. jejuni* and *tlp2* contributes to *in-vivo* colonization of the chicken gastrointestinal tract. The findings of this study not only highlight the significance of *tlp2* in *C. jejuni* pathogenesis but also elaborate on the complex mechanism by which iron regulates the chemotaxis in *C. jejuni* through Tlp2.

MATERIALS AND METHODS

Bacterial Strains, Media and Growth Conditions

Bacterial strains and plasmids used in this study are described in **Table 1**. *C. jejuni* used in this study are derivatives of strain 81–176 (WT) (Korlath et al., 1985) and NCTC 11168. *C. jejuni* strains were grown on Mueller-Hinton media (MH; Oxoid, Hampshire, United Kingdom) under microaerophilic conditions [(85% N_2 (v/v), 10% CO_2 (v/v) and 5% O_2 (v/v)] in a DG250 Microaerophilic Workstation (Microbiology International, Frederick, Maryland, United States) at 42°C. *E. coli* DH5α was used for plasmid propagation and cloning purposes and was routinely cultured on Luria-Bertani (LB) medium at 37°C overnight. Growth media was supplemented with appropriate antibiotics; chloramphenicol (10 μg/ml for *Campylobacter*; 20 μg/ml for *E. coli*) and kanamycin (30 μg/ml for *Campylobacter*; 50 μg/ml for *E. coli*) as required.

Generation and Complementation of *tlp2* Mutant

Recombinant DNA techniques were performed as per standard procedures (Sambrook et al., 1989). *C. jejuni tlp2* mutant was created by double crossover allelic exchange method as previously described (Rajashekara et al., 2009). Oligonucleotides used in the present study were synthesized from Integrated DNA Technologies (Skokie, IL, United States) and are listed in **Table 2**. Briefly, the gene of interest (*tlp2*) plus ∼ 1 kb flanking DNA was amplified by PCR from *C. jejuni* strain 81–176 genome. The purified PCR products were ligated into zeocin-resistant pZErO-1 (zero background cloning vector) (Invitrogen, Carlsbad, CA, United States), and the ligation product was transformed into Library Efficiency DH5α *E. coli* competent cells (Invitrogen) to generate the plasmid pZErO1-*tlp2*. The whole plasmid except the target gene was amplified by inverse PCR. Purified inverse PCR products were ligated either to a kanamycin resistant cassette (from pUC4K) or a chloramphenicol resistance cassette (from pUC4C), and the resulting suicide vector was electroporated into *C. jejuni*. Transformants were selected on MH agar supplemented with chloramphenicol or kanamycin. Individual clones were confirmed for deletion of the target gene by PCR. The *tlp2* mutant with kanamycin resistance was used in all the assays; except for reporter studies, in which case *tlp2* mutant with chloramphenicol resistance was used as reporter plasmid carries kanamycin resistance.

The complemented strain was created by amplifying coding regions of *tlp2* along with its potential promoter region by PCR using primers indicated in **Table 2**. The resulting fragment was cloned into *Sal*I-*Kpn*I digested pRY112 (Yao et al., 1993) and the complementation plasmid was introduced into the

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains	Relevant description	Source/Reference
C. jejuni 81–176 WT	Wild type strain of *C. jejuni*	Dr. Qijing Zhang
Δ*tlp2*	*C. jejuni* 81–176 derivative with deletion in *tlp2* gene; *tlp2*::kan	This study
Δ*tlp2-cm*	*C. jejuni* 81–176 derivative with deletion in *tlp2* gene; *tlp2*::cm	This study
tlp2 comp	*C. jejuni 81–176 tlp2* mutant complemented with wild type copy of *tlp2* on pRY112	This study
C. jejuni NCTC11168Δ*fur*	*C. jejuni* NCTC11168 derivative with deletion in *fur* gene; *fur*::tet	Dr. Jun Lin
WT P_{tlp2}-pMW10	*C. jejuni* 81–176 WT reporter strain carrying P_{tlp2}-pMW10	This study
Δ*tlp2* P_{tlp2}-pMW10	*C. jejuni* 81–176 Δ*tlp2* strain carrying P_{tlp2}-pMW10	This study
Δ*fur* P_{tlp2}-pMW10	*C. jejuni* 81–176 Δ*fur* strain carrying P_{tlp2}-pMW10	This study
WT P_{int}-pMW10	*C. jejuni* 81–176 WT reporter strain carrying $P_{tlp2-phoXint}$-pMW10	This study
E. coli DH5α	*E. coli* strain used for cloning	Invitrogen
Plasmids		
pZero-1	Cloning vector for making suicide vector; Zeo	Invitrogen
pUC4K	Source plasmid for kanamycin resistance gene; Kan	Amersham
pUC4C	Source plasmid for chloramphenicol resistance gene; Cm	This study
pMW10	Promoter shuttle vector; pMW10, Kan	Wosten et al., 1998
pRY112	*E.coli*-Campylobacter shuttle vector for complementation; Cm	Yao et al., 1993
pRK2013	Helper plasmid for complementation; Kan	Grabowska et al., 2011
pZero1-*tlp2*	pZero-1 containing the upstream and downstream sequences of *tlp2*; Zeo	This study
pZero1-Δ*tlp2*-kan	pZero1-*tlp2* with *tlp2* gene replaced by the pUC4K kan gene through inverse PCR; Zeo, Kan	This study
pZero1-Δ*tlp2*-cm	pZero1-*tlp2*-kan where kanwas replaced by the chloramphenicol gene; Zeo, Cm	This study
P_{tlp2}-pMW10	pMW10 carrying the *tlp2* promoter; Kan	This study
P_{int}-pMW10	pMW10 carrying the intergenic region between *tlp2* and *phoX*; Kan	This study

Cm, chloramphenicol resistance; Kan, kanamycin resistance; Zeo, Zeocin resistance.

Δ*tlp2* deletion mutant by biparental conjugation as described (Miller et al., 2000). Transconjugants were selected on MH agar supplemented with kanamycin and chloramphenicol and the resulting complementation strain was designated *tlp2* comp as listed in **Table 1**.

Chemotaxis Assay

To quantify chemotaxis, we adapted a modified capillary chemotaxis assay that quantitatively measures bacterial tactic responses (Mazumder et al., 1999; Cerda et al., 2003). The assay was previously used for quantifying chemotaxis in subsurface microaerophilic bacteria including *Campylobacter* (Mazumder et al., 1999; Chandrashekhar et al., 2015) and other *Epsilonproteobacteria*, such as *H. pylori* (Cerda et al., 2003, 2011). Briefly, *C. jejuni* wild type (WT), Δ*tlp2* mutant and the complemented strains were grown microaerobically at 42°C for 18 h on MH agar and resuspended in chemotaxis buffer (Phosphate Buffered Saline, PBS or Normal Saline, pH 7.4) and OD_{600} was adjusted to 0.5. A 100 μl volume of a solution of the compounds [All compounds at 0.1M except Pi (Inorganic Ventures, Christiansburg, VA, United States) at 1 mM and $FeSO_4$ (Sigma) at 0.1 mM] to be tested for chemotaxis response (buffer alone served as control) was aspirated through a 22 G stainless-steel needle (0.254 mm diameter × 20 mm long) into a 1 ml tuberculin syringe. The 0.1 M concentration of the compounds was selected based on previous studies and a series of preliminary experiments that showed that 100 mM resulted in the strongest chemotaxis response (Vegge et al., 2009; Tareen et al., 2010). A 100 μl of the OD_{600} adjusted bacterial suspension was drawn into a 200 μl disposable pipette tip and the needle-syringe system was fitted to the pipette tip in such a way that the needle was immersed into the bacterial suspension. The system was positioned horizontally and incubated at 42°C for 1 h. The needle-syringe system was then separated from the bacterial suspension containing pipette tip and contents of the syringe were 10-fold serially diluted in chemotaxis buffer, plated onto MH agar plates and incubated at 42°C under microaerophilic conditions to determine colony-forming units (CFUs). Relative Chemotaxis Ratio (RCR) toward a test compound was ascertained as a ratio between the numbers of bacteria entering the test needle-syringes to those in the control needle-syringes. A test compound was considered as an attractant if the RCR was ≥ 2 (Mazumder et al., 1999). Results were expressed as the mean of three independent assays. A mutant was considered deficient in chemotaxis toward a substrate if both the corresponding RCR value was significantly < 2 ($P < 0.05$) and the CFU of the mutants were significantly lower ($P < 0.05$) than those of the wildtype. A *C. jejuni* 81–176 *cheY* mutant which is incapable of directional movement (negative control) (Yao et al., 1997) and 0.1% porcine gastric mucin (positive control; Sigma) were also used to evaluate the integrity of the assay. To test the response to repellents, *C. jejuni* cultures were mixed with a repellent and the bacteria that entered the syringe, which in this instance contained only buffer, to escape the repellent were quantified as described above. To account for any methodological bias, capillary chemotaxis results were further verified by using the disk method (Vegge et al., 2009) for selected compounds.

TABLE 2 | Oligonucleotide primers used in this study.

Name	Sequence
Primers for gene deletion	
*tlp 2*F	ATATATGGTACCTTGCTACTAGTAT TTTGTTC
*tlp 2*R	AATTAACTCGAGCATAACCTTGT GGTACTATA
*tlp 2*F inv	ATATATGGATCCGAGAACA TGGTAGAGGCTTT
*tlp 2*R inv	ATATATGGATCCCCAGCATC TCTAAAATTCTT
Complementation primers	
tlp 2 comp F	AATGAAGTCGACAAATTATA ACGATATTAAGC
tlp 2 comp R	AATTAAGGTACCAAAACCTTT TCTTCTTAACA
Primers for RT-PCR and (q)RT-PCR	
Intergenic region P-180/181 (P1) F	ATAGCGTAGCTCAAATTGAT
Intergenic region P-180/181 (P1) R	AAGCATAGCAGCACTTAAAT
tlp2 control (P2) F	TGCAAATCTTGCTAAAACTA
tlp2 control (P2) R	GTCCAAATTCATCATTGCTT
phoX control (P3) F	GCTATGGATTTAACAAAACT
phoX control (P3) R	GTTAAACTGTCCTACATACA
phosR F	GCAAACATAATCATCACAACCAC
phosR R	GAGAGCAAGGATACAAAGAAGC
pstS F	CCTTATACAAACTGGAATCAAATC
pstS R	GACACATCACTCATTACAAGC
pstC F	CGCTTATGCTTTAGGTATGAC
pstC R	GCTGCCATCACCACTATC
Primers for promoter fusion reporter studies and primer extension assay	
*tlp2*_PF_F	ACA TTG ACA TCC CGG GTA TTT GCA GC
*tlp2*_PF_R	AAT CAG TGA GAT CTT CAA TTT TAC GC
CJJ81-180_PE_temp_F	GGG GGC AAA ATA ACA TTG ACA TCT AGA G
CJJ81-180_PE_temp_R	GCA TCT TGA CTA TCT AAC TGT TCT ATA GG
CJ81-180_PE_R1	ACC TAA AAT TAT CAA ACA CAC TAC TGC G
CJJ81-180_PE_R2	TAA TTT ATT TCA GCA TTC ACA ACT TCA TG
CJJ81-181_PE_temp_F	AAA CTG CAG GTA TCA CTC AAA TCA ATG
CJJ81-181_PE_temp_R	ACC TAG CAA ATC CTT ATC CTT AAG C
CJJ81-181_PE_R1	TTG CAA AAA AAG CCA CCA TAG AAC C
CJJ81-181_PE_R2	TTA AAA CCT TTG CTT CAT AAC CTT GTG G

Determination of the *tlp2* Transcriptional Levels With Reporter Gene Assays

The partial coding region of *tlp2* and the upstream region was amplified with *tlp2*-PF_F (*Sma*I) and *tlp2*-PF_R (*Bgl*II) primers and cloned into pMW10, a shuttle vector for *E. coli* and *C. jejuni,* containing a promoterless *lacZ* gene (Wosten et al., 1998). The plasmid was mobilized into *C. jejuni* WT and Δ*tlp2::Cm* strains by electroporation. The Δ*tlp2::Cm* strain was used for reporter studies since pMW10 carries kanamycin resistance. *The*Δ*tlp2::Cm* was generated as described above. β-Galactosidase assay was performed with *C. jejuni* strains harboring the *tlp2* promoter (P_{tlp2})-*lacZ* transcriptional fusion construct, as described previously (Wosten et al., 1998). To examine the effect of Pi and iron on *tlp2* transcription, reporter strains were incubated in MOPS and MEMα (Life technologies, Invitrogen) supplemented with Pi and $FeSO_4$ or $FeCl_3$ (Iron), respectively.

Additionally, reporter fusions were also created for the intergenic region between *tlp2* (*Cjj81176_0180*) and *phoX* (*Cjj81176_181*) to determine any potential promoter in the intergenic region. The intergenic region was amplified with specific primers listed in **Table 2** and cloned into pMW10 using the *BamH*I-*Xba*I sites. Reporter gene assays were carried out as described above.

Reporter gene assays were also carried out in the *C. jejuni* 81–176 Δ*fur* mutant. *C. jejuni* 81–176 Δ*fur* mutant was created by natural transformation of WT containing the (P_{tlp2})-*lacZ* transcriptional fusion construct with genomic DNA from *C. jejuni* NCTC11168 Δ*fur* mutant as described previously (Jeon et al., 2008; Gangaiah et al., 2009). Briefly, 1 ml of *C. jejuni* WT reporter strain was resuspended to an OD_{600} of 0.5. Approximately, 5 μg of genomic DNA from *C. jejuni* NCTC11168 Δ*fur* mutant was added and incubated for 4 h microaerobically. The bacteria were plated on MH plates supplemented with appropriate antibiotics and incubated microaerobically at 42°C for 48 h. The deletion of the *fur* gene in 81–176 was confirmed by PCR.

RNA Extraction and Reverse Transcriptase Overlapping PCR

Briefly, *C. jejuni* WT grown overnight in MH agar plate was scraped and resuspended to an OD_{600} of 0.05 in MEM-α or MH broth and grown up to mid log phase (6 h), respectively. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and quantified using NanoDrop ND-2000c spectrophotometer (Wilmington, DE, United States). cDNA synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen), was used as a template for PCR with a set of overlapping primers for the *tlp2*, *phoX,* and the 135 base pairs intergenic region between *tlp2* and *phoX*. (**Table 2**).

Primer Extension Assay

Primer extension assay was performed as described previously (Kim et al., 2011). Briefly, *C. jejuni* WT strain was grown for 6 h (mid-log phase) with shaking in MH broth at 42°C and harvested by centrifugation at 10,000 × *g* for 5 min. Total RNA was purified with TRIzol (Invitrogen) according to the manufacturer's instructions. Purified RNA was resuspended in sterile distilled RNase-free water, and the RNA concentration was determined by measuring the OD of the solution at 260 and 280 nm using NanoVue (GE Healthcare). A portion (10 pmol) of the PE_R primer was labeled with ^{32}P at the 5′ end by 10 U

of T4 polynucleotide kinase (Invitrogen) and 80 μCi of [γ-^{32}P] dATP for 30 min at 37°C. The labeling mixture was heated at 70°C for 10 min and purified with MicroSpin G-25 columns (GE Healthcare). The γ-^{32}P-end-labeled primer (0.5 pmol) was co-precipitated with 15 μg of total RNA by the addition of sodium acetate and absolute ethanol. The pellet was washed with 75% ethanol, dried at room temperature, and resuspended in 20 μl of 250 mM KCl, 2 mM Tris (pH 7.9), and 0.2 mM EDTA. The mixture was heated to 65°C and then was allowed to cool to room temperature for 1 h. After annealing, 50 μl of reaction solution containing 5 μg of actinomycin D, 700 μM deoxynucleoside triphosphates, 10 mM $MgCl_2$, 5 mM DTT, 20 mM Tris (pH 7.6), 30 U of RNasin (Promega), and 150 U of Superscript® III reverse transcriptase (Invitrogen) was added. The mixture was incubated at 42°C for 70 min and treated with 100 U of RNase T1 (Invitrogen) at 37°C for 15 min. The sample was ethanol precipitated after addition of 1.4 μl of 5M NaCl with 2.5 volumes of absolute ethanol and then washed with 75% ethanol. Sample was resuspended with 6 μl of formamide dye and 4 μl of Tris-EDTA (pH 8.0) buffer and then denatured at 90°C for 3 min. The samples were resolved on 6% polyacrylamide-8M urea gels, and the reverse transcription signals were analyzed by using BAS 2500 (Fuji Film). Primers, CJJ81176_180_PE and CJJ81176_181_PE (**Table 2**) were used for sequencing the upstream regions of *tlp2* and *phoX*, for transcription start site with a SequiTherm EXCELII DNA sequencing system (Epicenter).

Alkaline Phosphatase Assay

PhoX activity was determined as described previously (Drozd et al., 2011). Briefly, WT, Δ*tlp2,* and the *tlp2 comp* strains were grown overnight on MH plates with appropriate antibiotics. The cultures were gently scraped, washed and resuspended in MEM and incubated at 42°C microaerobically with shaking for 2 h. Cultures were then centrifuged for 10 min at 7000 × *g* and supernatant was removed. Cells were gently washed with 50 mM MOPS buffer (pH 7.4) (Sigma) and incubated with shaking at 42°C for 2 h following which, OD_{600} readings were taken. Cells were pelleted and resuspended in PNPP buffer containing 2 mM p-nitrophenyl phosphate (PNPP; Sigma) and incubated at 37°C. OD measurements at 550 nm and 420 nm were taken, and the phosphatase activity was calculated as described previously (Wosten et al., 2006). The assay was performed a total of three times with duplicate samples in each assay. Additionally, effect of iron on PhoX activity was assessed by supplementation of $FeSO_4$ at 40 μM concentration in MOPS buffer.

Nutrient Downshift Assay

The role of *tlp2* in *C. jejuni* survival under nutrient downshift was assessed using MEM-α as described previously (Gangaiah et al., 2010). Briefly, mid-log-phase cultures of WT, Δ*tlp2*, and the *tlp2 comp* strains were pelleted, washed twice and resuspended in MEM-α with OD_{600} adjusted to 0.05. The bacterial suspensions were incubated microaerobically at 42°C with shaking. Samples were taken over time, serially diluted (10-fold) in MEM-α media and plated on MH agar for determining CFU. The experiment was performed three times and the average for each time point was taken.

Quantitative Reverse Transcriptase PCR (qRT-PCR) Analysis of Phosphate Uptake Genes

The *C. jejuni* WT and Δ*tlp2* cultures were assessed for changes in expression of phosphate uptake genes (*phosR, pstC,* and *pstS*) (Wosten et al., 2006). Briefly, *C. jejuni* WT and Δ*tlp2* strains were grown to mid-log phase in MEM-α microaerobically, with shaking at 42°C. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). RNA and cDNA concentrations and purity were determined using NanoDrop ND-2000c spectrophotometer (Wilmington, DE, United States). Quantitative RT-PCR was performed with a SensiMixPlus SYBR RT-PCR kit (Quantace, Norwood, MA, United States) in a Mastercycler ep realplex2 thermal cycler (Eppendorf, Westbury, NY, United States). Gene specific primers (**Table 2**) used in this analysis have been described previously (Drozd et al., 2014). The relative levels of expression of target genes were normalized to 16S rRNA gene expression of the same strain. The relative fold changes in gene expression was calculated using the comparative threshold cycle (CT) method to yield fold-difference in transcript level compared to WT (Livak and Schmittgen, 2001). The qRT-PCR was performed a total of three times with duplicate samples in each assay.

Invasion and Intracellular Survival Assays

Invasion and intracellular survival of *C. jejuni* WT and Δ*tlp2* mutant in INT 407 cell line (human embryonic intestine cells, ATCC CCL 6) was assessed as described previously (Kassem et al., 2012). Briefly, mid-log phase grown bacterial cells were collected by centrifugation (5,000 × *g,* 10 min), washed twice with MEM containing 1% (v/v) FBS and resuspended in MEM. INT 407 cells (1.4×10^5 per well) in MEM with 10% (v/v) fetal bovine serum (FBS) were seeded in 24-well tissue culture plate and incubated for 18 h at 37°C with 5% CO_2. INT 407 cells were infected with multiplicity of infection (MOI) 100 for invasion and intracellular survival assays and incubated for 3 h at 37°C. Following 3 h of incubation with bacteria; cells were treated with gentamicin (150 μg/ml) and incubated for additional 2 h. After 2 h of incubation, the infected cells were rinsed three times with MEM, lysed with 0.1% (v/v) Triton-X 100, serially diluted in MEM and plated on MH agar. The percent invasion was calculated as follows: (no. of CFU recovered after lysis of INT 407 cells/CFU added to each well) × 100.

To assess survival of *C. jejuni* WT and Δ*tlp2* mutant in INT 407 cell line, following 2 h of gentamicin treatment, the infected cells were washed with MEM three times and covered with MEM containing gentamicin (10 μg/ml) and incubated for 24 h at 37°C. After 24 h of incubation, infected cells were washed with MEM, lysed and plated as described above. In parallel, we also cultured the supernatant of gentamicin treated monolayers to ensure the quality of the gentamicin protection assay.

Chicken Colonization Assay

Chicken colonization study was performed as described previously (Gangaiah et al., 2009). Briefly, 3 day-old specific pathogen free chickens (n = 6 for each group) were obtained from a local hatching facility (Food Animal Health Research Program, OARDC, Wooster, OH, United States). *Campylobacter* free chickens were inoculated orally with 10^4 CFU of the *C. jejuni* WT and Δ*tlp2* mutant strain in 200 μl of PBS (pH 7.4). Chickens were euthanized after 7 days post-inoculation and ceca, duodenum, jejunum, liver, spleen and bursa were collected aseptically, weighed, homogenized, serially diluted in PBS (pH 7.4) and plated on appropriate MH agar containing *Campylobacter* selective supplement with or without kanamycin to determine colony forming units (CFU). Plates were incubated at 42°C microaerobically and CFUs per gram of tissues were determined.

Statistical Analysis

Statistical significance of data generated in this study was determined using two tailed Student's t-test. Results of the promoter fusion assay were statistically analyzed using one way Anova with Dunett's multiple comparison posttests. Data from the chicken colonization experiment was analyzed using the Mann Whitney test. $P \leq 0.01$ or 0.05 (α level) was considered statistically significant.

RESULTS

The Δ*tlp2* Mutant Is Defective in Chemotaxis Toward Aspartate, Pyruvate, Pi and Iron

To assess the role of *tlp2* in *C. jejuni* chemotaxis, a deletion mutant was constructed with the coding region of *tlp2* being replaced with kanamycin resistance gene. Syringe capillary chemotaxis assays were performed to determine the chemotactic activity of *C. jejuni* WT, the Δ*tlp2* mutant and the complemented strains toward different substrates (**Table 3**). Substrates with RCR values > 2 and < 0.1 were considered as chemo attractants and repellants, respectively, for WT *C. jejuni* (Hugdahl et al., 1988; Cerda et al., 2003; Chandrashekhar et al., 2015). In addition, capillary assay showed strong chemotaxis of *C. jejuni* toward 0.1% porcine gastric mucin (RCR = 9.0), while a non-motile *cheY* mutant had an RCR below the detection limit (∼0) for some of the known attractants (Chandrashekhar et al., 2015). Compared to the WT, the Δ*tlp2* mutant was defective in chemotaxis toward aspartate (P = 0.0292) and pyruvate (P = 0.0010) with RCR values < 2 (**Figure 1**) (RCR values: aspartate: 3.81 for the WT and 1.45 for the Δ*tlp2* mutant; pyruvate: 2.96 for the WT and 0.33 for the Δ*tlp2* mutant) (Cerda et al., 2003). Even though the *tlp2* mutant showed RCR values less than 2 for isocitrate, succinate and propionate; they were not statistically significant (**Table 3**). Interestingly, Δ*tlp2* mutant also showed a chemotaxis defect toward Pi and iron ($FeSO_4$), compared to WT (**Figure 1**). We observed that $FeSO_4$, but $FeCl_3.6H_20$ (ferric iron source) and $(NH_4)_2SO_4$ (sulfate source) were not a chemoattractant for *C. jejuni*, based on the RCR indices for these compounds ($FeCl_3.6H_20$: RCR of 1.40 and $(NH_4)_2SO_4$: RCR of 0.40) (**Table 3**). The chemotaxis defect was restored to WT levels in the complemented strain; however, chemotaxis toward iron was partially restored in the complemented strain (**Figure 1**). Chemotaxis results were also confirmed by assessing chemotaxis

TABLE 3 | RCR values[a] for the WT and *tlp2* mutant for all compounds tested.

Chemicals tested	WT	Δ*tlp2*
Aspartate	3.81 ± 0.32	1.45 ± 0.35
L-glutamine	4.19 ± 0.77	2.93 ± 0.38
L-serine	2.04 ± 0.46	1.99 ± 0.82
Fumarate	9.32 ± 1.45	2.52 ± 0.79
Isocitrate	2.51 ± 0.41	1.5 ± 0.65[b]
Formate	4.41 ± 0.13	2.36 ± 1.05
Succinate	3.73 ± 1.2	1.2 ± 0.70[b]
Pyruvate	2.96 ± 0.566	0.33 ± 0.15
Propionate	2.97 ± 1.51	1.35 ± 0.30[b]
Inorganic phosphate	2.15 ± 0.46	0.4 ± 0.15
Deoxycholic acid	<0.1	<0.1
Cholic acid	<0.1	<0.1
$FeSO_4$	3.40 ± 0.58	0.66 ± 0.10
$FeCl_3.6H_20$[a]	1.4 ± 0.35	NT
$(NH_4)_2SO_4$[a]	0.1	NT

The results show the means and standard errors of three independent experiments. An RCR value of 2 or above indicates chemotaxis toward the test chemical (Mazumder et al., 1999). [a]The RCR value of the WT strain was <2.0, hence the compounds were not chemoattractants for C. jejuni 81–176 and were therefore not tested (NT) for chemotaxis with the mutant. [b]The RCR of these compounds are lesser than 2 for the tlp2 mutant, however, not significant statistically P > 0.05.

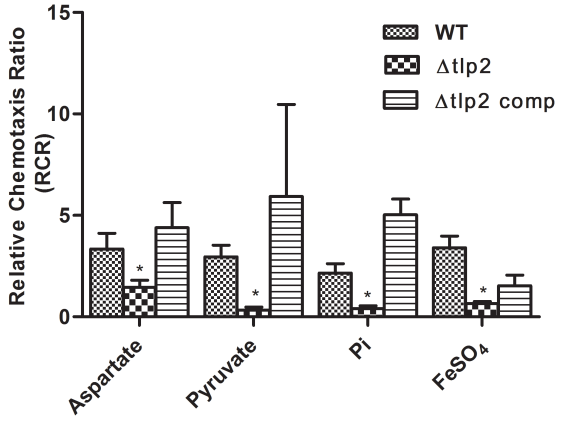

FIGURE 1 | The Δ*tlp2* mutant was significantly defective in chemotaxis toward aspartate, pyruvate, inorganic phosphate and ferrous sulfate. Chemotaxis was determined using the capillary method (Chandrashekhar et al., 2015) where an RCR-value of 2 or above indicates chemotaxis toward the test chemical. This graph represents only those compounds (out of 15 compounds) for which a defect in chemotaxis was observed in the Δ*tlp2* mutant. The complete results for chemotaxis toward all 15 compounds tested are listed in **Table 3**. *$P \leq 0.05$. The results show the means and standard errors of three independent experiments.

using the disk method for selected substrates such as aspartate and pyruvate (data not shown).

Iron Induces *tlp2* Promoter (P_{tlp2}) Activity

Decreased chemotaxis toward iron observed in the Δ*tlp2* mutant encouraged us to investigate the *tlp2* expression under different growth conditions. The level of *tlp2* transcription was quantified with β-galactosidase assays in presence of metal ions, such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} in MEM-α that does not contain these metals (van Vliet et al., 1998; Kim et al., 2011). Assay was performed in the presence of 20 μM $CuCl_2$, 40 μM $FeSO_4$, 40 μM $FeCl_3$, 40 μM $MnCl_2$, and 10 μM $ZnCl_2$. Since MEM-α media already has Ca^{2+} (1.8 mM) and Mg^{2+} (0.8 mM), we did not supplement the media with these two metal ions. Iron in both ferrous ($FeSO_4$) and ferric ($FeCl_3$) forms induced *tlp2* expression at 40 μM concentrations (**Figure 2A**), whereas other metals had no effect on the level of *tlp2* transcription (**Supplementary Figure S1A**). For Fe^{2+}, 40 μM was used based on the dose response assay (**Supplementary Figure S1B**) which showed best result at this concentration (**Supplementary Figure S1B**). Concentrations of iron as low as 5 μM $FeSO_4$ also significantly induced *tlp2* expression (**Supplementary Figure S1B**).

Similarly, the activity of P_{tlp2} was investigated in the presence of Pi due to the observed chemotaxis defect toward Pi (**Table 3** and **Figure 1**). MOPS buffer was used as a low phosphate medium for the incubation of the *C. jejuni* reporter strains (Gangaiah et al., 2009). The concentration of Pi added to MOPS

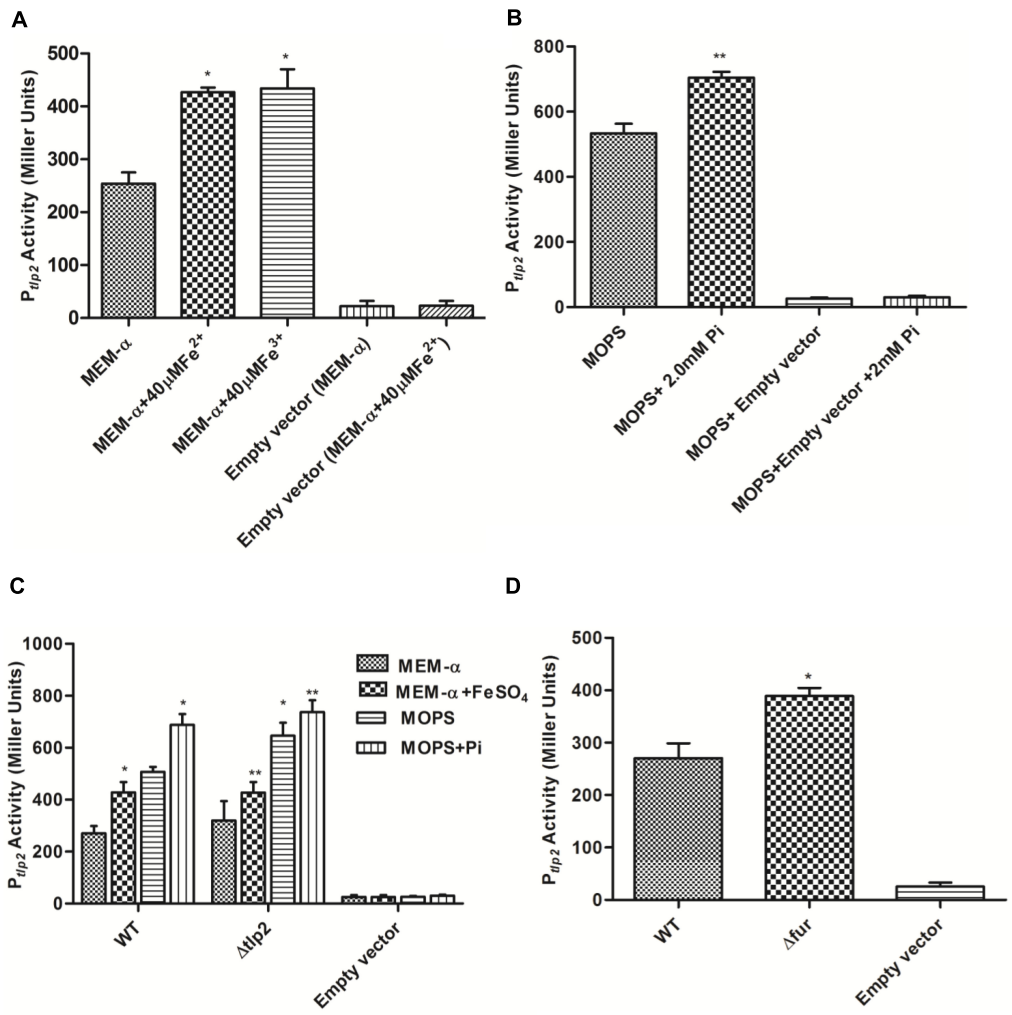

FIGURE 2 | β-galactosidase activity of *Campylobacter jejuni* WT carrying P_{tlp2}-*lacZ* transcriptional fusion construct. **(A)** β-galactosidase activity in the absence (uninduced) and presence of 40 μM $FeSO_4$ or $FeCl_3(H_2O)_6$ (induced) added to MEM-α. **(B)** β-galactosidase activity in the absence (uninduced) and presence of 2mM Pi (induced) added to MOPS. **(C)** β-galactosidase activity of the P_{tlp2}-lacZ fusion assays in the Δ*tlp2::Cm* mutant in the presence or absence of 40 μM $FeSO_4$ in MEM-α and in the presence or absence of 2mM Pi in MOPS. **(D)** β-galactosidase activity of the P_{tlp2}-lacZ fusion in the Δ*fur* mutant in MEM-α. The cells were incubated for 8 h before carrying out the assay. The results show the means and standard deviations of three independent experiments. $*P < 0.05$ where each group is compared with the WT reporter strain that is not induced (MEM-α or MOPS) and $**P < 0.05$ where each group is compared with the WT that is induced (with $FeSO_4$ or Pi).

buffer ranged from 1 to 3 mM but P_{tlp2} was most significantly induced in the presence of 2 and 3 mM of Pi (**Figure 2B** and **Supplementary Figure S1C**). Further, P_{tlp2} activity in the *tlp2* deletion mutant was also studied to assess the effect of the gene product on its promoter activity. Since pMW10 shuttle vector has a kanamycin resistant cassette, we created a Δ*tlp2* mutant with a chloramphenicol resistant cassette. We found that the *tlp2* expression was also induced in the Δ*tlp2* mutant in the presence of Pi and $FeSO_4$ similar to WT (**Figure 2C**). Even though the P_{tlp2} activity in Δ*tlp2* mutant was higher than the WT both in the presence or absence of Pi and Fe, the difference was not statistically significant. These results suggest that *tlp2* transcription is independent of Tlp2 protein levels in the cell.

Ferric uptake regulator protein (Fur) plays an important role in *C. jejuni* iron homeostasis (van Vliet et al., 1998). In addition, iron and Fur are shown to regulate *tlp* genes (Cj0262c; Tlp4 and Cj1110c; Tlp8) in *C. jejuni* (Butcher et al., 2012). We, therefore, investigated if *tlp2* expression is regulated by Fur. Interestingly, the P_{tlp2} activity was increased in a Δ*fur* mutant of *C. jejuni* 81–176 in MEM-α (**Figure 2D**). These observations revealed a role for *fur* in the regulation of *tlp2* expression. Since a *fur* mutation derepresses genes involved in iron acquisition in *C. jejuni* (Holmes et al., 2005), there will be over-accumulation of iron in the *fur* mutant. The increased levels of intracellular levels may increase the P_{tlp2} activity in the *fur* mutant.

The *tlp* and *phoX* Genes Are Co-transcribed

The *tlp2* gene (CJJ81176_180) is located upstream to *phoX* (CJJ81176_181) in the same orientation with an intergenic region of 135 base pairs (**Figure 3A**). Additionally, a previous study indicated that *phoX* gene (Cj0145), located immediately downstream of *tlp2*, is induced by iron and was also enriched in the CjFur ChIP-chip assay (Butcher et al., 2012). As the Δ*tlp2* mutant is defective in chemotaxis toward iron (Fe) and Pi, and the *tlp2* transcription is modulated by iron and Pi, we hypothesized that these two genes may be co-transcribed. To test this, total RNA was extracted from WT grown in MEM-α and analyzed by RT-PCR using primers designed to amplify a flanking region of the two genes. The results indicated that *tlp2* and *phoX* are co-transcribed (**Figure 3B**). In addition, an amplicon was also observed when WT was grown in nutrient rich MH broth (data not shown). These results implied that *phoX* is co-transcribed with *tlp2* under the conditions tested in this study.

Further, an intergenic region between *tlp2* and *phoX* was fused to the promoterless *lacZ* gene to confirm that the promoter activity observed was specific to P_{tlp2}. The reporter strains did not show any promoter activity; the promoter activity in the β-galactosidase assay was similar to that of the negative control (empty plasmid) (**Figure 3C**). Similar findings were observed when media were supplemented with iron or Pi (data not shown), confirming that there was no promoter in the intergenic region between *tlp2* and *phoX* under these tested conditions.

Furthermore, a primer extension analysis revealed a single transcription start site (TS) upstream to the *tlp2* gene (**Figures 3D,F**). The TS is located 53 bp upstream of the *tlp2* start codon with a ribosomal binding site located 12 bp upstream from the start codon. The -10 region was identified with the first T of the TATA box located 59 bp upstream of the start codon. Consistent with the results above, no transcription start site was observed in the 135 bp intergenic region between *tlp2* and *phoX* (**Figure 3E**). This result indicates that *tlp2* and *phoX* genes constitute an operon, and the transcription of *phoX* is dependent on the *tlp2* promoter and they are co-transcribed.

Alkaline Phosphatase Activity Is Increased in the Presence of Iron

A study investigating the regulatory potential of Fur of *C. jejuni* identified that *phoX* is activated by iron (Butcher et al., 2012). Therefore, the PhoX activity of *C. jejuni* WT was evaluated in MEM-α supplemented with 40 μM $FeSO_4$. The PhoX activity of the WT strain increased approximately four-fold in the presence of iron (**Figure 4**). Similarly, a higher PhoX activity was also observed in the Δ*tlp2* mutant in MEM-α in the presence of iron similar to the WT (**Figure 4**); however, this increase was not significant ($P \leq 0.09$). In the complemented strain, the PhoX activity was similar to the WT with or without iron (**Figure 4**). These results suggest that iron upregulates the PhoX activity in *C. jejuni* and potentially intersects the phosphate utilization pathway of *C. jejuni*.

Deletion of *tlp2* Affected Nutrient Stress Survival

The effect of a *tlp2* mutation on stress survival was monitored by comparing the growth of the Δ*tlp2* mutant strain to the WT *C. jejuni* in nutrient-limited conditions. The Δ*tlp2* mutant did not display any growth defect when grown in nutrient-rich MH broth (data not shown); however, the *tlp2* mutant on transition from nutrient rich MH broth to nutrient deficient MEM (without glutamine) exhibited survival defects in the late stationary phase especially 36 h and onward. The survivability of the *tlp2* mutant strain was decreased by one and more than two orders of magnitude at 36 and 60 h, respectively, as compared to the WT ($P < 0.05$) (**Figure 5**).

Deletion of *tlp2* Affected Intracellular Survival in Intestinal Epithelial Cells

The consequence of *tlp2* deletion on virulence-associated traits of *C. jejuni* was evaluated by the ability of Δ*tlp2* mutant to invade and survive within the human intestinal epithelial INT 407 cells (Candon et al., 2007). The Δ*tlp2* demonstrated similar invasion in INT 407 cells; however, the Δ*tlp2* mutant showed a higher intracellular survival, with almost 2 logs more bacteria recovered compared to the WT (**Figures 6A,B**).

The Δ*tlp2* Mutant Is Defective in Colonization of the Chicken Gastrointestinal Tract

To investigate the role of Tlp2 in colonization of *C. jejuni*, we investigated the colonization of Δ*tlp2* mutant and WT in different segments of the chicken intestine. The Δ*tlp2* mutant

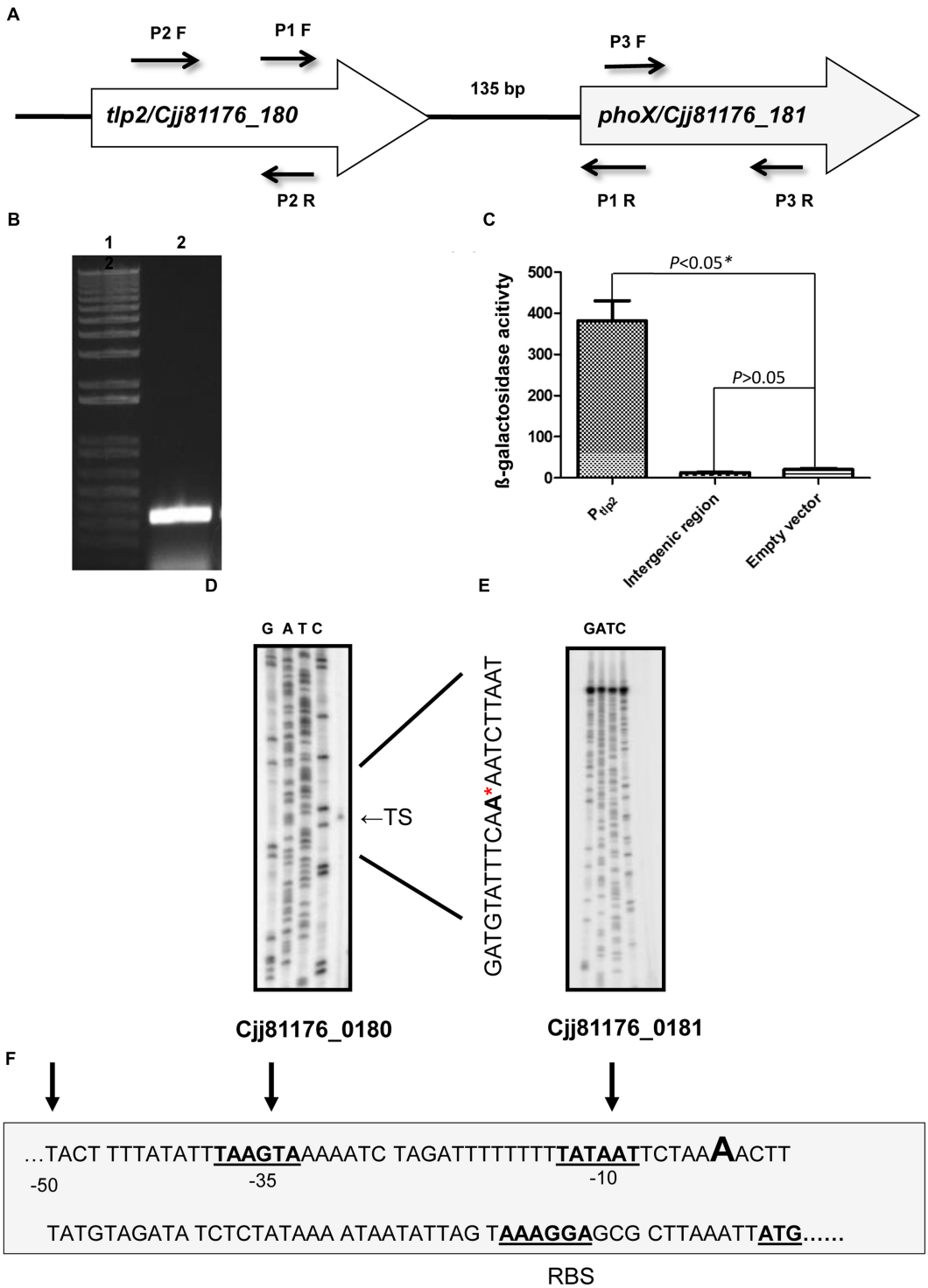

FIGURE 3 | (A) Genetic organization of *tlp2*. The *tlp2* gene (*cjj81176_180*) is located upstream of the *phoX* gene (*Cjj81176_181*) which encodes the alkaline phosphatase (PhoX) enzyme. The *tlp2* and *phoX* genes are separated by a 135 bp intergenic region. **(B)** Reverse Transcriptase overlapping PCR showing co-transcription of *tlp2* and *phoX*. Intergenic region was amplified with primer pair P1 F (forward) and R (reverse) in WT strain grown in MEM-α. Primers for the *tlp2* (P2 F and R) and *phoX* (P3 F and R) genes were included as control regions as well (data not shown). **(C)** The P_{int}-*lacZ* fusion showed no β-galactosidase activity compared to the P_{tlp2}–*lacZ* fusion in the WT strain. Determination of the transcriptional start site for *tlp2* **(D)** and *phoX* **(E)** by a primer extension assay. Only one transcriptional start site is seen upstream to *tlp2*, designated TS (Transcriptional Start) indicated with an arrowhead on the right and by the * in the sequence. No transcriptional start site was found in the region upstream to *phoX* **(F)**: The −10 and −35 elements of the P_{tlp2} are underlined and the ribosomal binding site is indicated as RBS.

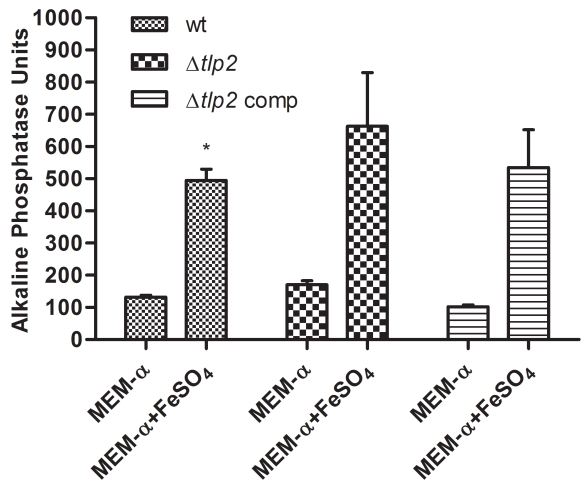

FIGURE 4 | *C. jejuni* WT and Δ*tlp2* strains shows a higher PhoX activity in the presence of iron (40 μM $FeSO_4$). The bacterial cultures grown in MEM-α in the presence or absence of 40 μM $FeSO_4$. The data is a mean of 3 independent trials. *$P \leq 0.05$.

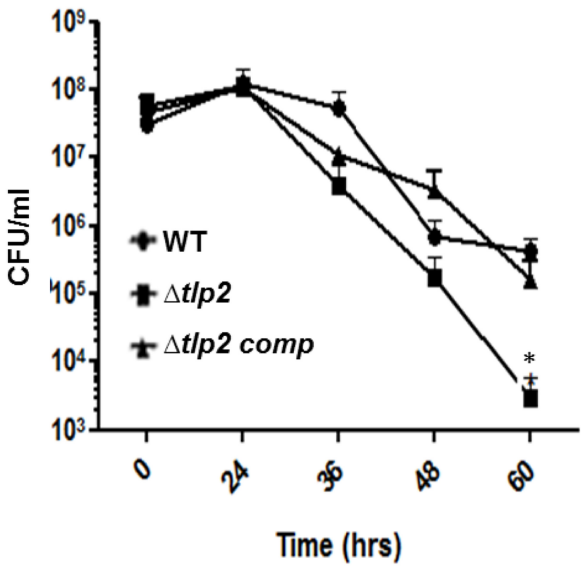

FIGURE 5 | Survival of Δ*tlp2* mutant under nutrient downshift. Survival under nutrient stress was assessed by resuspending MH agar grown bacterial cultures in MEM-α, and determining CFU at different time points. Each data point represents the mean ± SE of 3 independent experiments. *$P \leq 0.05$.

and WT were inoculated into 3-day old chicks orally (10^4 CFU/chicken), and bacterial burden was analyzed after 7 days of infection. Colonization of the *C. jejuni* WT strain in the chicken gastrointestinal tract (cecum and duodenum/jejunum) ranged from 4×10^7 to 2×10^8 CFU per gram of tissue; while in the Δ*tlp2* mutant varied from 2×10^2 to 8×10^3 CFU per gram of tissue. The Δ*tlp2* mutant showed a 4–5 logs decrease in cecal colonization compared to the WT (**Figure 7A**). The Δ*tlp2* mutant was not detected in the duodenum and colonization of the jejunum was also reduced by almost 4 logs (**Figures 7B,C**). However, the liver, spleen, and bursa showed no colonization by *C. jejuni* WT and Δ*tlp2* mutant. These findings suggest that the *tlp2* is essential for achieving optimal colonization in the proximal and distal segments of the gastrointestinal tract, including the cecum.

DISCUSSION

In this study, we characterized the role of *tlp2* in chemotaxis, stress survival, and colonization of the chicken gut. Our results indicated that *tlp2* is involved in chemotaxis toward aspartate, pyruvate, Pi, and iron. Promoter fusion assays revealed that iron, in the ferrous and ferric form induces the *tlp2* promoter activity. Iron is essential for *C. jejuni* colonization in the host as it is one of the limiting nutrients sequestered away from the pathogen by the host and the bioavailability of iron in the intestine is not very well understood (Naikare et al., 2006).

Predicted domain structure of the Tlp2 in the SMART database (Schultz et al., 1998) revealed a single periplasmic Cache_1 (Ca^{2+} channels and chemotaxis receptors) domain (Anantharaman and Aravind, 2000) and a cytoplasmic MCP signaling domain. Cache domain is found in the extracellular or periplasmic portions of chemoreceptors from Gram-positive and Gram-negative bacteria, and is associated with sensing of small molecules (Anantharaman and Aravind, 2000). The cache domains of *Pseudomonas aeruginosa* and *Vibrio cholerae* have been associated with chemotaxis toward amino acids (Nishiyama et al., 2012). The Cache domain is responsible for interaction with multiple ligands and thereby chemotaxis (Tasneem et al., 2005). Δ*tlp2* mutant shows decreased chemotaxis toward aspartate, pyruvate, iron and Pi. Additionally, Tlp2 shows 38% identity with the periplasmic region of the multiple ligand binding Tlp3 (Ccml) of *C. jejuni*, possessing a single cache domain which can potentially bind to multiple ligands with varying affinity (Rahman et al., 2014).

Studies in *S. oneidensis* and *G. metallireducens* report chemotaxis toward iron in the ferrous form (Childers et al., 2002; Bencharit and Ward, 2005). Iron is a redox active metal, and chemotaxis to iron suggests bacterial movement through reduced metal gradients toward potential electron acceptors (oxidized ferric form). Our observations in the WT (*C. jejuni* 81–176) strain show that it is chemotactic toward ferrous iron. The chemotactic response of *C. jejuni* toward iron (Fe^{2+}) can be explained as bacterial adaptation to the assimilatory requirement for iron, as it is an important constituent of iron sulfur proteins and other cellular processes (Bencharit and Ward, 2005). Comparably, the chemotactic response of *H. pylori* toward a metal ion (zinc) has been primarily attributed to the mechanism of nutrient acquisition by bacteria (Sanders et al., 2013).

A study in *C. jejuni,* employing CjFur ChIP-chip analysis, identified *cj0145* (*phoX*) as a novel gene in the Fur regulon in *C. jejuni*, which is activated by iron (Butcher et al., 2012). Much in line with the study above, we found in our study that PhoX activity in the WT is significantly increased in the

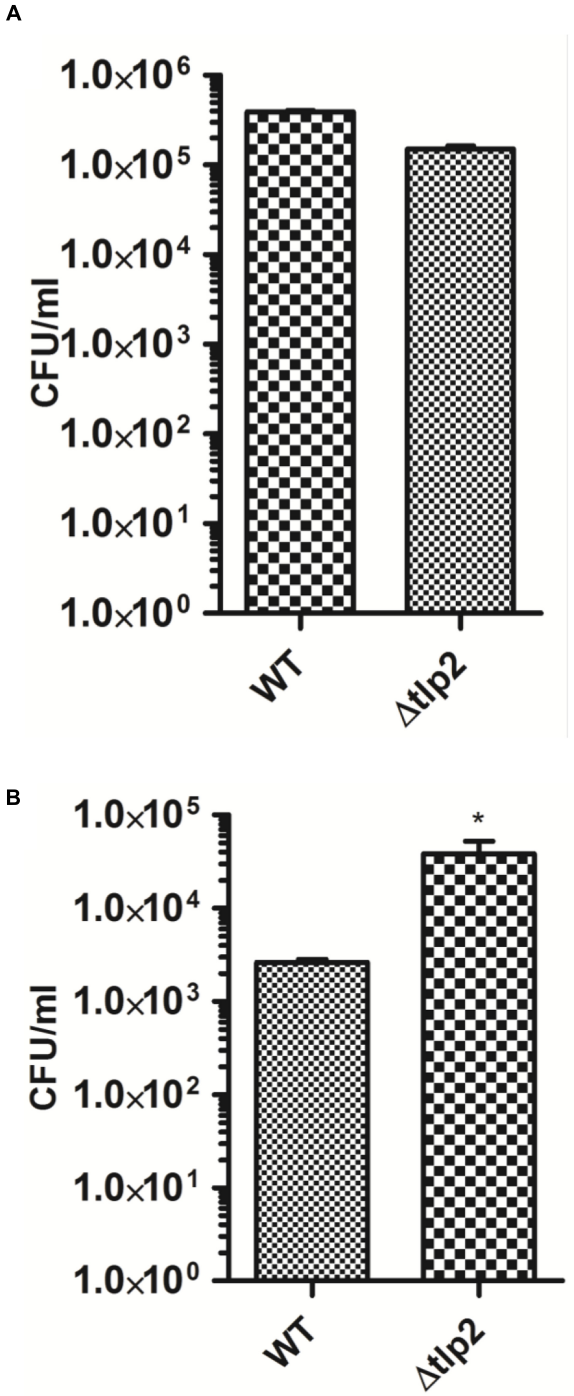

FIGURE 6 | The Δ*tlp2* mutant exhibits no invasion defect **(A)** and increased intracellular survival **(B)** in INT 407 human intestinal cells. Bacteria were infected at an MOI of 100 for both invasion (2 h of gentamicin treatment) and intracellular survival assays (24 h of further incubation). Each data point represents the mean ± SE of 3 independent experiments. $^*P \leq 0.05$.

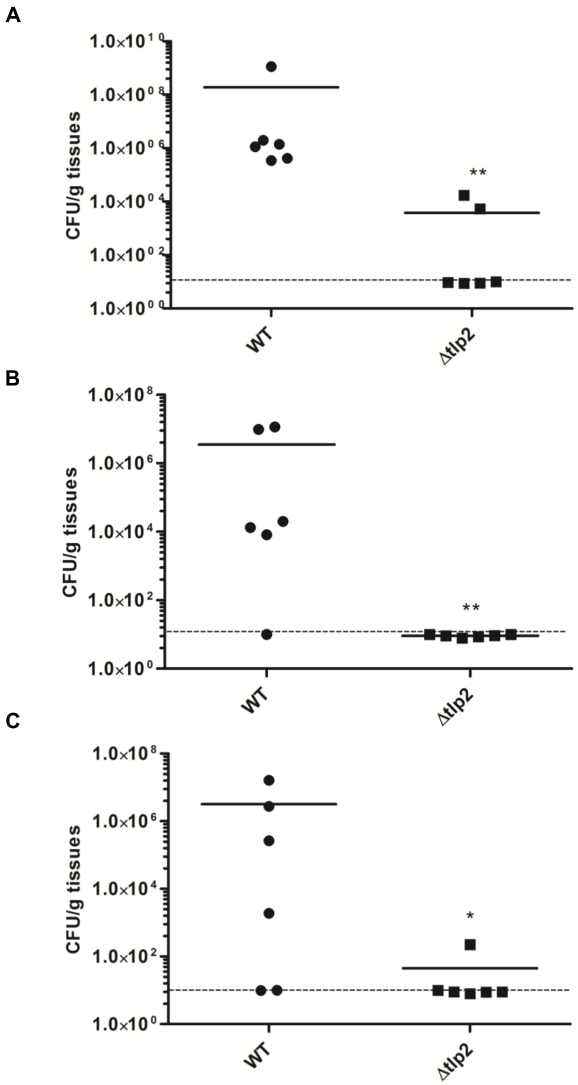

FIGURE 7 | Colonization of the Δ*tlp2* mutant in chicken gastrointestinal tract. The Δ*tlp2* mutant exhibited a significant defect in colonization of the **(A)** cecum **(B)** duodenum and **(C)** jejunum, compared to the WT. Each data point represents CFU/g of tissue. $^*P \leq 0.05$ and $^{**}P \leq 0.01$ Dotted line represents the detection limit.

presence of iron. A recent study on the *Pseudomonas fluorescens* PhoX revealed that iron is a cofactor required for enzyme activity, additionally implying that the bioavailability of iron affects bacterial phosphate uptake (Yong et al., 2014). Although a similar mechanism for increased PhoX activity in *C. jejuni* in the presence of iron can be envisioned, a further biochemical investigation on *C. jejuni* PhoX is needed to identify the precise role of iron in its enzymatic activity.

The sensing, uptake and utilization of inorganic phosphate in prokaryotes enables their ability to withstand conditions of phosphate deprivation. The Pi sensing or taxis has been studied in bacterial pathogens such as *Enterococcus cloacae* and *P. aeruginosa* under conditions of phosphate starvation, with two chemotactic transducers identified for Pi taxis in *P. aeruginosa*. The Pho regulon and the phosphate uptake system regulate Pi taxis in both bacteria (Kusaka et al., 1997; Wu et al., 2000).

C. jejuni being an enteric pathogen is subjected to its survival under low phosphate conditions in the chicken gastrointestinal tract. While the uptake and utilization of Pi in *C. jejuni* through the two-component PhosS/PhosR operon has been previously described (Wosten et al., 2006), nothing is known about Pi taxis in this microaerophile. In our study, *C. jejuni* WT is chemotactic toward Pi, whereas the Δ*tlp2* mutant displayed a decreased chemotaxis. The decreased cellular availability of Pi in the *tlp2* mutant was accompanied by an upregulation of the *phosR* (response regulator of Pho regulon) and the genes for phosphate uptake (*pstC* and *pstS*) which is normally induced in response to Pi limitation (**Supplementary Figure S2**) (Wosten et al., 2006). Additionally, the *tlp2* mutant's decreased survival under nutrient mediated stress (**Figure 5**) can be attributed to the Pi limiting conditions created due to decreased Pi taxis. Earlier studies have indicated that survival under low-nutrient stress is regulated by PPK1 mediated synthesis of poly-P from Pi (Candon et al., 2007; Gangaiah et al., 2009).

PhoX hydrolyzes phospho-organic compounds to Pi, a preferred phosphate source and a building block for poly-P in *C. jejuni* (Candon et al., 2007; Drozd et al., 2011). PhoX in *C. jejuni* is activated by the PhosS-PhosR two component system, under phosphate limiting conditions (Wosten et al., 2006). However, what remains to be investigated is whether PhosR also regulates the *tlp2* promoter activity in *C. jejuni*. The *tlp2* gene is located upstream to *phoX* in *C. jejuni*, and our investigation of *tlp2* transcriptional organization revealed that both genes are transcribed together from a single promoter (P_{tlp2}) located upstream to *tlp2*. These findings however, contradict a previous finding in *C. jejuni* 81116, where *phoX* was shown to be transcribed by a promoter located in the intergenic region of *tlp2* and *phoX*, when *C. jejuni* was grown in a chemically defined medium (Wosten et al., 2006). However, we could not observe any promoter activities in the intergenic region using a primer extension assay under our experimental conditions (**Figure 3**). Further, both strains possess 135 bps intergenic region between *tlp2* and *phoX*; however, showed 95.5% sequence similarity. Therefore, the disparity could be due to the different media and strains used in the two different studies.

The Δ*tlp2* mutant exhibited an increased intracellular survival in INT 407 cell monolayer than the WT strain. The group A Tlps 1, 4, 7 and 10 but not Tlp2, have been shown to play a role in *C. jejuni* invasion of human intestinal epithelial cells (Vegge et al., 2009; Hartley-Tassell et al., 2010; Tareen et al., 2010). *C. jejuni* is known to survive within epithelial cells and can be viable for up to 24 h (Watson and Galan, 2008). Studies have also indicated a role for iron acquisition in *C. jejuni* intracellular survival (Naikare et al., 2006). This therefore piqued our interest in identifying a role for a Tlp involved in chemotaxis toward iron, in *C. jejuni* survival within host cells. The results of our study showed that the deletion of *tlp2* increased intracellular recovery of *C. jejuni*. This was in contrary to our belief that deletion of *tlp2* would decrease the survival of *C. jejuni* in host cells, due to the decreased chemotaxis toward iron. It must however, be noted that intracellular *C. jejuni* undergo a metabolic reprogramming which affects their survival within epithelial cells (Svensson et al., 2009; Liu et al., 2012). The increased intracellular survival in the Δ*tlp2* mutant may indicate a dysregulation of cellular process which warrants further investigation.

The role of *tlp2* in tissue specific colonization of the chicken gastrointestinal tract was investigated. Mutation in *tlp2* resulted in a colonization defect in the cecum, with a more profound reduction seen in the duodenum and jejunum. Catabolism of amino acids such as aspartate and serine are essential for *C. jejuni* colonization of the avian gut (Guccione et al., 2008), as reflected by the *tlp1* mutant (aspartate chemoreceptor), which was severely impaired in colonization of the chicken ceca (Hartley-Tassell et al., 2010). The *tlp2* mutant demonstrated a decreased chemotaxis toward aspartate, which might explain the reduced colonization. The utilization of glutamine, glutathione and asparagine in *C. jejuni* 81–176 is associated to tissue-specific colonization of the murine intestine (Hofreuter et al., 2008). However, it is not known if the ability to metabolize these nutrients also supports tissue specific colonization in the chicken intestinal tract. Additionally, chemotaxis toward pyruvate and fumarate mediated by Tlp9 represents energy taxis in *C. jejuni*. Energy taxis is an essential driving force for *C. jejuni* for establishment during colonization of the host (Vegge et al., 2009). The chicken cecum represents an iron and phosphate limiting environment for *C. jejuni* and iron acquisition is known to be essential for *C. jejuni* colonization of the chicken (Naikare et al., 2006). It is not surprising to see that Δ*tlp2* mutant, defective in chemotaxis toward Pi and iron, is also defective in colonization of the chicken cecum, duodenum, and jejunum. These findings clearly indicate that *tlp2* contributes to *C jejuni* interaction with host cells, which is an important determinant for *C jejuni* pathogenesis and colonization of the chicken gastrointestinal tract.

In summary, the present study identifies a role for *tlp2* in *C. jejuni* chemotaxis, stress survival and colonization of the chicken gastrointestinal tract. Further, our findings indicate that iron regulates *tlp2*. The *tlp2* mutant was also defective in chemotaxis to Pi and showed increased PhoX activity. This suggests a possible cross-talk between iron and phosphate regulatory pathways, which needs further investigation. In addition, the increased PhoX activity in the presence of iron seen in *C. jejuni* indicates that iron may reduce the bioavailability of phosphate. Our findings in this study suggest a basis for future biochemical characterization of PhoX in *C. jejuni*.

ETHICS STATEMENT

Animal experiments were conducted according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), the Ohio State University. Chickens were housed at the Food Animal Health Research Program Animal Care Facility, which is fully accredited by AAALAC and the animals were supervised by a senior veterinarian. Infectious agents were administered using manual restraint for less than

one minute to minimize distress. Before necropsy, chickens were euthanized by carbon dioxide inhalation. This method is consistent with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

GR and KC designed the experiments. KC, SH, BJ, and SR performed the experiments and collected the data. KC, GR and VS analyzed the data. KC, GR, VS, and BJ wrote the paper.

FUNDING

This work was supported by the funds from Ohio Agricultural Research and Development Center (OARDC); The Ohio State University. KC was supported by a fellowship from the Indian Council of Agricultural Research (ICAR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Juliette Hanson for assistance with chicken colonization studies. We acknowledge Dr. Jun Lin (The University of Tennessee) for providing the *C. jejuni* NCTC11168 *fur* mutant strain and Dr. John Gunn (Ohio State University) for critically reading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02674/full#supplementary-material

REFERENCES

Anantharaman, V., and Aravind, L. (2000). Cache - a signaling domain common to animal Ca(2 +)-channel subunits and a class of prokaryotic chemotaxis receptors. *Trends Biochem. Sci.* 25, 535–537. doi: 10.1016/S0968-0004(00)01672-8

Bencharit, S., and Ward, M. J. (2005). Chemotactic responses to metals and anaerobic electron acceptors in Shewanella oneidensis MR-1. *J. Bacteriol.* 187, 5049–5053. doi: 10.1128/JB.187.14.5049-5053.2005

Braun, V., and Hantke, K. (2003). "Mechanisms of bacterial iron transport," in *Microbial Transport System*, ed. G. Winkelmann (Weinheim: Wiley-VCH Verlag GmBH & Co), 289–311.

Butcher, J., Sarvan, S., Brunzelle, J. S., Couture, J. F., and Stintzi, A. (2012). Structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10047–10052. doi: 10.1073/pnas.1118321109

Candon, H. L., Allan, B. J., Fraley, C. D., and Gaynor, E. C. (2007). Polyphosphate kinase 1 is a pathogenesis determinant in Campylobacter jejuni. *J. Bacteriol.* 189, 8099–8108. doi: 10.1128/JB.01037-1037

CDC. (2013). "Incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 u.s. sites, 1996–2012," in *Morbidity and Mortality Weekly Report*, ed. K. Charlotte (Washington, DC: U.S. Government Printing Office).

Cerda, O., Rivas, A., and Toledo, H. (2003). Helicobacter pylori strain ATCC700392 encodes a methyl-accepting chemotaxis receptor protein (MCP) for arginine and sodium bicarbonate. *FEMS Microbiol. Lett.* 224, 175–181. doi: 10.1016/S0378-1097(03)00423-3

Cerda, O. A., Nunez-Villena, F., Soto, S. E., Ugalde, J. M., Lopez-Solis, R., and Toledo, H. (2011). tlpA gene expression is required for arginine and bicarbonate chemotaxis in helicobacter pylori. *Biol. Res.* 44, 277–282. doi: /S0716-97602011000300009

Chandrashekhar, K., Gangaiah, D., Pina-Mimbela, R., Kassem, I. I., Jeon, B. H., and Rajashekara, G. (2015). Transducer like proteins of *Campylobacter jejuni* 81-176: role in chemotaxis and colonization of the chicken gastrointestinal tract. *Front. Cell. Infect. Microbiol.* 5:46. doi: 10.3389/fcimb.2015.00046

Chandrashekhar, K., Kassem, I. I., and Rajashekara, G. (2017). *Campylobacter jejuni* transducer like proteins: chemotaxis and beyond. *Gut Microbes* 8, 323–334. doi: 10.1080/19490976.2017.1279380

Childers, S. E., Ciufo, S., and Lovley, D. R. (2002). Geobacter metallireducens accesses insoluble Fe(III) oxide by chemotaxis. *Nature* 416, 767–769. doi: 10.1038/416767a.

Day, C. J., Hartley-Tassell, L. E., Shewell, L. K., King, R. M., Tram, G., Day, S. K., et al. (2012). Variation of chemosensory receptor content of *Campylobacter jejuni* strains and modulation of receptor gene expression under different in vivo and in vitro growth conditions. *BMC Microbiol.* 12:128. doi: 10.1186/1471-2180-12-128

Drozd, M., Chandrashekhar, K., and Rajashekara, G. (2014). polyphosphate-mediated modulation of *Campylobacter jejuni* biofilm growth and stability. *Virulence* 5, 680–690. doi: 10.4161/viru.34348

Drozd, M., Gangaiah, D., Liu, Z., and Rajashekara, G. (2011). Contribution of TAT system translocated PhoX to *Campylobacter jejuni* phosphate metabolism and resilience to environmental stresses. *PLoS One* 6:e26336. doi: 10.1371/journal.pone.0026336

Ernst, F. D., Bereswill, S., Waidner, B., Stoof, J., Mader, U., Kusters, J. G., et al. (2005). Transcriptional profiling of Helicobacter pylori Fur- and iron-regulated gene expression. *Microbiology* 151(Pt 2), 533–546. doi: 10.1099/mic.0.27404-27400

Food and Drug Administration, and HHS. (2014). Establishing a list of qualifying pathogens under the food and drug administration safety and innovation act: final rule. *Fed. Regist.* 79, 32464–32481.

Gangaiah, D., Kassem, I. I, Liu, Z., and Rajashekara, G. (2009). Importance of polyphosphate kinase 1 for *Campylobacter jejuni* viable-but-nonculturable cell formation, natural transformation, and antimicrobial resistance. *Appl. Environ. Microbiol.* 75, 7838–7849. doi: 10.1128/AEM.01603-1609

Gangaiah, D., Liu, Z., Arcos, J., Kassem, I. I, Sanad, Y., Torrelles, J. B., et al. (2010). Polyphosphate kinase 2: a novel determinant of stress responses and pathogenesis in *Campylobacter jejuni*. *PLoS One* 5:e12142. doi: 10.1371/journal.pone.0012142

Grabowska, A. D., Wandel, M. P., Lasica, A. M., Nesteruk, M., Roszczenko, P., Wyszynska, A., et al. (2011). *Campylobacter jejuni* dsb gene expression is regulated by iron in a Fur-dependent manner and by a translational coupling mechanism. *BMC Microbiol.* 11:166. doi: 10.1186/1471-2180-11-166

Guccione, E., Leon-Kempis Mdel, R., Pearson, B. M., Hitchin, E., Mulholland, F., van Diemen, P. M., et al. (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol. Microbiol.* 69, 77–93. doi: 10.1111/j.1365-2958.2008.06263.x

Harris, H. W., El-Naggar, M. Y., Bretschger, O., Ward, M. J., Romine, M. F., Obraztsova, A. Y., et al. (2010). Electrokinesis is a microbial behavior that requires extracellular electron transport. *Proc. Natl. Acad. Sci. U.S.A.* 107, 326–331. doi: 10.1073/pnas.0907468107

Hartley-Tassell, L. E., Shewell, L. K., Day, C. J., Wilson, J. C., Sandhu, R., Ketley, J. M., et al. (2010). Identification and characterization of the aspartate chemosensory receptor of *Campylobacter jejuni*. *Mol. Microbiol.* 75, 710–730. doi: 10.1111/j.1365-2958.2009.07010.x

Hendrixson, D. R., and DiRita, V. J. (2004). Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. *Mol. Microbiol.* 52, 471–484. doi: 10.1111/j.1365-2958.2004.03988.x

Hermans, D., Pasmans, F., Messens, W., Martel, A., Van Immerseel, F., Rasschaert, G., et al. (2012). Poultry as a host for the zoonotic pathogen *Campylobacter jejuni*. *Vector Borne Zoonotic Dis.* 12, 89–98. doi: 10.1089/vbz.2011.0676

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., et al. (2011). Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet. Res.* 42:82. doi: 10.1186/1297-9716-42-82

Hofreuter, D., Novik, V., and Galan, J. E. (2008). Metabolic diversity in *Campylobacter jejuni* enhances specific tissue colonization. *Cell Host Microbe* 4, 425–433. doi: 10.1016/j.chom.2008.10.002

Holmes, K., Mulholland, F., Pearson, B. M., Pin, C., McNicholl-Kennedy, J., Ketley, J. M., et al. (2005). *Campylobacter jejuni* gene expression in response to iron limitation and the role of fur. *Microbiology* 151(Pt 1), 243–257. doi: 10.1099/mic.0.27412-27410

Hugdahl, M. B., Beery, J. T., and Doyle, M. P. (1988). Chemotactic behavior of *Campylobacter jejuni*. *Infect. Immun.* 56, 1560–1566.

Jeon, B., Muraoka, W., Sahin, O., and Zhang, Q. (2008). Role of cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 52, 2699–2708. doi: 10.1128/aac.01607-1607

Kassem, I. I., Khatri, M., Esseili, M. A., Sanad, Y. M., Saif, Y. M., Olson, J. W., et al. (2012). Respiratory proteins contribute differentially to *Campylobacter jejuni's* survival and in vitro interaction with hosts' intestinal cells. *BMC Microbiol.* 12:258. doi: 10.1186/1471-2180-12-258

Kim, M., Hwang, S., Ryu, S., and Jeon, B. (2011). Regulation of perR expression by iron and PerR in *Campylobacter jejuni*. *J. Bacteriol.* 193, 6171–6178. doi: 10.1128/JB.05493-5411

Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C., and Robinson, R. A. (1985). A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J. Infect. Dis.* 152, 592–596. doi: 10.1093/infdis/152.3.592

Korolik, V. (2010). Aspartate chemosensory receptor signalling in *Campylobacter jejuni*. *Virulence* 1, 414–417. doi: 10.4161/viru.1.5.12735

Kusaka, K., Shibata, K., Kuroda, A., Kato, J., and Ohtake, H. (1997). Isolation and characterization of Enterobacter cloacae mutants which are defective in chemotaxis toward inorganic phosphate. *J. Bacteriol.* 179, 6192–6195. doi: 10.1128/jb.179.19.6192-6195.1997

Lertsethtakarn, P., Ottemann, K. M., and Hendrixson, D. R. (2011). Motility and chemotaxis in *Campylobacter* and *Helicobacter*. *Annu. Rev. Microbiol.* 65, 389–410. doi: 10.1146/annurev-micro-090110-102908

Lill, R. (2009). Function and biogenesis of iron-sulphur proteins. *Nature* 460, 831–838. doi: 10.1038/nature08301

Liu, X., Gao, B., Novik, V., and Galan, J. E. (2012). Quantitative proteomics of intracellular *Campylobacter jejuni* reveals metabolic reprogramming. *PLoS Pathog* 8:e1002562. doi: 10.1371/journal.ppat.1002562

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

Marchant, J., Wren, B., and Ketley, J. (2002). Exploiting genome sequence: predictions for mechanisms of Campylobacter chemotaxis. *Trends Microbiol.* 10, 155–159. doi: 10.1016/S0966-842X(02)02323-5

Mazumder, R., Phelps, T. J., Krieg, N. R., and Benoit, R. E. (1999). Determining chemotactic responses by two subsurface microaerophiles using a simplified capillary assay method. *J. Microbiol. Methods* 37, 255–263. doi: 10.1016/S0167-7012(99)00072-X

Miller, W. G., Bates, A. H., Horn, S. T., Brandl, M. T., Wachtel, M. R., and Mandrell, R. E. (2000). Detection on surfaces and in Caco-2 cells of *Campylobacter jejuni* cells transformed with new gfp, yfp, and cfp marker plasmids. *Appl. Environ. Microbiol.* 66, 5426–5436. doi: 10.1128/AEM.66.12.5426-5436.2000

Naikare, H., Palyada, K., Panciera, R., Marlow, D., and Stintzi, A. (2006). Major role for FeoB in *Campylobacter jejuni* ferrous iron acquisition, gut colonization, and intracellular survival. *Infect. Immun.* 74, 5433–5444. doi: 10.1128/IAI.00052-56

Nishiyama, S., Suzuki, D., Itoh, Y., Suzuki, K., Tajima, H., Hyakutake, A., et al. (2012). Mlp24 (McpX) of *Vibrio cholerae* implicated in pathogenicity functions as a chemoreceptor for multiple amino acids. *Infect. Immun.* 80, 3170–3178. doi: 10.1128/IAI.00039-12

Rahman, H., King, R. M., Shewell, L. K., Semchenko, E. A., Hartley-Tassell, L. E., Wilson, J. C., et al. (2014). Characterisation of a multi-ligand binding chemoreceptor ccml (tlp3) of *Campylobacter jejuni*. *PLoS Pathog* 10:e1003822. doi: 10.1371/journal.ppat.1003822

Rajashekara, G., Drozd, M., Gangaiah, D., Jeon, B., Liu, Z., and Zhang, Q. (2009). Functional characterization of the twin-arginine translocation system in *Campylobacter jejuni*. *Foodborne Pathog Dis.* 6, 935–945. doi: 10.1089/fpd.2009.0298

Reuter, M., and van Vliet, A. H. (2013). Signal balancing by the CetABC and CetZ chemoreceptors controls energy taxis in *Campylobacter jejuni*. *PLoS One* 8:e54390. doi: 10.1371/journal.pone.0054390

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanders, L., Andermann, T. M., and Ottemann, K. M. (2013). A supplemented soft agar chemotaxis assay demonstrates the Helicobacter pylori chemotactic response to zinc and nickel. *Microbiology* 159(Pt 1), 46–57. doi: 10.1099/mic.0.062877-62870

Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5857–5864. doi: 10.1073/pnas.95.11.5857

Svensson, S. L., Davis, L. M., MacKichan, J. K., Allan, B. J., Pajaniappan, M., Thompson, S. A., et al. (2009). The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. *Mol. Microbiol.* 71, 253–272. doi: 10.1111/j.1365-2958.2008.06534.x

Tareen, A. M., Dasti, J. I., Zautner, A. E., Gross, U., and Lugert, R. (2010). *Campylobacter jejuni* proteins Cj0952c and Cj0951c affect chemotactic behaviour towards formic acid and are important for invasion of host cells. *Microbiology* 156(Pt 10), 3123–3135. doi: 10.1099/mic.0.039438-39430

Tasneem, A., Iyer, L. M., Jakobsson, E., and Aravind, L. (2005). Identification of the prokaryotic ligand-gated ion channels and their implications for the mechanisms and origins of animal Cys-loop ion channels. *Genome Biol.* 6:R4. doi: 10.1186/gb-2004-6-1-r4

van Vliet, A. H., Wooldridge, K. G., and Ketley, J. M. (1998). Iron-responsive gene regulation in a *Campylobacter jejuni* fur mutant. *J. Bacteriol.* 180, 5291–5298.

Vegge, C. S., Brondsted, L., Li, Y. P., Bang, D. D., and Ingmer, H. (2009). Energy taxis drives *Campylobacter jejuni* toward the most favorable conditions for growth. *Appl. Environ. Microbiol.* 75, 5308–5314. doi: 10.1128/AEM.00287-289

Watson, R. O., and Galan, J. E. (2008). *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog* 4:e14. doi: 10.1371/journal.ppat.0040014

Wosten, M. M., Boeve, M., Koot, M. G., van Nuenen, A. C., and van der Zeijst, B. A. (1998). Identification of *Campylobacter jejuni* promoter sequences. *J. Bacteriol.* 180, 594–599.

Wosten, M. M., Parker, C. T., van Mourik, A., Guilhabert, M. R., van Dijk, L., and van Putten, J. P. (2006). The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system. *Mol. Microbiol.* 62, 278–291. doi: 10.1111/j.1365-2958.2006.05372.x

Wu, H., Kato, J., Kuroda, A., Ikeda, T., Takiguchi, N., and Ohtake, H. (2000). Identification and characterization of two chemotactic transducers for inorganic phosphate in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 3400–3404. doi: 10.1128/JB.182.12.3400-3404.2000

Yao, R., Alm, R. A., Trust, T. J., and Guerry, P. (1993). Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. *Gene* 130, 127–130. doi: 10.1016/0378-1119(93)90355-7

Yao, R., Burr, D. H., Doig, P., Trust, T. J., Niu, H., and Guerry, P. (1994). Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol. Microbiol.* 14, 883–893. doi: 10.1111/j.1365-2958.1994.tb01324.x

Yao, R., Burr Donald, H., and Guerry, P. (1997). CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol. Microbiol.* 23, 1021–1031. doi: 10.1046/j.1365-2958.1997.2861650.x

Yong, S. C., Roversi, P., Lillington, J., Rodriguez, F., Krehenbrink, M., Zeldin, O. B., et al. (2014). A complex iron-calcium cofactor catalyzing phosphotransfer chemistry. *Science* 345, 1170–1173. doi: 10.1126/science.1254237

Young, K. T., Davis, L. M., and Dirita, V. J. (2007). 'Campylobacter jejuni: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* 5, 665–679. doi: 10.1038/nrmicro1718

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 YK and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 January 2019
doi: 10.3389/fmicb.2018.03180

The Addition of Viriditec™ Aqueous Ozone to Peracetic Acid as an Antimicrobial Spray Increases Air Quality While Maintaining *Salmonella* Typhimurium, Non-pathogenic *Escherichia coli*, and *Campylobacter jejuni* Reduction on Whole Carcasses

*Dana Kristen Dittoe[1,2], Kristina Marie Feye[1,2], Bob Peyton[3], Drew Worlie[3], Michael J. Draper[3] and Steven C. Ricke[1,2]**

[1] Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [2] Center for Food Safety, University of Arkansas, Fayetteville, AR, United States, [3] TetraClean Systems LLC, Omaha, NE, United States

OPEN ACCESS

Edited by:
Jean-Christophe Augustin, École Nationale Vétérinaire d'Alfort, France

Reviewed by:
Xiang Yang, University of California, Davis, United States
Vasileios Englezos, Università degli Studi di Torino, Italy

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

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

Received: *18 October 2018*
Accepted: *07 December 2018*
Published: *08 January 2019*

Citation:
Dittoe DK, Feye KM, Peyton B, Worlie D, Draper MJ and Ricke SC (2019) The Addition of Viriditec™ Aqueous Ozone to Peracetic Acid as an Antimicrobial Spray Increases Air Quality While Maintaining Salmonella Typhimurium, Non-pathogenic Escherichia coli, and Campylobacter jejuni Reduction on Whole Carcasses. Front. Microbiol. 9:3180. doi: 10.3389/fmicb.2018.03180

Currently, the most utilized antimicrobial in processing facilities is peracetic acid, PAA; however, this chemical is increasingly recognized as a hazard to human health. Preliminary evidence suggests that ozone, when introduced in a specific manner, can reduce the noxious nature of PAA. Therefore, the objective of the current study was to evaluate the efficacy of TetraClean Systems aqueous ozone, O_3, in combination with PAA as an antimicrobial spray on whole chicken carcasses. This trial used 70 whole hen carcasses (7 treatments; 10 replications) that were inoculated in a 400 mL cocktail containing *Salmonella*, *Escherichia coli*, and *Campylobacter* (10^7 CFU/mL) and allowed to adhere for 60 min at 4°C for a final concentration of 10^5 to 10^6 CFU/g. The experimental 5 s (4×) spray treatments included: a no treatment negative control, TW; TW + O_3 (10 ppm), TW + PAA (50 ppm), TW + PAA (500 ppm), TW + O_3 + PAA (50 ppm), and TW + O_3 + PAA (500 ppm). During treatment application, ambient PAA vapor was measured with a ChemDAQ Safecide PAA vapor sensor. After treatment, carcasses were immediately rinsed in 400 mL of nBPW for 2 min. Following rinsing, the dot method was utilizing for enumeration with 10 μL of rinsate being serially diluted, plated on XLD and mCCDA agar, and incubated aerobically at 37°C for 24 h or microaerophilically at 42°C for 48 h. Log-transformed counts were analyzed using ANOVA in JMP 14.0. Means were separated using Tukey's HSD when $P \leq 0.05$. There was a significant treatment effect among *Salmonella*, *E. coli*, and *Campylobacter* counts, and a significant treatment effect among ambient PAA ($P < 0.05$). TW + O_3 + PAA (500 ppm), reduced *Salmonella* significantly compared to TW (5.71 and 6.30 log CFU/g).

Furthermore, TW + PAA (500 ppm), reduced the presence of *E. coli* significantly compared to TW or no treated control (5.57 and 6.18 log CFU/g). Also, TW + PAA (50 ppm), TW + PAA (500 ppm), and TW + O_3 + PAA (500 ppm) significantly reduced *Campylobacter* compared to carcasses not treated (4.80, 4.81, and 4.86 log CFU/g). Lastly, the addition of ozone significantly reduced the ambient PAA when O_3 was added to 500 ppm of PAA, as TW + O_3 + PAA (500 ppm) produced less ambient PAA than TW + PAA (500 ppm) (0.052 and 0.565 ppm). In conclusion, the addition of ozone to PAA may demonstrated the ability to effectively reduce ambient PAA, thus increasing employee safety.

Keywords: poultry, spray cabinet, aqueous ozone, peracetic acid, pathogenic reduction, ambient peracetic acid vapor

INTRODUCTION

Currently, the United States poultry industry utilizes peroxyacetic acid, also known as peracetic acid (PAA), to decontaminate poultry within poultry processing facilities. The disinfectant, PAA, is a product of the reaction between acetic acid and hydrogen peroxide. It is a colorless acid with a strong odor. The bactericidal effect of PAA is due to it being a strong oxidizing agent of the cell membrane and other cell components (Oyarzabal, 2005). However, this chemical is corrosive and unstable. PAA is one of the most common antimicrobials used in poultry processing facilities, as it is applied in the chillers (pre-chiller, chiller, and post-chiller), part dips, spray cabinets, in and out bird washes at concentrations typically ranging from 200 to 2,000 ppm; however, it has been known to be a hazard to human health (National Academies of Science [NAS], 2010). PAA is reported to be an irritant to the upper respiratory tract, eye, and skin (Merka and Urban, 1978; Fraser and Thorbinson, 1986; Janssen, 1989; Janssen and van Doorn, 1994). Direct contact in the eye and skin can be avoided if the proper personal protective equipment (PPE) is worn, but there are limited approaches to protect the upper respiratory tract from the vapors emitted from PAA (American Thoracic Society, 1996).

Currently, there is no OSHA (Occupational Safety and Health Administration) limit on the acute or long-term exposure limit of PAA during shifts for employees. However, other governing bodies have set limits and guidelines for the exposure of PAA vapor. In 2014, the American Conference Governmental Hygienists (ACGIH) set a threshold limit of 0.04 ppm as the 15-minute Short Term Exposure Limit (STEL; American Conference of Governmental Industrial Hygienists [ACGIH], 2016). Furthermore, the National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances (NAC/AEGLL Committee), during an 8 h exposure time, set AEGL-1, 2, and 3 limits to 0.17, 0.51, and 1.3 ppm of PAA vapor, respectively (National Academies of Science [NAS], 2010), with firm limits of total exposure time for AEGL-1 and 2 limits at 0.17, and 0.51 ppm (National Academies of Science [NAS], 2010). An exposure at AEGL-1 produces noticeable discomfort, and irritation, with reversible effects upon removal from exposure site. AEGL-2 exposure produces irreversible or other long-lasting serious health conditions and may impair one's ability to escape. Lastly, an AEGL-3 exposure results in life-threatening health conditions and can result in death. As of 2015, NIOSH published a draft Immediately Dangerous to Life or Health (IDLH) value for 0.64 ppm (National Institute for Occupational Safety and Health [NIOSH], 2015).

As there are no current strategies employed to reduce the ambient PAA in a processing facility, there is a significant need to develop and easily implement measures to prevent PAA vapor exposure. One novel approach is to utilize the commercial aqueous ozone product (Viriditec^{TM}, TetraClean Systems LLC, Omaha, NE) to distribute aqueous ozone directly to PAA, as preliminary evidence suggests, the addition of ozone can reduce the noxious nature of PAA (data not shown).

Previously, chlorine was utilized as the primary sanitizer in processing facilities but has been replaced in the last decade with PAA. Studies have demonstrated that 85 ppm of PAA has the capability to reduce the incidence of *Salmonella* and *Campylobacter* by 92 and 43% on poultry carcasses when applied in a commercial poultry chiller (Bauermeister et al., 2008b). Whereas, 30 ppm of chlorine was only capable of reducing *Salmonella* and *Campylobacter* by 43 and 13%, when used in a poultry chiller (Bauermeister et al., 2008b). Furthermore, PAA has been shown to mitigate *Staphylococcus* spp., *Listeria* spp., and generic *Escherichia coli* more than 5-log CFU regardless of the food source being evaluated (Brinez et al., 2006). Bauermeister et al. (2008a) reported the reduction of *Salmonella* and *Campylobacter* to be greater in carcasses chilled in solutions containing 200 ppm of PAA compared to those chilled in 30 ppm chlorine, ≈1 log reduction. Therefore, it is imperative to mitigate the noxious nature of PAA without reducing the bactericidal effects of PAA in poultry processing facilities. Thus, it was the objective of the current experiment to evaluate the efficacy of a commercial aqueous ozone (O_3) alone or in combination with PAA on reducing ambient PAA and poultry pathogens when applied as an antimicrobial spray on whole chicken carcasses.

MATERIALS AND METHODS

Viriditec^{TM} Aqueous Ozone Generation

TetraClean's Viriditec^{TM} aqueous ozone system has been characterized as a patented technology that utilizes Nanobubble

Technology to combine water and ozone to yield aqueous ozone. In the current study, the system produced ozone gas which was injected into a water stream and further infused through the systems patented configuration and mixing technology. A Q46H/64 Dissolved Ozone Monitor (Analytical Technologies Industries, Collegeville, PA, United States) was utilized to measure the specific ozone levels generated from the Viriditec^{TM} aqueous ozone system. The result was an aqueous ozone solution that contained 10 ppm of dissolved ozone in solution.

Carcass Procurement and Indigenous Pathogen Screening

A total of 70 whole hen carcasses (7 treatments; 10 replicates) with an average weight of 1749.87 g were obtained from a free-range poultry facility immediately after processing and were void of any antimicrobial treatments prior to the onset of the current experiment. A review by the institutional animal care and use committee (IACUC) was exempted because the birds were raised in an off-campus commercial farm operation and the current study was restricted to microbiological evaluation of bird carcasses selected for study. Immediately following evisceration, on the same day all 70 carcasses were shipped on ice and upon arrival at the University of Arkansas Center for Food Safety one carcass was screened for the background indigenous presence of *Salmonella*, *E. coli*, and *Campylobacter*. The remaining carcasses were stored at 4°C refrigeration until the onset of the study which began within 24 h post-slaughter.

Inocula Preparation and Inoculation

Prior to the study, a frozen stock of *Salmonella* Typhimurium (UK-1), *E. coli* (J53), and *Campylobacter jejuni* were streaked to isolation on respective media and incubated either aerobically at 37°C for 24 h or microaerophilically at 42°C for 48 h. Subsequently, one isolated colony from the incubated plates were streaked onto fresh medium and incubated under the previously mentioned conditions. Simultaneously, an isolated colony was streaked onto Xylose Lysine Deoxycholate (XLD; HiMedia, West Chester, PA, United States) and modified Charcoal-Cefoperazone-Deoxycholate agar (mCCDA; HiMedia, West Chester, PA, United States) for confirmation and incubated either aerobically at 37°C for 24 h or microaerophilically at 42°C for 48 h. Following confirmation, isolated colonies from the incubated media were then transferred to 40 mL of fresh Luria-Bertani Broth and Mueller Hinton Broth (Hardy Diagnostics, Irving, TX, United States) and incubated under previously mentioned conditions in a shaking incubator at 200 rpm for 12 to 16 h. The resulting cultures of 3×10^7 CFU/mL of *Salmonella* Typhimurium (UK-1), *E. coli* (J53), and *C. jejuni*, respectively.

Directly following the overnight (12 to 16 h) incubation of the cultures, the cultures were spun down at 18,000 g for 5 min, decanted, and then washed twice in 1 × Phosphate Buffered Saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per 1 L, with the pH adjusted to 7.4 with HCl). After the final wash, the pellet was re-suspended in 400 mL of sterile PBS.

The carcasses were inoculated in a 400 mL cocktail containing 3×10^7 CFU/mL of *Salmonella* Typhimurium (UK-1), *E. coli* (J53), and *C. jejuni*. *Salmonella*, *E. coli*, and *Campylobacter* inocula were allowed to adhere at 4°C for 60 min for a final attachment of 10^6, 10^6 and 10^5 CFU/g. Following the attachment period, the whole carcass weights were recorded, and the treatments were administered. The carcasses were independently placed into a spray cabinet constructed from a modified refrigerator (Model No. FFTR1814LW2, Fridgaire, Miami, FL, United States) with four pressure nozzles that administered 500 mL of treatments via a high-pressurized spray (15 psi). The treatment was applied 4 × with 5 s on 5 s off for a total duration of 20 s treatment application. The treatments utilized in the current study were: a no-treatment negative control, tap water (TW); TW + O_3 (10 ppm), TW + PAA (50 ppm), TW + PAA (500 ppm), TW + O_3 + PAA (50 ppm), and TW + O3 + PAA (500 ppm). The commercial PAA utilized in the current study was Spectrum (FMC, Philadelphia, PA, United States). To reduce cross contamination, treated carcasses were placed into individual sterile poultry rinse bags (Nasco, Fort Atkinson, WI, United States) and allowed to rest for 2 min.

Microbial Analysis

After the appropriated resting period, 400 mL of neutralizing Buffered Peptone Water (nBPW; 20.0 g of buffered peptone, 7 g of refined soy lecithin or equivalent, 1.0 g of sodium thiosulfate, 12.5 g of sodium bicarbonate, per 1 L of DI water; USDA Food Safety and Inspection Service, 2016) was poured directly on top and inside the carcasses. The carcasses were then manually agitated for 2 min in an 180° arcing motion. The carcasses were aseptically removed, discarded, and the subsequent rinsate was utilized for downstream analysis.

Salmonella, *E. coli*, and *Campylobacter* Enumeration

Rinsates were aliquoted to 15 mL conical tubes (VWR, Radnor, PA, United States) and subsequently 20 μL of rinsate was serially diluted to 10^{-6} in 180 μL of 1 × PBS via a flat bottom 96 well plate. The dot method was utilized in the current study where 10 μL of the rinsate was plated on XLD and mCCDA, allowed to dry completely, inverted, and incubated aerobically at 37°C for 24 h or microaerophilically at 42 °C for 48 h, respectively. On XLD, only colonies with black centers were considered as *Salmonella* and yellow colonies with surrounding yellow color change were considered as *E. coli*. On mCCDA, colony forming units with a silver metallic sheen were considered as *C. jejuni*.

Ambient PAA

To measure the ambient PAA vapor emitted from the treatment solution application, a SafeCide ChemDAQ sensor and meter was utilized (ChemDAQ Inc., Pittsburgh, PA, United States). The sensor was located directly outside the modified spray cabinet and measurements were recorded in real-time for each treatment application ($n = 10$; $N = 70$).

Statistical Analysis

Each carcass was randomly assigned to a treatment prior to the onset of the study. The CFU of *Salmonella*, *E. coli*,

and *Campylobacter* were log transformed and reported on a CFU of bacteria per gram of chicken basis (CFU/g). The data were analyzed using One-Way ANOVA in JMP 14.0 (SAS Institute Inc., Cary, NC, United States). Means were separated using Tukey's Protected HSD with a significant level of $P \leq 0.05$.

RESULTS

Quantification of *Salmonella*, *E. coli*, and *Campylobacter* Recovered From Treated Carcasses

In the current experiment, there was a treatment effect for *Salmonella, E. coli,* and *Campylobacter* recovered from the treated inoculated carcasses ($P < 0.05$). No treatments significantly reduced the concentration of *Salmonella* Typhimurium (UK-1) on whole carcasses compared to untreated carcasses (6.10 log CFU/g of *Salmonella*, **Figure 1**, $P = 0.0476$). However, those treated with TW + 500 ppm PAA + O_3 (5.71 log CFU/g of *Salmonella*) had significantly lower log CFU per gram of *Salmonella* than those treated with TW alone (6.30 log CFU/g of *Salmonella*). Carcasses treated with TW + 500 ppm PAA + O_3 (5.71 log CFU/g of *Salmonella*) did numerically possess the lowest log CFU/g of *Salmonella* compared to all other treatments. However, the treatment of both TW + PAA and TW + PAA + O_3 did not differ significantly in recovered *Salmonella* (6.05, 5.86, 5.96, and 5.71 log CFU/g of *Salmonella*).

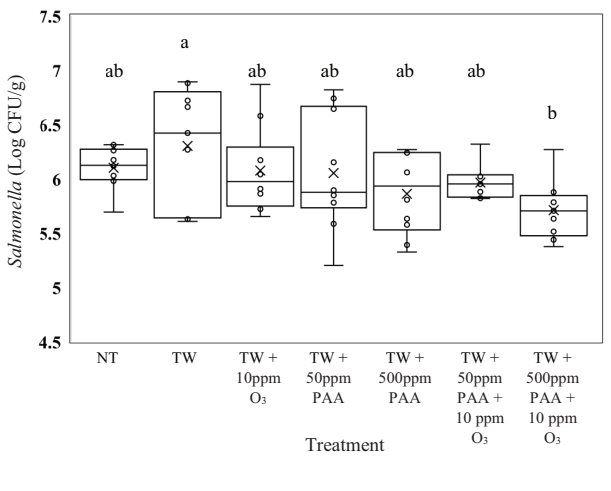

FIGURE 1 | The effect of applying peracetic acid (PAA) alone and in combination with aqueous ozone at 10 ppm on the mean log CFU/g of *Salmonella* Typhimurium UK-1 on whole hen carcasses[1,2]. Carcasses were inoculated with 10^7 CFU/mL of *Salmonella* for a final attachment of 10^6 CFU/g of *Salmonella* Typhimurium UK-1. Birds were then placed in a modified spray cabinet to be treated for 5 s (4×) via a low pressurized spray. Immediately after, birds were rinsed in 400 mL of neutralizing buffered peptone water (nBPW) and subsequently plated to determine load of *Salmonella*. [1]$N = 67$, $n = 10$, $P = 0.0476$. [2]Means with different superscripts are considered significantly different (a,b).

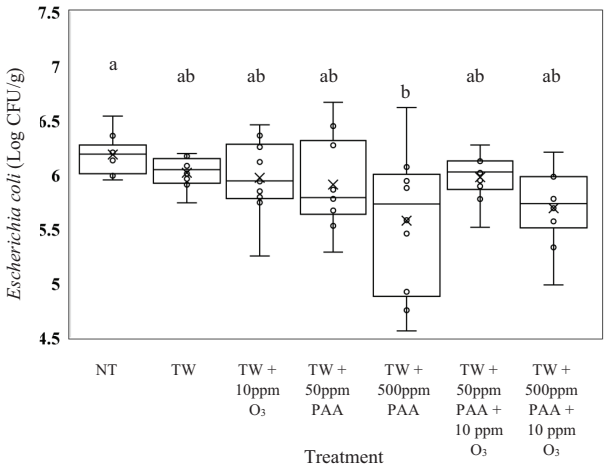

FIGURE 2 | The effect of applying PAA alone and in combination with aqueous ozone at 10 ppm on the mean log CFU/g of *Escherichia coli* J53 on whole hen carcasses[1,2]. Carcasses were inoculated with 10^7 CFU/mL of *E. coli* for a final attachment of 10^6 CFU/g of *E. coli* J53. Birds were then placed in a modified spray cabinet to be treated for 5 s (4×) via a low pressurized spray. Immediately after, birds were rinsed in 400 mL of nBPW and subsequently plated to determine load of *E. coli*. [1]$N = 68$, $n = 10$, $P = 0.0126$. [2]Means with different superscripts are considered significantly different (a,b).

Unlike the recovered *Salmonella*, *E. coli* (J53) recovered from the rinsates of carcasses treated with TW did not exhibit significantly higher counts compared to any of the other treated carcasses (**Figure 2**, $P = 0.0126$). Carcasses treated with TW + 500 ppm of PAA (5.57 log CFU/g of *E. coli*) yielded a lower load of *E. coli* than those not treated (6.18 log CFU/g of *E. coli*). Similar to *Salmonella*, the recovery of *E. coli* did not differ from carcasses treated with TW + PAA and TW + PAA + O_3, regardless of PAA concentration.

The recovered load of *C. jejuni* (log CFU/g of *Campylobacter*) was greatest in carcasses not treated (**Figure 3**; $P = 0.0006$). The *C. jejuni* recovered from carcasses not treated (5.20 log CFU/g of *C. jejuni*) did not differ from carcasses treated with TW, TW + O_3 and TW + 50 ppm PAA + O_3 (4.97, 5.00, and 4.96 log CFU/g of *C. jejuni*). Further, the lowest load of *C. jejuni* was recovered from carcasses treated with TW + 50 ppm PAA, TW + 500 ppm PAA, and TW + 500 ppm PAA + O_3 (4.80, 4.81, and 4.86 log CFU/g of *C. jejuni*) which were significantly different from the untreated control.

Quantification of PAA Vapor From Treated Carcasses

From the current experiment, it was determined that there was significant treatment effect on the production of ambient PAA (ppm) (**Figure 4**; $P < 0.0001$). Further, it was demonstrated that the greatest production of ambient PAA was derived from the treatment solution TW + 500 ppm PAA (0.565 ppm of

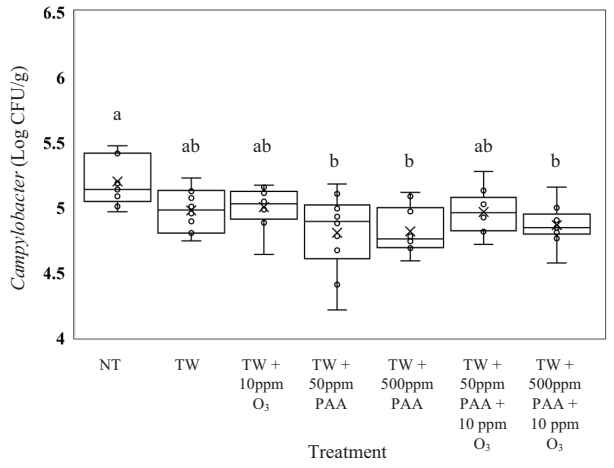

FIGURE 3 | The effect of applying PAA alone and in combination with aqueous ozone at 10 ppm on the mean log CFU/g of *Campylobacter jejuni* on whole hen carcasses[1,2]. Carcasses were inoculated with 10^6 CFU/mL of *C. jejuni* for a final attachment of 10^5 CFU/g of *C. jejuni*. Birds were then placed in a modified spray cabinet to be treated for 5 s (4×) via a low pressurized spray. Immediately after, birds were rinsed in 400 mL of nBPW and subsequently plated to determine load of *Campylobacter*. [1]$N = 69$, $n = 10$, $P = 0.0006$. [2]Means with different superscripts are considered significantly different (a,b).

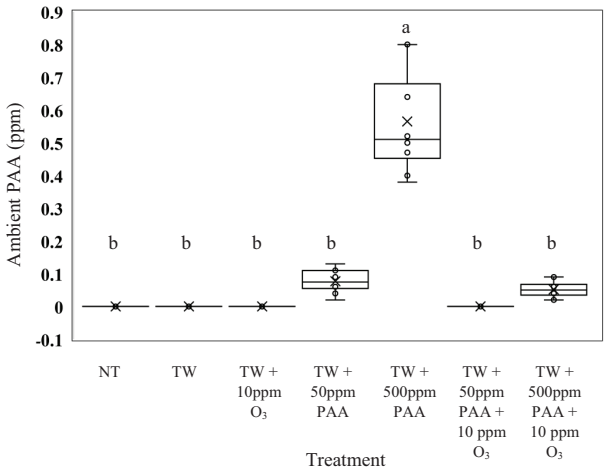

FIGURE 4 | The effect of applying PAA alone and in combination with aqueous ozone at 10 ppm on whole hen carcasses on the mean ppm of the surrounding ambient PAA[1,2]. Carcasses were inoculated with *Salmonella* Typhimurium UK-1, *E. coli* J53, and *C. jejuni*. Birds were then placed in a modified spray cabinet to be treated for 5 s (4×) via a low pressurized spray. While the treatments were being applied, a ChemDaq SafeCide ambient PAA monitor and sensor, located outside the modified spray cabinet, was utilized to determine ppm of ambient PAA. [1]$N = 70$, $n = 10$, $P < 0.0001$. [2]Means with different superscripts are considered significantly different (a,b).

Ambient PAA). The treatment solutions consisting of, NT, TW, TW + O_3, and TW + 50 ppm PPA + O_3 did not produce any ambient PAA; however, the ambient PAA produced from those treatments was not different that the ambient PAA produced off of the treatments: TW + 50 ppm PAA and TW + 500 ppm PAA + O_3 (0.011 and 0.008 ppm of ambient PAA).

DISCUSSION

Impact of Sanitizer Treatments on *Salmonella*, *E. coli*, and *Campylobacter* Inocula

In the current study, the addition of Viriditec^{TM} aqueous ozone to a commercial PAA was utilized to determine if the addition of aqueous ozone possessed synergistic affects in mitigating three Gram-negative bacteria, commonly associated with poultry. Previously, it has been suggested that Gram-negative bacteria may be more sensitive to ozone than Gram-positive bacteria due to the greater presence of peptidoglycan in the cell wall of Gram-positive bacteria. Rey et al. (1995) demonstrated the resistance to aqueous ozone was enhanced when N-acetyl glucosamine, a constituent of the peptidoglycan of bacterial cell walls, was present (pH 3 to 7).

In addition, the utilization of ozone has been demonstrated to possess the ability to disrupt the cell membrane and induce membrane permeability in *Salmonella* and *E. coli* spp., respectively, thus weakening the bacterial cell wall and ultimately leading to cell death (Komanapalli and Lau, 1996; Dave, 1999). However, in the current study, there was no difference on the reduction of any of the Gram-negative bacteria, *Salmonella, E. coli*, or *Campylobacter*, between the use of tap water or aqueous ozone (10 ppm) when sprayed for 5 s (4×) on whole hen carcasses.

The lack of effect of aqueous ozone, alone, may have been in part due to the short duration of the treatment application (5 s; 4×) utilized in the current study. Previous research has generally utilized aqueous ozone for a longer duration of exposure, when evaluating bactericidal effects of food matrices. Gertzou et al. (2016) determined the addition of gaseous ozone for 1 h at 10 ppm to fresh chicken legs extended their shelf life 4 days more than the control when packaged in polyamide/polyethylene (PA/PE) packaging. Others have seen various levels of gaseous ozone (1, 0.1, and 33 ppm) applied for 5, 20, and 9 min, respectively, to be effective at reducing *L. monocytogenes* in water, fish, and poultry samples (Fisher et al., 2000; Vaz-Vehlo et al., 2001; Muthukumar and Muthuchamy, 2013). Previously, aqueous ozone (4.5 ppm) in poultry chillers has demonstrated the potential to significantly mitigate total aerobes, psychrotrophs, coliforms, fecal coliforms, and *Salmonella* (78, 37, 91, 91, and 81%, respectively) on chicken broiler carcasses that had been chilled for 45 min compared to those not chilled (Sheldon and Brown, 1986). When aqueous ozone (0.5 to 6.5 ppm) was applied to poultry meat, in a separate study, it reduced the load of *Salmonella* Enteritidis by 0.6 to 4 log CFU (Dave, 1999).

However, ozone has demonstrated to be more effective in reducing bacteria when suspended in pure water than in food products (Khadre et al., 2001). In agreement with the current study, where aqueous ozone did not have an effect on mitigating pathogen load when utilized alone at 10 ppm, Fabrizio et al. (2002) determined that the spray application of distilled water, 10 ppm aqueous ozone, 10% trisodium phosphate (TSP), 2% acetic acid (AA), 20 ppm sodium hypochlorite, electrolyzed oxidizing water (pH 2.4 to 2.7, 1,150 mV ORP, 50 ppm free CL) on chicken whole carcasses did not have an effect on *Salmonella* Typhimurium load on day 0. However, Fabrizio et al. (2002) did report that the submersion of electrolyzed oxidizing water, TSP, AA, and aqueous ozone reduced *Salmonella* Typhimurium to levels of detection only after selective enrichment on whole chicken carcasses. The submersion of whole carcasses in aqueous ozone has also demonstrated the potential to reduce total aerobic bacteria on d 0 and reduce *E. coli* and total coliforms on d 7 compared to whole carcasses not treated (Fabrizio et al., 2002). Thus, the complete submersion of carcasses may prove to have a greater bactericidal effect than sprays may have, as observed in the current study.

In the current study when PAA was utilized alone, both concentrations of PAA, 50 and 500 ppm, reduced *C. jejuni* load, but only 500 ppm reduced *E. coli,* and no concentration of PAA reduced *Salmonella* compared to the control. Although PAA has been demonstrated to be an effective antimicrobial in previous research, mitigating pathogens by 2 logs or greater (Bauermeister et al., 2008a), the current research did not demonstrate the same efficacy. In fact, the current study demonstrated no treatments were capable of exhibiting practical reductions of pathogen load of 1 log or greater. Unlike the current research, Bauermeister et al. (2008a) demonstrated that when PAA is applied in the chiller at 200 ppm *Salmonella* and *Campylobacter* load are reduced roughly below 2 and 2.5 log CFU when artificially inoculated with 10^6 CFU/mL of *Salmonella* and *Campylobacter*, respectively. However, as with aqueous ozone, the application method may play a part in the differences in pathogen reduction.

Other short duration antimicrobial treatments of poultry meat with PAA have demonstrated little consistency. Del Río et al. (2007) demonstrated that when chicken legs were dipped in solutions containing 220 ppm PAA (Inspexx 100, Ecolab, St. Paul, MN, United States) for 15 s, *Enterobacteriaceae* and coliforms were reduced 0.24 ± 0.19 and 0.28 ± 0.84, respectively, on d 0; however, the reduction of bacteria on legs dipped in PAA was not significantly different than the legs treated with water. In contrast, Nagel et al. (2013) found that the post-chill application of PAA at concentration 400 and 1,000 ppm for 20 s had the potential to reduce the load of *Salmonella* Typhimurium and *C. jejuni* on artificially contaminated chicken breasts by 2 log CFU/mL.

Although, aqueous ozone and PAA, alone, did not mitigate pathogens as previous studies have shown, the combination of aqueous and PAA demonstrated an additive effect. This additive effect may be in part due to the byproducts of PAA and O_3. As PAA is the equilibrium product of acetic acid and hydrogen peroxide, when it dissociates acetic acid and hydrogen peroxide molecules are released. Hydrogen peroxide in aqueous solution is then capable of partially dissociating to hydroperoxide anion (HO_2^-) which is very reactive to ozone (Taube and Bray, 1940). Further, as acetic acid directly affects the pH, ozone is stabilized as ozone is more stable at a low pH (Khadre et al., 2001).

Overall, the current study demonstrated that the reduction of pathogens while utilizing the modified spray cabinet, was significant, it was not extensive. Previously, it has been demonstrated that bacteria reside not only on the exposed muscle surfaces, but within the feather follicles (Barnes and Impey, 1968). Thus, creating difficulties for antimicrobial treatments to properly disinfect poultry carcasses. This may explain the small reductions seen in the current study. This was also seen in a study performed by Sheldon and Brown (1986), who demonstrated less than a 1 log reduction of total aerobic bacteria, psychrotrophs, coliforms and fecal coliforms, and *Salmonella* when broiler carcasses were chilled in aqueous ozone for 45 min compared to those not chilled.

Decomposition of PAA Vapor

In the current study, the addition of aqueous ozone to PAA reduced the ambient PAA emitted when carcasses were treated in a modified spray cabinet. There is limited research on the proposed mechanism behind the reduction of PAA vapor, however, the authors have two proposed hypotheses to describe reduction in ambient PAA vapor. First, PAA (CH_3COOOH) is formed from the equilibrium of hydrogen peroxide (H_2O_2 or OH) and acetic acid (CH_3COOH). From the reaction of the acetic acid radical (Reaction 1) and ozone (O_3, Reaction 2) result in the formation of the peroxyacetic acid radical which disproportionates (Reaction 3) to produce 70% hydrogen peroxide (H_2O_2) and other products: formaldehyde, glyoxylic acid, glycolic acid, and organic peroxides (Sehested et al., 1992) as seen in the following reactions:

$$OH + CH_3COOH \rightarrow {}'CH_2COOH + H_2O$$

$${}'CH_2COOH + O_3 \rightarrow {}'OOCH_2COOH$$

$$2'OOCH_2COOH \rightarrow 70\%\ H_2O_2 + \text{other products}$$

The second explanation for the loss of ambient PAA vapor is that the ozone is being "robbed" an oxygen from the PAA to reduce it to acetic acid in the gas state, preventing the OH radical formation as seen in the following reaction:

$$CH_3OOOH + O_3 \rightarrow CH_3OOH + 2O_2$$

CONCLUSION

In conclusion, the combination of 10 ppm of aqueous ozone, Viriditec^{TM}, and 500 ppm of PAA has the potential to mitigate the presence of *Salmonella* Typhimurium (UK-1), *E. coli* J53, and *C. jejuni*. Furthermore, the combination of 10 ppm of aqueous ozone with 500 ppm of PAA demonstrated the ability of ozone to reduce the ambient PAA

vapor by 90%, when compared to 500 ppm of PAA alone. Thus, the application of TetraClean's product Viriditec^{TM} has the ability to enhance the safety for poultry processing employees. Although the current study demonstrated the promising capabilities of aqueous ozone and PAA, in combination, future research is necessary to develop an understanding of the impact the combination of aqueous ozone and PAA has on the shelf life of processed poultry and the subsequent changes in the microbiome.

AUTHOR CONTRIBUTIONS

All authors significantly contributed to the work of the current study. KF, DD, and SR designed and prepared the current study with the assistance from DW, BP, and MD. DD and DW conducted the experiments. DD analyzed the data and constructed the manuscript with the assistance from KF, DW, BP, MD, and SR.

ACKNOWLEDGMENTS

DD would like to knowledge the Graduate College at the University of Arkansas for its support through the Distinguished Academy Fellowship and the continued support from the Cell and Molecular Biology Program and Department of Food Science at the University of Arkansas. Furthermore, the authors would like to acknowledge TetraClean Systems LLC for its support and assistance in completing this research.

REFERENCES

American Conference of Governmental Industrial Hygienists [ACGIH] (2016). *Annual TLVs® (Threshold Limit 4 Values) and BEIs® (Biological Exposure Indices) Booklet*. Cincinnati, OH: ACGIH® Signature Publications.

American Thoracic Society (1996). *Respiratory Protection Guidelines. American Thoracic Society: Medical Section of the American Lung Association*. Available at: https://www.thoracic.org/statements/resources/eoh/resp1-13.pdf [accessed October 16, 2018]

Barnes, E. M., and Impey, C. (1968). Psychrophilic spoilage bacteria of poultry. *J. Appl. Bacteriol.* 31, 97–107. doi: 10.1111/j.1365-2672.1968.tb00345.x

Bauermeister, L. J., Bowers, J. W. J., Townsend, J. C., and McKee, S. R. (2008b). Validating the efficacy of peracetic acid mixture as an antimicrobial in poultry chillers. *J. Food Prot.* 71, 1119–1122. doi: 10.4315/0362-028X-71.6.1119

Bauermeister, L. J., Bowers, J. W. J., Townsend, J. C., and McKee, S. R. (2008a). The microbial and quality properties of poultry carcasses treated with peracetic acid as an antimicrobial treatment. *Poult. Sci.* 87, 2390–2398. doi: 10.3382/ps.2008-00087

Brinez, W. J., Roig-Sagues, A. X., Hernandez Herrero, M. M., Lopez-Pedemonte, T., and Guamis, B. (2006). Bactericidal efficacy of peracetic acid in combination with hydrogen peroxide against pathogenic and non pathogenic strains of *Staphylococcus* spp., *Listeria* spp. and *Escherichia coli*. *Food Contr.* 17, 516–521. doi: 10.1016/j.foodcont.2005.02.014

Dave, S. A. (1999). *Efficacy of Ozone Against Salmonella Enteritidis in Aqueous Suspensions on Poultry Meat*. MSc. thesis, The Ohio State University, Columbus, OH.

Del Río, E., Muriente, R., Prieto, M., Alonso-Calleja, C., and Cpita, R. (2007). Effectiveness of trisodium phosphate, acidified sodium chlorite, citric acid, and peroxyacids against pathogenic bacteria on poultry during refrigerated storage. *J. Food Prot.* 70, 2063–2071. doi: 10.4315/0362-028X-70.9.2063

Fabrizio, K. A., Sharma, R. R., Dermirci, A., and Cutter, C. N. (2002). Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce *Salmonella* species on poultry. *Poult. Sci.* 81, 1598–1605. doi: 10.1093/ps/81.10.1598

Fisher, C. W., Lee, D., Dodge, B.-A., Hamman, K. M., Robbins, J. B., and Martin, S. E. (2000). Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 66, 1405–1409. doi: 10.1128/AEM.66.4.1405-1409.2000

Fraser, J. A. L., and Thorbinson, A. (1986). *Fogging Trials with Tenneco Organics Limited (30th June, 1986) at Collards Farm*. Warrington: Solvay Interox.

Gertzou, I. N., Drosos, P. E., Karabagias, I. K., and Riganakos, K. A. (2016). Combined effect of ozonation and packaging on shelf life extension of fresh chicken legs during storage under refrigeration. *J. Food Sci. Technol.* 53, 4270–4277. doi: 10.1007/s13197-016-2421-7

Janssen, P. J. M. (1989). *Acute Inhalation Toxicity Studies of Proxitane 1507 in Male Rats (I)*. Report No. S. 8906, Int. Doc. No. 56645/25/89. Weesp: Duphar B.V.

Janssen, P. J. M., and van Doorn, W. M. (1994). *Acute Inhalation Toxicity Study with Proxitane AHC in Male and Female Rats*. Report No. S. 9408, Int. Doc. No. 56345/48/94. Brussels: Solvay.

Khadre, M. A., Yousef, A. E., and Kim, J.-G. (2001). Microbiological aspects of ozone applications in food: a review. *J. Food Sci.* 66, 1242–1252. doi: 10.1111/j.1365-2621.2001.tb15196.x

Komanapalli, I. R., and Lau, B. H. S. (1996). Ozone-induced damage of *Escherichia coli* K-12. *Appl. Environ. Biotechnol.* 46, 610–614. doi: 10.1007/s002530050869

Merka, V., and Urban, R. (1978). Study of inhalation toxicity of performic, peracetic and perpropionic acid in mice. *J. Hyg. Epidemiol. Microbiol. Immunol.* 20, 54–60.

Muthukumar, A., and Muthuchamy, M. (2013). Optimization of ozone in gaseous phase to inactivate *Listeria monocytogenes* on raw chicken samples. *Food Res. Int.* 54, 1128–1130. doi: 10.1016/j.foodres.2012.12.016

Nagel, G. M., Bauermeister, L. J., Bratcher, C. L., Singh, M., and McKee, S. R. (2013). *Salmonella* and *Campylobacter* reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. *Int. J. Food Microbiol.* 165, 281–286. doi: 10.1016/j.ijfoodmicro.2013.05.016

National Academies of Science [NAS] (2010). *Chapter 7: Peracetic Acid-Acute Exposure Guideline Levels. In: Acute Exposure Guideline Levels for Selected Airborne Chemicals*, Vol. 8. Available at: http://www.epa.gov/opptintr/aegl/pubs/peracetic_acid_final_volume8_2010.pdf

National Institute for Occupational Safety and Health [NIOSH] (2015). *Immediately Dangerous to Life or Health (IDLH) Value Profile for Peracetic Acid. External Review Draft*. Available at: https://www.cdc.gov/niosh/docket/review/docket156a/pdfs/g1-013-peracetic-acid-cas-79-21-0.pdf

Oyarzabal, O. A. (2005). Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *J. Food Prot.* 68, 1752–1760. doi: 10.4315/0362-028X-68.8.1752

Rey, R. P., Sellés, A. N., Baluja, C., and Otero, M. L. (1995). Ozonation kinetics of glucosamine and N-acetyl glucosamine in aqueous medium. *Ozone Sci. Eng.* 17, 463–467. doi: 10.1080/01919510508547349

Sehested, K., Corfitzen, H., Holcman, J., and Hart, E. J. (1992). Decomposition of ozone in aqueous acetic acid solutions (pH 0-4). *J. Phys. Chem.* 96, 1005–1009. doi: 10.1021/j100181a084

Sheldon, B. W., and Brown, A. L. (1986). Efficacy of ozone as a disinfectant for poultry carcasses. *J. Food Sci.* 51, 305–309. doi: 10.1111/j.1365-2621.1986.tb11116.x

Taube, H., and Bray, W. C. (1940). Chain reactions in aqueous solutions containing ozone, hydrogen peroxide, and acid. *J. Am. Chem. Soc.* 62, 3357–3373. doi: 10.1021/ja01869a027

USDA Food Safety and Inspection Service (2016). *FSIS Notice 41-16. June* 8, 2016. Washington D.C: United States Department Of Agriculture.

Vaz-Vehlo, M., Duarte, G., McLauchlin, J., and Gibbs, P. (2001). Characterization of *Listeria monocytogenes* isolated from production lines of fresh and cold-smoked fish. *J. Appl. Microbiol.* 91, 556–562. doi: 10.1046/j.1365-2672.2001.01425.x

Conflict of Interest Statement: BP, DW, and MD are employed by the company TetraClean Systems LLC, Omaha, NE, United States.

The remaining 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: 23 January 2019
doi: 10.3389/fmicb.2018.03280

Developments in Rapid Detection Methods for the Detection of Foodborne *Campylobacter* in the United States

Steven C. Ricke[1], Kristina M. Feye[1], W. Evan Chaney[2], Zhaohao Shi[1], Hilary Pavlidis[2] and Yichao Yang[3]*

[1]Department of Food Science, Center of Food Safety, University of Arkansas, Fayetteville, AR, United States, [2]Diamond V, Cedar Rapids, IA, United States, [3]Department of Poultry Science, University of Arkansas, Fayetteville, AR, United States

OPEN ACCESS

Edited by:
Eugenia Bezirtzoglou, Democritus University of Thrace, Greece

Reviewed by:
Spyridon Ntougias, Democritus University of Thrace, Greece
Christi Swaggerty, United States Department of Agriculture, United States
Birce Mercanoglu Taban, Ankara University, Turkey

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

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

Received: *23 July 2018*
Accepted: *17 December 2018*
Published: *23 January 2019*

Citation:
Ricke SC, Feye KM, Chaney WE, Shi Z, Pavlidis H and Yang Y (2019) Developments in Rapid Detection Methods for the Detection of Foodborne Campylobacter in the United States. Front. Microbiol. 9:3280. doi: 10.3389/fmicb.2018.03280

The accurate and rapid detection of *Campylobacter* spp. is critical for optimal surveillance throughout poultry processing in the United States. The further development of highly specific and sensitive assays to detect *Campylobacter* in poultry matrices has tremendous utility and potential for aiding the reduction of foodborne illness. The introduction and development of molecular methods such as polymerase chain reaction (PCR) have enhanced the diagnostic capabilities of the food industry to identify the presence of foodborne pathogens throughout poultry production. Further innovations in various methodologies, such as immune-based typing and detection as well as high throughput analyses, will provide important epidemiological data such as the identification of unique or region-specific *Campylobacter*. Comparable to traditional microbiology and enrichment techniques, molecular techniques/methods have the potential to have improved sensitivity and specificity, as well as speed of data acquisition. This review will focus on the development and application of rapid molecular methods for identifying and quantifying *Campylobacter* in U.S. poultry and the emergence of novel methods that are faster and more precise than traditional microbiological techniques.

Keywords: *Campylobacter*, rapid detection, characterization, poultry, molecular technology

INTRODUCTION

Campylobacter species *Campylobacter jejuni* and *Campylobacter coli* are etiological agents of campylobacteriosis, which is a significant foodborne disease in the United States (Humphrey et al., 2007; Horrocks et al., 2009; Bolton, 2015). The Centers for Disease Control and Prevention (CDC) estimates that approximately 9% of foodborne illnesses in the United States originate from *Campylobacter* spp., with 15% of campylobacteriosis cases leading to hospitalization (Scallan et al., 2011). In recent years, despite other reservoirs for campylobacteriosis, chicken and other poultry products remain the primary contributors to campylobacteriosis. *Campylobacter* is not generally pathogenic in adult birds and is considered a commensal microorganism of the gastrointestinal microbiota; therefore, once *Campylobacter* successfully colonizes a few birds,

it rapidly asymptomatically disseminates throughout the flock and is extremely difficult to track (Horrocks et al., 2009).

As a result, the status of *Campylobacter* in the bird is a significant concern to poultry producers as colonized birds often reach the processing plant undetected and lead to foodborne illness. As the demand for more poultry meat and faster line speeds increases in the United States, it will become important to develop rapid and reliable detection methods that enable real-time decision making for producers. Unfortunately, the poultry industry still relies on traditional microbiology-based approaches, which are time consuming and have relatively high limits of detection. In order to understand the relationship between traditional strategies to monitor for *Campylobacter* and the poultry industry, a critical review of the available culture methods in animal production, and specifically poultry production in the United States, was written by Huang et al. (2015). Huang and colleagues highlighted numerous issues associated with traditional culturing methods, such as media bias and time-to-data acquisition; however, the authors did not discuss alternative methods in depth (Huang et al., 2015). Non-culture-based methods should alleviate the concerns associated with microbiological approaches and enable the rapid assessment of the prevalence and even the absolute quantification of *Campylobacter* in poultry matrices. This review will focus on rapid, non-commercial molecular methods for the detection of *Campylobacter* and its potential use in U.S. poultry production systems.

CHALLENGES TO THE IDENTIFICATION AND DETECTION OF *CAMPYLOBACTER*

Campylobacter jejuni and *C. coli* have the highest rate of foodborne-related clinical campylobacteriosis, and as a result, the detection and identification of *Campylobacter* are dedicated almost exclusively to these two species. Traditional microbiological culture methods have evolved over time to include the use of selective media and the optimization of growth conditions and antibiotic support to reduce co-cultured species of microorganisms. Media use guidelines necessitate the elevated incubation temperature (42°C) and a microaerophilic atmosphere to favor the growth of the thermophilic *Campylobacter*. Additionally, several antibiotics can be employed that repress the growth of non-*Campylobacter* species while simultaneously supporting the growth of naturally antibiotic-resistant isolates of *Campylobacter* (Eberle and Kiess, 2012). Once successfully isolated from poultry matrices, the further confirmation of the identification of *Campylobacter* includes phenotypic differentiation such as biotyping, serotyping, and multilocus enzyme electrophoresis discussed in detail by Eberle and Kiess (2012).

Despite the continued refined options for the isolation of *Campylobacter*, challenges remain, which reduce the efficiency of these methodologies. Unlike other foodborne pathogens like *Salmonella*, *Campylobacter* exhibits dynamic and malleable physiological and metabolic biological characteristics that can actively interfere with the sensitivity and specificity of culture-dependent methods. Evidence for this plasticity emerged during the assessment of multiple metabolic traits using an extensive panel of biochemical tests to evaluate multiple *Campylobacter* species (On et al., 1996). Data revealed significant and unique phenotypic diversity (On et al., 1996). This metabolic fluidity is further supported by evidence suggesting extensive genetic diversity and genomic instability in *C. jejuni* poultry isolates that may be environmentally influenced (Wassenaar et al. 1998; Wilson et al., 2010). Another good example of this plasticity is that when *Campylobacter* becomes stressed when exposed to psychrotrophic conditions during refrigeration and freezing, *Campylobacter* becomes viable but non-cultivable (VBNC), which renders it unable to be detected using many traditional microbiological techniques (Tholozan et al., 1999; Ziprin et al., 2003; Castro et al. 2018). Metabolically driven strain to strain variation in the expression of virulence factors makes targeting these factors problematic for the detection of *Campylobacter* using a single methodology becomes problematic (Hofreiter et al., 2008). While it remains unclear as to how the strain-specific diversity of *Campylobacter* impacts the growth and recovery of the pathogen on selective media, it is highly likely that bias and changes in sensitivity and specificity are fluid and environmentally driven through unknown biological interactions.

Consequently, there are significant challenges in culturing *Campylobacter* on selective and/or differential media that arise in the presence of other microorganisms, which can likely influence the metabolism of *Campylobacter*. Also, the diversity of poultry-specific matrices may also induce biochemical changes to *Campylobacter*, which further obscure isolation and identification. In order to assess whether or not microbiologically diverse and complex matrices influence the identification of *Campylobacter*, Oakley et al. (2012) compared five different commercialized selective *Campylobacter* media for the ability to isolate *Campylobacter* from broiler fecal samples. Oakley and colleagues then compared the sequenced colonies from selective media to the pooled fecal samples using 16S rRNA tagged-pyrosequencing. Sequencing results indicated that 0.04% of the total fecal microbial community was *Campylobacter*. Comparing *Campylobacter*-specific media to the sequencing results of the individual colonies indicated that 88–97% of the putative colonies were in fact *Campylobacter* (Oakley et al., 2012). Therefore, when taken together, the specificity of *Campylobacter*-selective media when isolating the pathogen from complex matrices necessitates doubt. Additional data from other studies revealed that specific isolation procedures and culture media influence the diversity of *Campylobacter* species recovered from samples. For example, Ugarte-Ruiz et al., (2013) used eight different isolation protocols, with or without enrichment, followed by culture on selective media. Data indicated that the isolation method used by researchers influenced *Campylobacter* species isolated from poultry samples (Ugarte-Ruiz et al., 2013). Temperature, media, time, and enrichment all influence the ability to isolate *Campylobacter*.

Perhaps, the most significant data that lend themselves to further doubting the use of selective and/or differential media

for the isolation of *Campylobacter* emerged with data from next-generation sequencing studies. In a more recent study, Kim et al. (2017a) compared poultry carcass rinsate directly with microbiome sequencing and then to the sequences of the pooled colonies recovered from commercial *Campylobacter* on selective media. Despite the results of selective media, the *Campylobacter* was not a predominant bacterium identified in the rinsate microbiota despite dominating the *Campylobacter* selective plates. While initially not surprising, at different stages of poultry processing, there were a significant range of non-*Campylobacter* bacteria that cocultured on selective media. Common cocultured genera include *Oscillospira, Acinetobacter, Enterococcus,* and *Bacillus* (Kim et al., 2017a). Therefore, data indicate that environmental influences, such as those found at different stages of poultry processing, drive the challenges associated with culturing of *Campylobacter* on selective media. As a result, the traditional culture-based microbiological techniques for the detection of *Campylobacter* in poultry processing must be scrutinized and alternative methods need development. The following sections will focus on the available molecular methods that enable more specific and sensitive detection and quantitation of *Campylobacter* spp. from food and poultry samples.

RAPID DETECTION OF *CAMPYLOBACTER*—GENERAL CONCEPTS

Over the past few decades, the rapid, culture-independent detection of bacterial pathogens has become increasingly routine. For the detection of foodborne pathogens, there are two classes of technologies used for the fast identification of pathogens: immune and nucleic acid-based methodologies. The immune-based detection methodologies exploit the affinity of antibodies for specific target antigens found on the surface of the desired microorganism. The use of nucleic acid-based technologies recognizes unique and highly specific DNA or RNA sequences that can either be sequenced, amplified and visualized on a gel, or otherwise differentiated for detection, quantification, and molecular typing (Yolken, 1988; Manfreda and De Cesare, 2005; Maciorowski et al., 2006; Mandal et al., 2011; Gharst et al., 2013; Park et al., 2014; Välimaa et al., 2015; Baker et al., 2016; Chen and Park, 2016; Zeng et al., 2016). Both immune and nucleic acid-based approaches are summarized in **Table 1** (Immune) and **Table 2** (Nucleic-acid). Because these methods can be quick and accurate, kits are actively being commercialized to detect foodborne pathogens. Also, once commercialized, immune and nucleic acid-based assays have the potential to become high throughput. High-throughput assays that are simple to use with a fast turn-around time are imperative for any technology to truly be successful in displacing common microbiological techniques used in poultry monitoring systems. Additionally, having a significant level of repeatability and reliability will also become important to satisfy regulatory bodies that monitor poultry productions. While these approaches have their weaknesses, namely time, specificity, and the limits of detection and quantification, it likely is a necessary step to modernize the food industry. By modernizing monitoring strategies industry-wide, novel insight may fill knowledge gaps associated with the transmission of foodborne diseases (Hannson et al., 2016). Finally, by creating a faster monitoring regime, real-time management decisions that are economical while safeguarding the food supply are possible (Hannson et al., 2016).

As a society, we are at the precipice of significant changes arising from the innovations associated with next-generation sequencing and proteomic technologies. Such innovations not only impact molecular genetics and biology but can also be used to improve immune-based technologies. These changes will definitively improve the monitoring of food supplies for pathogens, as well as a host of other opportunities for the poultry industry. Importantly, as the genomes of particular species continue to

TABLE 1 | Detection of *Campylobacter* spp. using various immunological techniques.

Target epitope	Organism	Type	Author; Notes
Monoclonal assays			
Flagellin	*Campylobacter* spp.	Monoclonal antibodies	Nachamkin and Hart (1986)
Outer membrane protein	*Campylobacter* spp.	Monoclonal antibodies	Lamoureux et al. (1997)
15-kDa cell surface protein	*C. jejuni/C. coli*	Monoclonal antibodies	Kawatsu et al. (2008)
Two *C. jejuni*/one *C. coli* epitope	*C. jejuni/C. coli*	Monoclonal antibody 33D2	Heo et al. (2009)
Lipopolysaccharide antigens	*C. jejuni*	Monoclonal antibodies	Brooks et al. (1998)
Hippurate hydrolase	*C. jejuni*	Monoclonal antibodies	Steele et al. (2002)
Outer membrane protein	*C. jejuni*	Monoclonal antibodies	Qian et al. (2008)
Nonconventional assays			
Surface antigen	*Campylobacter* spp.	Enzyme-linked fluorescent assay	Reis et al. (2018); VIDAS® 30 system
Surface antigen	*C. jejuni/C. coli*	Microplate EIA assay	Granato et al. (2010); Premier CAMPY EIA kit
Surface antigen	*C. jejuni/C. coli*	Microplate EIA assay	Granato et al. (2010); ProSpectT Campylobacter EIA kit
Surface antigen	*C. jejuni/C. coli*	Lateral-flow EIA assay	Granato et al. (2010); ImmunoCARD STAT! CAMPY kit
Surface antigen	*C. jejuni*	Cotton swab colorimetric assay	Alamer et al. (2018); swabs contain colored nanobeads with monoclonal antibody cocktails
Surface antigen	*C. jejuni*	Biosensor	Masdor et al. (2016); biosensor with sandwich assays
Surface antigen	*C. jejuni*	Single-chain variable fragment antibodies	Nzuma et al. (2018); detection with IMS-qPCR

be identified, the knowledge of foodborne pathogen biology and plasticity will co-evolve. This may be particularly important for an organism such as *Campylobacter,* which can exist as VBNC. Being able to understand specific environmental cues for triggering the VBNC status of *Campylobacter* may help researchers understand its survival patterns and metabolic dependencies. Said knowledge is exploitable as it will naturally lead to improved detection techniques targeted to detect VBNC *Campylobacter*. However, there are also drawbacks to this approach (**Table 3**). Currently, drawbacks include assay cost and the requirement for higher-skilled workers, and the requirement to update present and future technologies may be problematic. The following sections will describe the current rapid detection and identification methods for foodborne *Campylobacter* in poultry.

TABLE 2 | Advantages and disadvantages of selected detection methods.

	Advantages	Disadvantages
Conventional techniques		
Selective plating	Inexpensive	Cannot culture VBNC state cells
	Well-established	Variable specificity
	Can customize antibiotic makeup	Can be affected by culturing methods
Immune-based		
ELISA	Can perform many samples at once	Loss of sensitivity and specificity in mixed cultures
	Several different possible techniques (direct, indirect, sandwich)	Cross-reactivity between closely related species
	Can change selectivity based on targeted epitopes	False positives from complex matrices
Flow cytometry	Multiple parameters analyzed	Expensive, specialized equipment
	Single cell analysis	Requires highly trained personnel to prepare, run, and analyze data
	High specificity	Relatively slow
PCR-based		
Conventional PCR	Better specificity than plating	Non-specific binding of similar DNA
	Can be combined with other assays such as ELISA	Must be optimized
	Relatively simple and quick	Can only be used for presence/absence
Multiplex PCR	Assay multiple species at once	Requires highly specific primers
	Higher throughput than conventional PCR	Difficult to optimize
	Less costly than running multiple assays	False negatives/positives
qPCR	High sensitivity	Complex matrices may include inhibitors
	Used for rapid detection	Require highly specific primers
	May be multiplexed	Cannot differentiate live/dead cells
	EMA/PMA may be used to help distinguish dead cells	
dPCR	Cheaper than qPCR	Cannot differentiate live/dead cells
	Less vulnerable to inhibitors than qPCR	Greater chance of false positives than qPCR
	No calibration or internal controls required	
Sequencing		
16S rRNA	Highly conserved region found in all bacteria	High cost of equipment
	Able to distinguish species using variable regions	Relative abundance may be skewed by copy number
	Can be used on non-culturable bacteria	Possible species level resolution issues
Whole genome sequencing	Open access of many databases	High cost of equipment
	High discrimination	Specialized training required
	Can detect antimicrobial resistances and virulence genes	Varied interpretation of data

TABLE 3 | Recent studies in whole genome sequencing (WGS) of *Campylobacter* spp.

Sequencing type	Organism	Topic	Sample; Country; Author; Notes
Illumina	*C. jejuni/coli*	Antimicrobial resistance profiling	Clinical, meats, ceca isolates; USA; Zhao et al. (2016) Identified antimicrobial resistance genes to predict phenotypic resistance.
Illumina	*C. jejuni/coli*	Antimicrobial resistance profiling	Poultry isolates; USA; Whitehouse et al. (2018) Identified antimicrobial resistance genes to predict phenotypic resistance.
Illumina	*C. jejuni*	Antimicrobial resistance profiling	Clinical, poultry isolates; Estonia; Mäesaar et al. (2018) Used WGS and MLST to analyze antimicrobial resistance in strain types.
Illumina	*C. jejuni*	Antimicrobial resistance profiling	Poultry isolates; Europe; Leekitcharoenphon et al. (2018) Examined fluoroquinolone resistance in poultry isolates from 12 European countries.
Illumina	*Campylobacter* spp.	Comparative analysis	Poultry, bird isolates; USA; Lawton et al. (2018) Compared using MALDI-TOF MS as a rapid method to identify *Campylobacter* isolates to species level.
Ion torrent	*C. jejuni*	Comparative analysis	Clinical, animal, environmental isolates; France; Thépault et al. (2018) Studied pathogen source attribution of campylobacteriosis in France using WGS and MLST.

(Continued)

TABLE 3 | Continued

Sequencing type	Organism	Topic	Sample; Country; Author; Notes
Illumina	*C. jejuni/coli*	Epidemiology	Chicken liver pâté; Sweden; Lahti et al. (2017) Outbreak of campylobacteriosis due to undercooked chicken liver pate.
Illumina/ wgMLST	*C. jejuni*	Epidemiology	Clinical, poultry, bovine isolates; Israel; Rokney et al. (2018) Used WGS and wgMLST to screen for virulence genes in Isreaeli *C. jejuni* isolates.
Illumina	*C. jejuni*	Genome sequence	Poultry isolates; USA; Taveirne et al. (2017) Sequenced three *C. jejuni* strains from naturally colonized farm-raised chickens.
Illumina/ SMRT	*C. jejuni*	Genome sequence	Poultry isolates; USA; Sacher et al. (2018) Sequenced three phage-propagating strains of *C. jejuni*.
Illumina/ SMRT	*C. coli*	Genome sequence	Poultry isolate; USA; Ghatak et al. (2017) Discovered type VI secretion system and antimicrobial resistance genes in plasmid of *C. coli* YH502.
Ion Torrent	*C. jejuni/coli*	Genome sequence	Chicken sushi; Japan; Asakura et al. (2017) Sequenced two isolates associated with an outbreak due to consumption of undercooked chicken sushi.
Illumina	*C. jejuni*	Genome wide association study	Clinical, animal, environmental isolates; Canada; Buchanan et al. (2017) Identified gene markers associated with clinically related *C. jejuni* isolates.
Illumina	*C. jejuni/coli*	Stress resistance	Clinical, poultry isolates; UK; O'Kane and Connerton (2017) Analyzed aerotolerant *C. coli* strain.

IMMUNE-BASED ASSAYS FOR *CAMPYLOBACTER*

The immune-based methodology for the detection of foodborne pathogens has been well established and includes enzyme-linked immunosorbent assays (ELISA), flow cytometry, and quantitative immunofluorescence, among others (Yolken, 1982, 1988; Maciorowski et al., 2006; Bordeaux et al., 2010; Oyarzabal and Battie, 2012; Baker et al., 2016; Alahi and Mukhopadhyay, 2017). Both monoclonal and polyclonal antibodies can be manufactured specifically to detect pathogen-specific epitopes. Additionally, antibodies can be modified, which commonly includes the conjugation of various detection systems, such as horseradish peroxidase, to improve the detection sensitivity and specificity of various target epitopes (Yolken, 1982; Preiner et al., 2014; Janda et al., 2016; Alahi and Mukhopadhyay, 2017).

Immunological methods dedicated to the detection and quantification of *Campylobacter* spp. have been extensively reviewed by Oyarzabal and Battie (2012) and will only be briefly discussed in the current review. Much of the early research focused on the discovery of conserved, non-species-specific *Campylobacter* antigens targeted by monoclonal antibodies, such as lipopolysaccharides, flagellin, and other protein antigens for detection immunoassays (Nachamkin and Hart, 1986; Lamoureux et al., 1997; Brooks et al., 1998; Kawatsu et al., 2008). An example of this was the work conducted by Nachamkin and Hart (1986) where researchers generated murine monoclonal antibodies to one of two distinct *Campylobacter* flagellin epitopes, and in turn became capable of detecting *Campylobacter* spp. and also differentiate *C. jejuni* or *coli*. However, as with most antibody-based biotechnology, there is significant cross-reactivity with *C. jejuni* and *C. coli* as their genetic divergence is not significant (**Table 1**). Other methodologies include those developed by Steele et al. (2002). Steele and colleagues used an ELISA to screen 11 monoclonal antibodies against *C. jejuni* hippurate hydrolase, or hipO, an enzyme specific to *C. jejuni*. They were able to identify several antibodies that demonstrated a high affinity for *C. jejuni* when exposed to cell extracts from *Campylobacter* and non-*Campylobacter* bacterial species. Research continued to evolve to reduce the cross-reactivity as time progressed. Qian et al. (2008) focused on a major outer membrane protein epitope that specifically targeted *C. jejuni*. Importantly, once researchers isolated the high-affinity *C. jejuni* antibody, they located the epitope by mapping it to a 13-amino-acid polypeptide and confirmed it by demonstrating that alteration of any of the amino acids contained within that epitope reduces antibody binding affinity. Further advancements also included the ability to generate monoclonal antibodies that bind to extremely conserved, linear epitopes not impacted by heat-killing *Campylobacter*, thus enabling the detection of the species in potentially thermophilic environments like a scalder (Heo et al., 2009).

Since the development of immunological-based methods to detect *Campylobacter,* several commercial immunoassays have come on the market and have been compared with non-immunological detection methods (**Table 1**). Again, such steps are necessary as the continued improvement of technology lends itself to more sensitive and specific assays, which then require validation for the poultry industry. A classic example of the validation of commercial kits that demonstrated superior performance as compared to conventional methods was described by Granato et al. (2010). While the kits were specific for clinical diagnostics, the methodology of comparing the gold standards (qPCR and microbiology) versus newer technology (EIA) is demonstrative of the rigor required for the validation of kits in the poultry industry. Granato and colleagues compared

conventional *Campylobacter* microbiological culturing techniques with three commercial *Campylobacter* enzyme immunoassay (EIA) kits as well as a real-time polymerase chain reaction (qPCR). Remarkably, all three EIA commercial kits demonstrated sensitivity and specificity above 98% as compared to 94.1% for the culture methods (Granato et al., 2010). Necessarily, if employing these methods in the poultry industry is the goal, similar metrics must be met to demonstrate that faster methods do not sacrifice sensitivity and specificity. Additionally, as previously mentioned, repeatability is important. In the case of EIA assays, Regnath and Ignatius (2014) also observed a similar agreement to Granato and colleagues when comparing EIA and culture methods for the detection of *C. jejuni* and *coli* from stool samples.

However, there are significant limitations associated with immune-based detection methods. Further comparisons of commercial kits with traditional microbiological and molecular techniques have suggested that despite advancements, cross-reactivity may occur with *Campylobacter* species leading to false positives. These species are typically not the commonly assayed *C. jejuni* and *C. coli*. As a result, some commercial immunoassays may elicit false positives and undefined variability for clinical samples (Myers et al. 2011; Floch et al., 2012; Couturier et al., 2013; Gharst et al., 2013). This lack of sensitivity may result in some evidence suggesting that for poultry samples, qPCR assays may be more sensitive than a commercial enzyme-linked fluorescent assay when tested on chilled and frozen broiler carcasses (Reis et al., 2018).

Challenges associated with matrix-induced loss of sensitivity and specificity have led to the innovation of the technology leading to self-contained immune-based biosensors and nano-based assays. These biosensors convert the binding activities of the antibody into an electrical signal that is more precise for the assessment of the pathogen in a mixed culture (O'Connell et al., 2000; Willner, 2002; Reshetilov, 2005; Wei et al., 2007; Huang et al., 2010; Masdor et al., 2016; Alahi and Mukhopadhyay, 2017; Asal et al., 2018). In a more recent study, Masdor et al. (2017) covalently attached rabbit polyclonal antibodies to gold chips and developed a surface plasma resonance (SPR) sensor platform for *C. jejuni* detection. When the sensor chips were assessed for the limit of detection (LOD), a sandwich format using a polyclonal antibody improved LOD compared to the direct assay. Nano-based immunoassays are potentially practical, user friendly detection strategies for the poultry industry, which can actively be adapted to various poultry-specific applications. For example, Alamer et al. (2018) developed a cotton swab immunoassay for potential use in poultry processing plants by immersing swabs into different colored nanobead-conjugated *Campylobacter jejuni*-specific monoclonal antibody cocktails. The change in color intensity of the swab was captured by a smartphone and quantitated with the National Institute of Health ImageJ computer program. They were able to achieve a LOD of 10 CFU per mL with no observable cross-reactivity. The concept of a cotton swab-based pathogen colorimetric assay would be attractive for assessing contamination in remote sites of poultry processing plants and offer immediate results for making in-plant decisions regarding control measures.

Another potential direction for the improvement of immunoassays would be the use of proteomics to identify ideal target epitopes for monoclonal antibodies to enhance the resolution of differentiating between different strains and species epithets of *Campylobacter*. As *Campylobacter* is genomically and metabolically fluid, this is extremely important for the next generation of EIA development. Rodrigues et al. (2016) used proteomics to demonstrate that there were differences in the cadre of proteins expressed between microaerophilic and aerobic culture conditions. Along these lines, Turonova et al. (2017) uncovered differences in protein expression when *C. jejuni* was switched from the stationary to the exponential growth phase. The application of proteomics to immune responses may prove to be especially helpful to find novel antibodies. Mehla and Ramana (2017) employed computational analyses based on informatics databases of the *Campylobacter* genome to identify and predict immunogenic epitopes that could stimulate B- and T-cell activity and serve as antigens for vaccine construction. It is not difficult to imagine that similar data mining approaches could be used to identify antigens for specific monoclonal antibody generation and EIA development that would enable optimal sensitivity and specificity. While easier said than done, data could then be used to develop antibodies that are robust to matrix inhibition and target highly specific and conserved epitopes.

Advances in proteomics-based antibody production technologies will improve both sensitivity and specificity as well as provide alternative sources for hybridoma-based antibody production. Alternative antibody production systems include development of alternative antibody sources such as cloning antibody variants into yeast or bacterial cultures, and perhaps even creating polyclonal antibodies in transgenic plant expression systems (Berghman et al., 2005; Baker et al., 2016). New opportunities exist that will be revolutionary for the poultry industry. For example, Nzuma et al. (2018) recently constructed recombinant single-chain fragment variable (scFv) *C. jejuni* antibodies with a scFv antibody phage-display library and spleen mRNA from *C. jejuni* immunized rabbits and subsequently purified the antibodies. The resulting purified *C. jejuni* scFv antibody was covalently bound to paramagnetic beads for use in an immunomagnetic separation (IMS) capture system, which was ran in parallel with qPCR, for the detection of *C. jejuni* successfully. The continued evolution of this technology may even allow for the real-time monitoring of poultry processing, which would be a momentous achievement. In the future, achieving a more precise identification of suitable epitopes with advanced bioinformatics makes it even more conceivable that immunoassay improvement will dramatically eliminate most of the current shortcomings associated with current assays.

CONVENTIONAL PCR AND *CAMPYLOBACTER*

Molecular methods for identifying and detecting foodborne pathogens have become more sensitive as comprehensive genomic

data continue to be generated from foodborne pathogens (Gharst et al., 2013; Park et al., 2014; Baker et al., 2016). One of the most valuable techniques to be employed by the food safety industry, and science as a whole, is polymerase chain reaction (PCR). The use of PCR is the foundation for numerous technologies. At its core, PCR uses specific oligonucleotides to bind to either unique genes carried by individual species or a single sequence that exhibits sequential or length variation and amplifies it. Therefore, PCR exploits the genomic nuances of a particular organism to differentiate it from unrelated and related organisms (Eisenstein, 1990; Carrino and Lee, 1995; Hill, 1996). A selected group of PCR primers, their gene targets, and experimental samples is presented in **Table 4**.

Numerous PCR-based approaches have been successful for the detection of *Campylobacter* species commonly found in poultry. While significant epidemiological evidence suggests that there are mainly two clinically important isolates of *Campylobacter*, the more advancements achieved in genomics and molecular biology, the more it will become necessary to differentiate between non-classical isolates of *Campylobacter*. A central target for differential PCR assays includes the 16S rDNA gene. Early efforts by Giesendorf et al. (1992) focused on PCR primers that targeted the variable regions of the 16S rRNA genes of three *Campylobacter* species: *C. jejuni*, *coli*, and *lari*. By doing so, researchers enabled a lower detection limit to approximately 12 colony forming units (CFU) as compared to traditional methods. In order to further distinguish between non-*C. jejuni* and *coli* species, Linton et al. (1996) successfully identified 16S rRNA sequences specific for five different *Campylobacter* species, namely: *C. upsaliensis*, *helveticus*, *fetus*, *hyointestinalis*, and *lari*. Researchers targeted PCR oligonucleotides for each species epithet along with a genus-specific *Campylobacter* oligonucleotide, which were created and validated. Taking a different approach, Uyttendaele et al. (1995) also targeted 16S rRNA to detect *C. jejuni* by combining selective enrichment and nucleic acid sequence-based amplification (NASBA) of RNA to shorten the assay time of food samples from 6 days to 26 h.

Other gene targets have also been used over the years, which exploit the carriage of gene-specific differences in closely related *Campylobacter* species. Oyofo et al. (1992) developed PCR assays based on the species-specific upstream regions of the flagellin genes *flaA* and *flaB* to differentiate *C. jejuni* and *C. coli*. *Campylobacter* spp. and non-*Campylobacter* genera were included in the analysis to evaluate the specificity of detection. Using a purified template, the assay achieved an overall detection sensitivity of 98.5%, which included the discrimination between *C. jejuni* and *C. coli* despite the similarities in flagellin gene sequences. Studies such as those conducted by Oyofo and colleagues are important as mixed poultry matrices include other, perhaps undefined, *Campylobacter* species as well as members of the family *Enterobacteriaceae* that share the same gene. Therefore, targeting an upstream region of the *flaA* and *flaB* genes to successfully identify and differentiate *C. jejuni* and *C. coli* is crucially important for the poultry industry. As a result, multiple labs continued to target the *flaA* and *flaB* genes, including Rasmussen et al. (1996), who created oligonucleotide probes hybridized to a microtiter plate targeting both flagellin genes to improve sensitivity (less than two cells for *C. jejuni*) and specificity of the detection of *Campylobacter* in chicken fecal samples.

Even though PCR is an improvement as compared to traditional microbiology-based culture methods, the successful and specific detection of *Campylobacter* in mixed matrices continues to be a challenge. This variability is influenced by several factors, such as the presence of non-culturable bacteria (Leskinen and Lim, 2008), polymerase inhibitors, fecal material (Loge et al., 2002), and low quantities of cells existing in a large volume of sample. Innovations to PCR-based techniques have addressed many of those limitations, such as reducing inhibitors, including an enrichment step before the PCR, and coupling the PCR assay with other assays like EIA to enhance assay sensitivity (Park et al., 2014). For example, by combining PCR with ELISA, researchers detected *Campylobacter* in environmental water samples that were below the limit of detection of conventional cultural methods (Sails et al., 2002).

On et al. (2013) emphasized that as new *Campylobacter* spp. are discovered, it is critical to revalidate existing PCR assays for *C. jejuni* and *C. coli* to reconfirm species specificity and avoid false positive results. Multiple clinical laboratories evaluated 31 different *Campylobacter* PCR assays to detect and differentiate *Campylobacter* species. The overall results for sensitivity (number of true positives/true + false positives) ranged from 0 to 100% and specificity (number of true negatives/true negatives + false positives) ranged from 55 to 100%. The authors concluded with the recommendation that as the taxon numbers for *Campylobacter* increase, it is critical for diagnostic laboratories dependent on PCR analyses to stay in touch with changes in taxon designations and to obtain the type of strains of new taxa to validate against in-house PCR methods. Campylobacter are extremely fluid organisms and require continuous innovation for their detection. Additionally, it will become increasingly important for testing to become integrative between clinical, epidemiological, and veterinary medicine in order to be all inclusive.

MULTIPLEX PCR DETECTION OF *CAMPYLOBACTER*

Multiplex PCR is a quick and reliable method for determining the presence or absence of multiple gene targets within a single sample. This approach has been established for use in *Campylobacter* for the rapid identification of several *Campylobacter* species epithets as well as *Campylobacter* and non-*Campylobacter* efficiently. Therefore, it is an attractive methodology for prevalence testing in the poultry industry as one sample can yield multiple identifications quickly and efficiently.

Multiplex PCR assays for the detection of *Campylobacter* spp. have proven useful for the identification of multiple species in food production samples. Zhao et al. (2001) developed a multiplex PCR to differentiate *Campylobacter jejuni* and *C.*

TABLE 4 | Gene targets for the detection of *Campylobacter* spp. using various polymerase chain reaction (PCR) techniques.

Target gene	Organism	Primers	Sample; Country; Author; Notes
Traditional and multiplex PCR			
16S rRNA	*Campylobacter* spp.	3'-ACCTTGTTACGACTTCACCCCA-5' 5'-GAGAGTTTGATCCTGGCTCAG-3'	Chicken wings; Netherlands; Giesendorf et al. (1992)
16S rRNA	*Campylobacter* spp.	5'-AATTCTAATACGACTCACTATAGGGAGAGTGTGACTGATCATCCTCTCA-3' 5'-GACAACAGTTGGAAACGACTGCTAATA-3'	Poultry products, dairy products, red meat, vegetables; Belgium; Uyttendaele et al. (1995)
16S rRNA	*Campylobacter* spp.	5'-GGATGACACTTTTCGGAGC-3' 5'-CATTGTAGCACGTGTGTC-3'	Fecal samples; Australia; Linton et al. (1996), Huq et al. (2014)
256bp fragment	*Campylobacter* spp.	5'-AGAACACGCGGACCTATATA-3' 5'-CGATGCATCCAGGTAATGTAT-3'	Poultry, beef, pork samples; USA; Jackson et al. (1996a,b), Zhao et al. (2001) Multiplex capable, see text for details
Flagellin -	*C. jejuni/coli*	5'-ATGGGATTTCGTATTAAC-3'	Fecal samples; USA; Oyofo et al. (1992)
flaA		5'-GAACTTGAACCGATTTG-3'	
Flagellin -	*C. jejuni/coli*	5'-CCAAATCGGTTCAAGTTCAAATCAAAC-3'	Fecal samples; Denmark; Guerry et al. (1990), Rasmussen et al. (1996)
flaA, flaB		5'-CCACTACCTACTGAAAATCCCGAACC-3'	
Heat shock protein -	*C. jejuni*	5'-CAAGTTGCTACAATCTCAGCCA-3'	Water samples; USA; Park et al. (2011)
hsp60		5'-GATAACACCATCTTTGCCCACT-3'	Multiplex capable, see text for details
Hippuricase -	*C. jejuni*	5'-GACTTCGTGCAGATATGGATGCTT-3'	Fecal samples; Denmark; Persson and Olsen (2005)
hipO		5'-GCTATAACTATCCGAAGAAGCCATCA-3'	Multiplex capable, see text for details
Cytolethal distending toxin -	*C. jejuni*	5'-AGGACTTGAACCTACTTTTC-3'	Broiler carcasses, vegetable samples; Brazil; Asakura et al. (2007), Carvalho et al. (2013)
cdt		5'-AGGTGGAGTAGTTAAAAACC-3'	Multiplex capable, see text for details
Aspartokinase -	*C. coli*	5'-GGTATGATTTCTACAAAGCGAG-3'	Fecal samples; Denmark; Linton et al. (1997), Persson and Olsen (2005)
asp		5'-ATAAAAGACTATCGTCGCGTG-3'	Multiplex capable, see text for details
Lipid A acyltransferase -	*C. upsaliensis*	5'-CGATGATGTGCAAATTGAAGC-3'	Biochemical assays; Japan; Yamazaki-Matsune et al. (2007)
lpxA		5'-TTCTAGCCCCTTGCTTGATG-3'	Multiplex capable, see text for details
qPCR and dPCR			
16S rRNA	*Campylobacter* spp.	5'-CTGCTTAACACAAGTTGAGTAGG-3' 5'-TTCCTTAGGTACCGTCAGAA3'	Chicken carcass rinses; Denmark; Josefsen et al. (2004), Josefsen et al. (2010); **PMA-PCR**
Fragment of *C. jejuni*	*C. jejuni*	5'-CTGAATTTGATACCTTAAGTGCAGC-3' 5'-AGGCACGCCTAAACCTATAGCT-3'	Viable/dead cells; Norway; Nogva et al. (2000), Rudi et al. (2005); **EMA-PCR**
16s rRNA	*Campylobacter* spp.	5'-GGATGACACTTTTCGGAGC-3' 5'-CATTGTAGCACGTGTGTC-3'	Fecal samples; UK; Logan et al. (2001); **qPCR**
ATP binding protein -	*C. jejuni/coli*	5'-AGTGCCGATAAAGGCTCATCA-3'	Poultry, fish, beef, pork, milk, vegetable samples; Spain; Bonjoch et al. (2009); **qPCR**
cje0832		5'-ACTCGTCGAGCTTGAAGAATACG-3'	
VS1 gene	*C. jejuni*	5'-GAATGAAATTTTAGAATGGGG-3' 5'-GATATGTATGATTTTATCCTGC-3'	Chicken, milk, water; China; Yang et al. (2006); **qPCR**
Hippuricase -	*C. jejuni*	5'-TCCAAAATCCTCACTTGCCATT-3'	Poultry processing water; USA; He et al. (2010), Rothrock Jr et al. (2013); **ddPCR**
hipO		5'-TGCACCAGTGACTATGAATAACGA-3'	
Cytochrome c oxidase -	*C. jejuni*	5'-TGGTCTAAGTCTTGAAAAAGTGGCA-3'	Broiler neck-skin; Slovenia; Toplak et al. (2012), Papic et al. (2017); **ddPCR**
ccoN		5'-ACTCTTATAGCTTTTCAAATGGCATATCC-3'	

coli and to confirm presumptive *Campylobacter* isolates on blood agar plates. The isolates recovered from the blood agar plates were from retail chicken, turkey, pork, and beef products. While most colonies were confirmed as being either *C. jejuni* (53.6%) or *C. coli* (41.3%), the remaining colonies apparently were other *Campylobacter* spp. not identified by the species-specific multiplex PCR. As several of the meat samples yielded more than one *Campylobacter* species, researchers concluded that because of the likelihood of mixed species contamination, it is critical to select more than one colony per plate for *Campylobacter* identification and subtyping. Data from the study also emphasized the need for expanding the multiplex PCR design to cover more *Campylobacter* species and to develop culture-independent methods.

Despite the importance of purely culture-independent methods being developed, typically, culturing *Campylobacter* may be a crucial first step to its identification in complex samples. Multiplex assays have the ability to very specifically determine if *Campylobacter* is present. The importance of being able to identify multiple *Campylobacter* species directly from single, yet diverse samples is supported by Inglis and Kalischuk (2003) when they compared four different *Campylobacter* media with direct PCR detection without the pre-enrichment of bovine fecal samples. The genus level identification for *Campylobacter* was generally more sensitive (8% improvement) as compared to a broad survey of microbiological isolation techniques that included four different media and three different incubation temperatures. While the detection of *C. lanienae* by PCR was more sensitive than microbiological isolation, this was not the case for *C. jejuni.* The authors suggested that this variability in isolation prevalence of *Campylobacter* spp. on selective media could be due to an inherent bias of selective media. Specifically, researchers theorized that in the case of *Campylobacter*, the media used may specifically enhance the growth and isolation of *C. jejuni* and *C. coli* and select against the less defined species epithets that may be present.

In another study, Huq et al. (2014) also saw an improved sensitivity using a multiplex PCR to identify *C. concisus*, *C. jejuni*, and *C. coli* in spiked human fecal samples from clinical gastroenteritis cases as compared to traditional microbiological techniques utilizing an antibiotic-free Columbia blood agar using the micron filtration technique. However, evidence suggests that depending on the sample, using selective media prior to PCR can improve sensitivity. For example, Persson and Olsen (2005) demonstrated that human stool samples spiked with *C. jejuni* and *C. coli* that were plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) improved the sensitivity of the PCR assay to 10^2 cells per ml of stool from 10^5 cells per ml of stool with direct multiplex PCR detection alone. In short, comparisons in sensitivity between direct PCR and culture methods versus selective enrichment-PCR combinations are likely a result of viability status of *Campylobacter* spp. in different samples and the caveats therein. Meaning, various samples contain different inhibitors present in the sample and can augment the sensitivity and specificity of the assay. Additionally, enrichment may overcome cell counts below the limit of detection, which may be important for monitoring poultry in the pre-harvest or peri-harvest setting. As a result, developing standard protocols will likely require independent validation for the type of sample being tested. While many of the aforementioned methodologies are clinically based, the complexity of human stool samples and the importance of highly specific and precise molecular identification of *Campylobacter* are essential.

Unlike immune-based methods that have challenges associated with the identification of closely related species, molecular PCR-based approaches may be able to offer significantly more detailed refinement and precision. Evidence suggests that in poultry, methods may need to go beyond classical genus identification, specifically *C. jejuni* and *C. coli*. The identification of *Campylobacter* may need to include the less characterized species like *C. lari*, *C. upsaliensis*, and *C. fetus* as well as other important foodborne pathogens simultaneously (Wang et al., 2002; Klena et al., 2004). Yamazaki-Matsune et al. (2007) expanded the range of *Campylobacter* by developing a multiplex PCR for six individual species of *Campylobacter*. Researchers designed oligonucleotides targeting *lpxA* in combination with previously published primers (16S rRNA, 23S rRNA, *ask*, *cstA*, *glyA*, and *cj0414*) to differentiate between *C. coli*, *C. fetus*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. Other laboratories have developed and applied a multiplex PCR for *Campylobacter* spp. using oligonucleotides to target the cytolethal distending toxin (*cdt*) genetic subunits (Carvalho et al. 2013; Kamei et al., 2016). Another example of this methodology that may be important for the poultry industry was conducted by Park et al. (2011). Park and colleagues developed multiplex a PCR assay that could detect several pathogens in a single reaction, including *Campylobacter*, *E. coli*, and *Salmonella* Typhimurium simultaneously. In a later study, Raja et al. (2016) developed a multiplex PCR for simultaneous detection of *Campylobacter jejuni* and *Listeria monocytogenes* in chicken meat.

The sensitivity and specificity of PCR, as well as the detection capabilities of the technology, are further improved with the use of nested PCR. Nested PCR starts with a single, long amplicon containing all of the genes of interest, which is then amplified in a second reaction. This overcomes difficulties like secondary structure and specificity challenges associated with two very closely related species or sub-species. This technique can be combined with a multiplex assay to differentiate sub-species of *Campylobacter*. Miller et al. (2007) further improved the genomic resolution by designing a nested multiplex assay with oligonucleotides targeting the nitrate reductase (*nap*) locus with flanking *napA* and *napB* primer sets for simultaneously distinguishing the two *C. jejuni* species: subsp. *jejuni* and subsp. *doylei*. Specific nuances between the two subspecies of *Campylobacter* resided in nitrate metabolism, where subspecies *doylei* is unable to reduce nitrate. Further, based on DNA microarray analyses, it was validated that *napA* and *napB* were either missing or their sequences were unique as compared to the subspecies *jejuni napA* and *napB*. Consequently, subsp. *doylei* strains failed to amplify with the *napA* internal primer set, but could amplify with the flanking *napA* primers and result in a smaller amplicon due to a deletion within *napA*.

Further refinement of this method became possible after sequencing data of the subsp. *doylei napA* and *napB* revealed that while all subsp. *doylei* possessed identical *napA* deletions, some strains also contained *napB* deletions compared to others where *napB* was intact. Primers were then redesigned to exploit that difference and improve the detection of these subspecies.

As with other methods, there are drawbacks to the technology, as delineated in **Table 2**. Despite the attractiveness of using multiplex PCR to simultaneously detect several *Campylobacter* spp., Park et al. (2014) have pointed out that there are limitations associated with involving more than 5–6 primer pairs in a single reaction with less than 100 bp differences between amplicons. The amplification of amplicons not adhering to those guidelines is difficult using a standard agarose gel electrophoresis. Additional primer sets to the mix also reduce the ability to optimize thermocycling as the T_m ranges between the oligonucleotides will likely become more difficult to control. Several approaches have been developed to overcome this issue, from changing the percentage of agarose to adjusting the buffers for electrophoresis. This has resulted in some researchers being able to overcome these limitations. To achieve the simultaneous detection of nine different foodborne pathogens, including *C. jejuni*, by multiplex PCR, Villamizar-Rodríguez et al. (2015) combined the enrichment of microbial populations using a universal pre-enrichment medium followed by PCR amplification with nine different primer pairs, with one unique oligonucleotide pair for each respective foodborne pathogen. To circumvent the difficulties with agarose gel electrophoresis, the authors differentiated the various sizes by capillary electrophoresis. When cultural and multiplex PCR results were compared for various spiked food matrices, coincident values ranged from 78 to 92%.

REAL-TIME QUANTITATIVE PCR (qPCR) AND *CAMPYLOBACTER*

An evolution in PCR technologies for the absolute quantification of pathogens and nucleic acid sequences became possible once intercalating fluorescent dyes that could be detected with specialized laser-imaging systems were introduced. Fluorescent dyes accumulate with each cycle with an intensity that is directly proportional to the amount of target template DNA. This technology is referred to as real-time PCR or quantitative PCR, which are both collectively known as qPCR (Higuchi et al., 1993; Park et al., 2014; Kralik and Ricchi, 2017). The increased sensitivity of fluorescence detection and the implementation of specialized cameras reduce the time it takes to detect a specific gene segment (Mackay, 2004). Fluorescence can either be introduced to the reaction through non-specific dyes that bind to the DNA, such as SYBR green, or by using probes with different fluorescent labels, which is commonly referred to as the TaqMan system. The use of fluorescent probes or melt temperatures allows for multiplexing based on fluorophore emission spectra (Wittmer et al., 2001). The speed and specificity of qPCR enabled the widespread implementation of its use for rapid detection and quantification of foodborne pathogens.

The technical aspects of qPCR and its application to the food industry have been reviewed recently (Chapela et al., 2015; Kralik and Ricchi, 2017); therefore, the following discussion will focus on the development of qPCR for *Campylobacter* in poultry production systems. Yang et al. (2003) developed a qPCR assay based on *VS1* of *C. jejuni* for quantitation in naturally contaminated poultry, milk, and environmental water samples. Without using culture-based enrichment, researchers achieved a sensitivity of 1 CFU in poultry breast, drumstick, and other poultry samples. This was statistically different from culture-based detection methods. Importantly, all poultry samples that tested positive for *Campylobacter* using qPCR were also positively identified on *Campylobacter* selective medium. However, some of the culture negative samples were qPCR positive, further highlighting the difficulties of traditional culture-based methods for the detection of *Campylobacter* in complex matrices.

As with other methods delineated in this review, a significant challenge exists in the detection of *Campylobacter* in diverse samples like ceca due to inhibitors present in the matrices or other unforeseen biological challenges. One way to overcome this inhibition was executed by Lund et al. (2004), who overcame qPCR inhibition in poultry fecal samples by using magnetic beads to separate the DNA. In order to control the uneven loss of DNA, researchers added a known amount of *Yersinia ruckeri* to the unsorted ceca, which is a fish pathogen not native to chickens, as an internal control for DNA isolation and qPCR amplification. Researchers did not identify significant differences in performance between qPCR and conventional selective enrichment culturing techniques nor a bias in DNA isolation. Similar to Lund and colleagues, Rudi et al. (2004) used paramagnetic beads for both *C. jejuni* isolation and DNA purification from chicken cecal and fecal samples. They achieved detection between 2 and 25 CFU from spiked cecal and fecal contents, and reduced the total assay time to less than 4 h. The ability to identify *Campylobacter* in ceca rapidly is essential for the poultry industry.

Multiplex qPCR has emerged to enable the simultaneous detection of all major poultry-specific *Campylobacter* genera within a single sample. While multiplex qPCR can be challenging, Chapela et al. (2015) pointed out that qPCR is actually fairly amendable to multiplexing as sequence-specific probes can be labeled with different fluorophores. This enables the sample to be assayed with multiple probes within a single reaction well. Even non-specific SYBR green can still differentiate genes by generating distinct melt curves for each gene that can be tracked and analyzed. For example, Park et al. (2011) evaluated water samples using different melting temperatures to quantify *C. jejuni* (80.1°C), *E. coli* O157:H7 (83.3°C), and *Salmonella* Typhimurium (85.9°C) in a single reaction. Both the qPCR and culturing methods indicated a reduction in viable cells in spiked watershed samples after 7 days of cold storage (4°C). In a later study, Barletta et al. (2013) successfully took a similar approach to differentiate *Campylobacter*, *Salmonella*, and *Shigella* with a SYBR green-based multiplex qPCR analysis of spiked stool samples.

As with conventional PCR, the ability to differentiate between various species of *Campylobacter* using qPCR and multiplexed

qPCR is important. To distinguish four different *Campylobacter* spp., Bonjoch et al. (2009) used two regulatory gene primers from the *bipA* gene for detection of *C. upsaliensis* and *C. lari* and the adenosine triphosphate (ATP)-binding protein CJE0832 for *C. coli* and *C. jejuni*. They analyzed a wide range of meat, fruit, and vegetable samples (200 total) including minced chicken, chicken drumsticks, and turkey legs. The samples were screened with both the qPCR assay and a cultural method. No false negative results were detected and 100% of the *Campylobacter* positive samples were confirmed. Likewise, when Reis et al. (2018) compared qPCR *Campylobacter* with an immunoassay and conventional PCR on frozen and chilled broiler carcasses, the qPCR results were more sensitive.

Several refinements have improved the qPCR methods for the detection and quantitation of *Campylobacter*, which are translatable to the poultry industry. As with other techniques, researchers have actively evaluated whether or not combining qPCR with enrichment or quantitation methods improves detection. The specific quantitation of *Campylobacter* relative to CFUs was improved by Banting et al. (2016), which is important as quantitation and prevalence both matter to the poultry industry. Banting and colleagues combined qPCR with a miniaturized most probable number (MPN), which is a standard for bacterial quantitation in the poultry industry. In this study, water samples were added to microtiter plates and were incubated, and then analyzed for *Campylobacter via* qPCR. For *Campylobacter* positive samples, additional qPCR assays were conducted to identify specific *Campylobacter* spp. Based on one of the specific qPCR assays, *Campylobacter* was estimated to be as low as less than 2 MPN per 300 ml and *C. jejuni*, *C. coli*, and *C. lari* were the most frequent isolates. The assay time can be further reduced by using a non-selective medium to increase the growth rate as demonstrated with *Salmonella* (Kim et al., 2017b). A key to these MPN approaches is to lower the limits of detection and to recover injured cells. As the miniaturization of MPN and qPCR combinations continues to evolve, along with incorporation of automation, opportunities will be offered to develop high-throughput assays that can be used by the food industry to process much larger sample volumes efficiently.

A key disadvantage of qPCR and other PCR-based technologies is that they cannot differentiate live versus dead cells as they quantify all nucleic acids present that are able to anneal to the oligonucleotides (Kralik and Ricchi, 2017). In order to overcome this potential issue, Kralok and Ricchi suggest either using a pre-enrichment growth stage to recover viable cells, using RNA, or using florescent dyes that penetrate and exploit the physiology of dead cells. The use of fluorescent dyes is increasingly popular as they can penetrate dead cell walls and degradation by dyes. Not surprisingly, the choice of dyes matters. For example, while Rudi et al. (2005) found a favorable comparison between ethidium monoazide (EMA) and propidium monoazide (PMA), Flekna et al. (2007) determined that EMA reduced the presence of viable genomic DNA in live *Campylobacter jejuni* and *Listeria monocytogenes*. Consistent results for PMA also appear to be variable for *Campylobacter* live-cell detection. Josefsen et al. (2010) compared a PMA-qPCR assay with the isolation of *C. jejuni* from poultry rinsates on modified charcoal cefoperazone deoxycholate (mCCDA) and Abeyta-Hunt-Bark agar plates. Researchers found a correlation between the live-dead qPCR and traditional microbiological plating techniques of 0.844 R^2. This correlation came into question when Pacholwicz et al. (2013) evaluated naturally contaminated carcasses with traditional microbiological enumeration against PMA-qPCR. Researchers did not detect a concordant relationship between the two methods, leading them to suspect that inadequate concentrations of PMA lead to an insufficient repression of dead cell DNA.

As a result, some controversy remains as to which fluorescent dye is the best choice to quantify live populations of *Campylobacter jejuni*. Some studies, such as that of Seinige et al. (2014), observed concordant data with EMA- and PMA-qPCR and traditional microbiological culturing techniques. Krüger et al. (2014) compared EMA with PMA and demonstrated that EMA was insufficiently active in less metabolically active *Campylobacter* cells. Researchers noted that PMA worked across a broad range of *Campylobacter* metabolic states equally, though it was less efficient in inhibiting DNA from dead cells as compared to EMA across the board. Based on this variability, Krüger et al. (2014) suggested incorporating principles of method for quantification, which reflect intact and infectious *Campylobacter*. Given the importance of distinguishing viable versus dead *Campylobacter* in poultry production systems, further refinement of this technique is needed if the methods are going to be incorporated into processing plants.

Other limitations also exist for qPCR, as described in Table **2**. Bias in detection and quantitation can occur with various platforms and extraction methods, as well as primer sensitivity and specificity. Caution must be used when extracting DNA from complex organic matrices, such as fecal material and carbohydrate-rich foods, as inhibitors can coprecipitate with the DNA. Therefore, it is important to customize extraction protocols to reduce contaminants that could interfere with PCR amplification (Pontiroli et al., 2011; Park et al., 2014). Methodologies to reduce inhibitory compound contamination include selecting an extraction procedure that minimizes interference or adding in a pre-enrichment step to allow bacteria to grow and then dilute out the inhibitors (Pontiroli et al., 2011; Park et al., 2014; Kralik and Ricchi, 2017). This consideration is true even for commercial DNA extraction kits. For example, when Kawase et al. (2014) compared two commercial fecal DNA extraction kits on human feces to pathogen DNA, they detected higher recovery of target genes and improved detection consistently with one kit versus the other. This was true for both samples spiked with pathogens as well as naturally contaminated samples from outbreak patients.

DIGITAL PCR (dPCR) AND *CAMPYLOBACTER*

Baker (2012) defined that digital PCR (dPCR) utilizes the fraction of negative replicates to determine the absolute copy number of a gene target as calculated using a Poisson statistical algorithm. This is accomplished by separating a sample into

a large number of small reaction chambers. The resulting number of positive versus negative reactions reveals the exact number of copies of a particular gene in the test sample after amplification is complete, unlike qPCR which follows fluorescence intensity during amplification (Baker, 2012). Therefore, dPCR is significantly more precise than qPCR. Another advantage of dPCR over qPCR is that quantification is less vulnerable to enzyme amplification inhibitors present in the sample matrix (Baker, 2012; Huggett et al., 2015; Papic et al., 2017). Variations of dPCR include droplet dPCR (ddPCR), where titrated emulsions of oil, water, and stabilizing chemicals generate droplets that can be placed into tubes in a thermocycler, followed by analysis on a droplet machine (Baker, 2012).

Poultry and *Campylobacter*-based applications for dPCR have been relatively limited thus far. Rothrock et al. (2015) used a ddPCR approach to quantify *Salmonella* spp., *C. jejuni*, and *L. monocytogenes* in water samples from commercial poultry processing facilities. The researchers collected water samples from the scalder and chiller tanks at three time points: prior to carcasses entering the processing plant, midday, and at the end when the last carcasses had been processed. This study was conducted over a three-day period. The same primers and probes were used for both qPCR and ddPCR assays. The data from these assays were compared with those of conventional culture methods for each pathogen. In general, more pathogens were detected at each sampling point by ddPCR than either cultural recoveries or qPCR. In particular, ddPCR detected *C. jejuni* and *L. monocytogenes* in both the scalder and chiller tank water samples throughout the day, even when they were not detected in the culture samples. This could be due to an enhanced sensitivity or due to picking up the residue of dead *Campylobacter.* More recently, Papic et al. (2017) compared qPCR and dPCR for the quantification of *C. jejuni* in broiler neck-skin samples from poultry processing plants in conjunction with the ISO Standard Plate Count Method. There was a statistically average agreement among all three methods. It was also noted that dPCR yielded an overestimate that they attributed to the high number of false positive outcomes. Another explanation could be that with the enhanced sensitivity of detection, the dPCR picks up residues of dead *Campylobacter* post sanitation. Regardless, the potential use of dPCR and ddPCR in the poultry industry is intriguing.

GENOTYPING OF *CAMPYLOBACTER*

Accurate methods for identifying and classifying *Campylobacter* isolates that have a short turnaround time are becoming important for rapidly identifying the source of infection, the vehicle for transmission, and the incidence of campylobacteriosis (Dingle et al., 2002). Consequently, genotyping approaches have been implemented for the major foodborne pathogens including *Salmonella*, pathogenic *E. coli*, and *Campylobacter* (Eberle and Kiess, 2012; Gharst et al., 2013; Park et al., 2014; Baker et al., 2016). Early genetic-based identification of *Campylobacte*r spp. focused on applying DNA homology comparisons for classifying and typing *Campylobacter* isolates. For example, Roop et al. (1984) used DNA homology to differentiate 84 strains of catalase-positive *Campylobacter* isolates, classifying them into seven separate DNA homology groups and correlating these groups to biochemical and physiological characteristics.

Recent advances in multiplex qPCR technology have enabled even more sensitive and specific genotyping. Banowary et al. (2015, 2018) employed a high resolution melt (HRM) curve analysis method based on the use of fluorescent DNA binding dyes. This technology differentiates *Campylobacter* PCR product sequence variation and calculates an average HRM genotype confidence percentage. Initial tests with human clinical and chicken swab samples using segments of the hippuricase gene (*hipO*) for *C. jejuni* and the aspartokinase (*asp*) gene for *C. coli* as primer sources allowed for differentiation of the two species in these sample matrices without requiring enrichment (Banowary et al., 2015). The HRM approach may offer more resolution for determining discrimination capability among primers. For example, Banowary et al. (2018) compared two multiplex PCR-HRM methods (mPCR1-HRM and mPCR2-HRM primers from *cadF* and *gpsA* genes, respectively) to detect and distinguish 24 poultry isolates and three reference strains of *C. jejuni* and *C. coli.* They found them to be more discriminatory than the *hipO* and *asp*-based primers used in their previous study (Banowary et al., 2015) and the mPCR1-HRM could differentiate *C. coli* intra-species variation and mPCR2-HRM *C. jejuni* intra-species variation.

Later developments in molecular technologies led to more advanced genotyping methodologies for *Campylobacter* spp. By the early 2000s, several different genotyping approaches had become available for *Campylobacter* including flagellin (*fla*) gene typing, pulsed-field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD), AFLP (amplified fragment length polymorphism), multiplex PCR-RFLP analysis, and multi-locus sequencing typing (Wassenaar and Newell, 2000; Eberle and Kiess, 2012). These were described in detail in several previous reviews (Wassenaar and Newell, 2000; Eberle and Kiess, 2012; Taboada et al., 2013) and will not be discussed in the current review.

NEXT-GENERATION SEQUENCING OF *CAMPYLOBACTER*

Since its inception, next generation sequencing (NGS) methods have rapidly been adopted for both the qualitative assessment of poultry systems and the epidemiological tracking of foodborne outbreaks. This technology consists of whole genome sequencing (WGS) actively being used to elucidate the individual genomes of foodborne pathogens during outbreaks as well as the complex metagenomics of the microbiomes associated with foodborne pathogens (Park et al., 2014; Allard, 2016; Cao et al., 2017; Sekse et al., 2017; Taboada et al., 2017; Ronholm, 2018). Furthermore, the use of benchtop sequencers, like nanopore, only increases the ability of the industry, researchers, and monitoring bodies alike to evaluate the genomics of species in real time (Llarena et al., 2017). The use of WGS has become

the preferred diagnostic and surveillance approach for the United States and global food safety regulatory agencies (Taboada et al., 2017).

Certainly, the laboratory advancements in sequence technologies have led to a decrease in costs, and the increased ease of accompanying bioinformatics programs has ushered WGS to the forefront of pathogen monitoring. The increase open access of the data and analytics platforms has led to the rapid dissemination of information available for foodborne pathogens (Sekse et al., 2017; Taboada et al., 2017; Ronholm, 2018). A list of recent research in the whole genome sequencing of *Campylobacter* spp. is presented in **Table 3**. Several challenges remain, such as the cost, educational burden for data analysis and interpretation, quality, and speed of sequencing (Metzker, 2010; Park et al., 2014; Pennisi, 2016, 2017; Llarena et al., 2017). The NGS databases for foodborne pathogens continue to grow. This improves the data resolution and tracking of outbreaks and makes characterizing taxonomical relatedness within an outbreak more precise. In fact, the use of NGS to pinpoint outbreaks is already common and shows an unusual robustness to the methodology used (Llarena et al., 2017). By focusing on allelic variation and differentiation between isolates, researchers can trace outbreaks with significant discriminatory power (Llarena et al., 2017). The scope and success of WGS approaches can be appreciated in the study of Llarena et al., 2017, with recent advances described in **Table 2**.

Importantly, the data gained go beyond bacterial identification. Early studies on sequencing of the *Campylobacter* genome revealed the presence of hypervariable regions contained within the loci of genes involved with surface structure biosynthesis or modification (Parkhill et al., 2000). What seems to be unique for the *Campylobacter* genome is that there are very few insertion, phage, or tandem repeat sequences. The usage of these data has been important as it has led to a number of applications for further analyses and characterization of the genus. For example, Sheppard and Maiden (2015) demonstrated that *C. jejuni* and *C. coli* evolution was highly dependent on recombination events. These recombination events resulted in distinct lineages emerging and other large-scale genomic interspecies introgression between the two species. Gilbert et al. (2018) demonstrated that even though *C. fetus* lineages can be genetically divergent, there was WGS evidence that homologous recombination still occurred between individual *C. fetus* found in the same host. *Campylobacter* WGS has also been used in a number of studies for epidemiology characterizations including surveillance and outbreak detection, as well as phylogenetic antimicrobial resistance analyses (Biggs et al., 2011; Revez et al., 2014; Cha et al., 2016; Clark et al., 2016; Zhao et al., 2016; Llarena et al., 2017; Joensen et al., 2018).

Data from *Campylobacter* WGS have also been used to resolve and develop diagnostic assays. Jansen van Rensburg et al. (2016) used access to published WGS data to evaluate a duplex qPCR *mapA-ceuE* assay for *C. jejuni* and *C. coli* to determine how inclusive it was for all potential isolates. They used *in silico* analyses of the *mapA* and *ceuE* primer and probe sequences against 1,713 genetically diverse *C. jejuni* and *C. coli* genomes. Using the proposed primer and probe-sets, researchers demonstrated that 99.7% of the isolates tested were correctly identified. Brzozowska, et al. (2018) used a *C. jejuni* subtyping database that included data from 24,000 isolates to identify prevalent subtypes. The resulting 166 sequenced genomes were used to identify clinically associated biomarkers of *C. jejuni*. After identification of marker genes, the selected biomarkers were validated against numerous clinical and non-clinical *C. jejuni* genomes. From there, 25 genes were putatively identified as robust diagnostic biomarkers for clinical *C. jejuni* subtypes. The authors concluded that combinations of these marker genes could be used for clinical diagnostic identification of *C. jejuni* isolates that represent a significant public health risk. Furthermore, Neal-McKinney et al. (2018) used WGS in combination with mutiplex qPCR to develop a diagnostic tool for *C. jejuni* isolates that encode *cst-II* or *cst-III*-sialyltransferase. These genes are of interest because they add sialic acid to the O-antigen of lipooligosaccharide (LOS), which mimics the host's gangliosides and has been associated with Guillain-Barre syndrome. Combining WGS to detect the genes and qPCR to screen a library of *C. jejuni* poultry field and clinical isolates along with *in silico* analyses to screen *C. jejuni* genomes revealed that the most *C. jejuni* species could produce LOS. Therefore, an assay based on these genes could be used to predict the potential of GBS from *C. jejuni* isolates. This relationship can be further exploited as the frequency of GBS-related genes may vary between isolates. The LOS gene, *wlaN,* appears to occur at a fairly low frequency (2 out of 16 *C. jejuni* and *C. coli* isolates) based on WGS profiles conducted by Cantero et al. (2018). Therefore, to further understand the relationships between copy numbers of biomarkers and the onset of GBS, more WGS data sets will need to elucidate this phenomenon. While much of the aforementioned studies are not targeted just to poultry, the patterns discovered and the tools developed will improve the food safety monitoring of poultry.

Further studies have applied WGS to poultry isolates of *Campylobacter* spp. Pendleton et al. (2013) compared PFGE (pulse-field gel electrophoresis) and *flaA* typing with WGS on a Roche 454 sequencing platform to assess discrimination capabilities among the three methods to detect *Campylobacter* from conventional and pasture flock poultry. There was no correlation between the different typing methods, but WGS appeared to be the most discriminatory and provided additional data such as the genome size and GC content. More importantly they also detected substantial evidence of genomic rearrangements within these *Campylobacter* isolates. Based on the WGS data, researchers noted that there were multiple copies of interspaced elements suggesting the potential for a high frequency of recombination events. This type of data is something likely to be missed by other non-genomic typing methods. Other WGS studies based on poultry *Campylobacter* spp. isolates have provided evidence for plasmid-mediated horizontal genetic transfer. Ghatak et al. (2017) generated a complete genomic sequence of a retail poultry *C. coli* isolate, revealing the presence of a mega-plasmid that carried several virulence factors and

antibiotic resistance elements, including the plasmid-containing type IV secretion system gene.

Finally, as compared to standard detection and quantitation techniques in food safety, NGS enables researchers to evaluate other important factors for *Campylobacter* zoonosis. Evidence for horizontal spread of antibiotic resistance in *Campylobacter* spp. has been found in other *Campylobacter* isolates beyond what was discovered by Ghatak and colleagues. Florez-Cuadrado et al. (2017) conducted WGS on erythromycin resistant *C. coli* isolates from turkeys and found that *erm*(B) clustered with genes associated with aminoglycosides and tetracycline resistance. The potential for multiple horizontal transmission events was based on comparative genomic analysis where identical *erm*(B) genes were detected among *Campylobacter* from turkeys, *Streptococcus suis* from pigs, and *Enterococcus faecium* and *Clostridium difficile* from humans. Given the documented risk of antimicrobial resistance genetic element dissemination, WGS should be helpful for the large-scale surveillance and epidemiological tracking of isolates from the poultry industry. As part of the National Antimicrobial Resistance Monitoring System (NARMS), Whitehouse et al. (2018) sequenced 589 *Campylobacter* isolates from retail poultry meats. From this data set, researchers were able to identify 10 antimicrobial resistant genes. They further demonstrated a general consensus between isolate genotypes and resistance phenotypes. As more isolates are characterized by WGS, general patterns of antimicrobial resistance and novel virulence genes of interest should continue to emerge, representing the possibility of horizontal transmission. Data therein can be used to epidemiologically track the function of geographical dissemination, update poultry production and retail practices, and uncover novel relationships to improve food safety.

CONCLUSIONS

While conventional culture methodology remains a mainstay for *Campylobacter* detection and quantitation, the consistency and reliability across various poultry production matrices are problematic. Because of the variability in *Campylobacter* species phenotypes and genotypes, more informative characterizations are needed to provide a more complete assessment of potential risk. Issues such as the existence of VBNC *Campylobacter* in different poultry production environments and the prevalence of certain virulence genes need to be taken into account when assessing and enumerating *Campylobacter* populations.

The use of high-throughput rapid microbiological methods in the poultry industry is not without challenges. New methods require sufficient validation and verification for sample type applications collected pre- or post-harvest. Generally, new methods undergo validation and certification *via* accreditation organizations such as the International Association of Analytical Communities (AOAC), wherein they are validated against "gold standard" reference methods to demonstrate equivalency or enhanced performance and fit-for-purpose use. In order to be considered attractive to the industry, new technology must be properly validated for application and use, as well as present improvements in accuracy, speed, economy, and user-friendliness. Finally, the quantitation of pathogens is becoming more important to establish baselines for assessing risk and predicting the effectiveness of overall prevention and management strategies.

Irrespective of the method chosen by the poultry industry, the specific methods and protocols need to be chosen based on food matrices, convenience, time, and cost. Immunoassays are beneficial when working with intact bacterial cells and the use of advanced proteomics should help with antigen target refinement. The improvements in PCR such as multiplex qPCR and dPCR technology should enhance their utility for routine high throughput detection assays in poultry production. The continued mining of WGS *Campylobacter* databases will allow the industry to become more comprehensive and innovative in the development of novel assays targeted to improving the sensitivity and specificity of PCR strategies. Technological improvements in WGS such as single-molecule sequencing without amplification are promising as they are capable of generating much longer reads with much less sample (Park et al., 2014; Lüth et al., 2018). However, as Lüth et al. (2018) pointed out, the standardization of wet laboratory methodology for pathogen typing, bioinformatic analyses and data storage, and mechanisms for worldwide sharing are needed, otherwise variability will make the data useless. This would seem to be particularly true for a foodborne pathogen such as *Campylobacter,* which exhibits considerable genetic variability. This variability makes it somewhat unpredictable from a transmission and detection standpoint. Enhanced and expanded WGS databases should lead to more effective surveillance and epidemiology of *Campylobacter* outbreaks and prevention.

AUTHOR CONTRIBUTIONS

SR and KF wrote the manuscript. WC, HP, and YY wrote parts of the manuscript and provided feedback. KF and SR provided the final edits. ZS created the figures and provided edits.

REFERENCES

Alahi, M. E. E., and Mukhopadhyay, S. C. (2017). Detection methodologies for pathogen and toxins: a review. *Sensors* 17, 1–20. doi: 10.3390/s17081885

Alamer, S., Eissa, S., Chinnappan, R., and Zourob, M. (2018). A rapid colorimetric immunoassay for the detection of pathogenic bacteria on poultry processing plants using cotton swabs and nanobeads. *Microchim. Acta* 185, 1–10. doi: 10.1007/s00604-018-2696-7

Allard, M. W. (2016). The future of whole-genome sequencing for public health and the clinic. *J. Clin. Microbiol.* 54, 1947–1948. doi: 10.1128/JCM.00046-16 and 10.1128/JCM.00081-16

Asal, M., Özen, O., Şahinler, M., and Potatoğlu, I. (2018). Recent developments in enzyme, DNA and immuno-based biosensors. *Sensors* 18, 1–16. doi: 10.3390/s18061924

Asakura, M., Samosornsuk, W., Taguchi, M., Kobayashi, K., Misawa, N., Kusumoto, M., et al. (2007). Comparative analysis of cytolethal distending toxin (cdt)

genes among *Campylobacter jejuni*, *C. coli* and *C. fetus* strains. *Microb. Pathog.* 42, 174–183. doi: 10.1016/j.micpath.2007.01.005

Asakura, H., Takahashi, N., Yamamoto, S., and Maruyama, H. (2017). Draft genome sequence of *Campylobacter jejuni* CAM970 and *C. coli* CAM962, associated with a large outbreak of foodborne illness in Fukuoka, Japan, in 2016. *Genome Announc.* 5, e00508–e00517. doi: 10.1128/genomeA.00508-17

Baker, C. A., Rubinelli, P. M., Park, S. H., and Ricke, S. C. (2016). Immuno-based detection of shiga toxin-producing pathogenic *Escherichia coli* – a review on current approaches and potential strategies for optimization. *Crit. Rev. Microbiol.* 42, 656–675. doi: 10.3109/1040841X.2015.1009824

Baker, M. (2012). Digital PCR hits its stride. *Nature Meth.* 9, 541–544. doi: 10.1038/nmeth.2027

Banowary, B., Dang, V. T., Sarker, S., Connolly, J. H., Chenu, J., and Groves, P. (2018). Evaluation of two multiple PCR-high resolution melt curve analysis methods for differentiation of *Campylobacter jejuni* and *Campylobacter coli* intrasepecies. *Avian Dis.* 62, 86–93. doi: 10.1637/11739-080417-Reg.1

Banowary, B., Dang, V. T., Sarker, S., Connolly, J. H., Chenu, J., Groves, P., et al. (2015). Differentiation of *Campylobacter jejuni* using multiplex-PCR and high resolution melt curve analysis. *PLoS One* 10:e0138808. doi: 10.1371/journal.pone.0138808

Banting, G. S., Braithwaite, S., Scott, C., Kim, J., Jeaon, B., Ashbolt, N., et al. (2016). Evaluation of various *Campylobacter*-specific quantitative PCR (qPCR) assays for detection and enumeration of *Campylobacteraceae* in irrigation water and wastewater via a miniaturized most-probable-number-qPCR assay. *Appl. Environ. Microbiol.* 82, 4743–4756. doi: 10.1128/AEM.00077-16

Barletta, F., Mercado, E. H., Lluque, A., Ruiz, J., Cleary, T. G., and Ochoa, T. J. (2013). Multiplex real-time PCR for detection of *Campylobacter*, *Salmonella*, and *Shigella*. *J. Clin. Microbiol.* 51, 2822–2829. doi: 10.1128/jcm.01397-13

Berghman, L. R., Abi-Ghanem, D., Waghela, S. D., and Ricke, S. C. (2005). Antibodies: an alternative for antibiotics? *Poult. Sci.* 84, 660–666. doi: 10.1093/ps/84.4.660

Biggs, P. J., Fearnhead, P., Hotter, G., Mohan, V., Collins-Emerson, J., Kwan, E., et al. (2011). Whole-genome comparison of two *Campylobacter jejuni* isolates of the same sequence type reveals multiple loi of different ancestral lineage. *PLoS One* 6:e27121, 1–14. doi: 10.1371/journal.pone.0027121

Bolton, D. J. (2015). *Campylobacter* virulence and survival factors. *Food Microbiol.* 48, 99–108. doi: 10.1016/j.fm.2014.11.017

Bonjoch, X., Calvo, L., Soler, M., Ruiz-Rueda, O., and Garcia-Gil, J. (2009). A new multiplexed real-time PCR assay to detect *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*. *Food Anal. Methods* 3, 40–46. doi: 10.1007/s12161-009-9110-3

Bordeaux, J., Welsh, A. W., Agarwal, S., Killiam, E., Baquero, M. T., Hanna, J. A., et al. (2010). Antibody validation. *BioTechniques* 48, 197–209. doi: 10.2144/000113382

Brooks, B., Mihowich, W. J. G., Blais, B. W., and Yamazaki, H. (1998). Specificity of monoclonal antibodies to *Campylobacter jejuni* lipopolysaccharide antigens. *Immunol. Investig.* 27, 257–265.

Buchanan, C. J., Webb, A. L., Mutschall, S. K., Kruczkiewicz, P., Barker, D. O. R., Hetman, B. M., et al. (2017). A genome-wide association study to identify diagnostic markers for human pathogenic *Campylobacter jejuni* strains. *Front. Microbiol.* 8, 1–9. doi: 10.3389/fmicb.2017.01224

Brzozowska, N., Goulay, J., O'Sullivan, A., Buchanan, F., Hannah, R., Stewart, A., et al. (2018). Characterizing genetic circuit components in *E. coli* towards a *Campylobacter jejuni* biosensor. *bioRxiv*. doi: 10.1101/290155

Cantero, G., Correa-Fiz, F., Ronco, T., Strube, M., Cerdà-Cuéller, M., and Pedersen, K. (2018). Characterization of *Campylobacter jejuni* and *Campylobacter coli* broiler isolates by whole-genome sequencing. *Foodborne Pathog. Dis.* 15, 145–152. doi: 10.1089/fpd.2017.2325

Cao, Y., Fanning, S., Proos, S., Jordan, K., and Srikumar, S. (2017). A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Front. Microbiol.* 8, 1–16. doi: 10.3389/fmicb.2017.01829

Carrino, J. J., and Lee, H. H. (1995). Nucleic acid amplification methods. *J. Microbiol. Methods* 23, 3–20. doi: 10.1016/0167-7012(95)00024-F

Carvalho, A. F., Silva, D. M., Azevedo, S. S., Piatti, R. M., Genovez, M. E., and Scarcelli, E. (2013). Detection of CDT toxin genes in *Campylobacter* spp. strains isolated from broiler carcasses and vegetables in São Paulo, Brazil. *Brazilian J. Microbiol.* 44, 693–699. doi: 10.1590/S1517-83822013000300005

Castro, A. G. S. A., Dorneles, E. M. S., Santos, E. L. S., Alves, T. M., Silva, G. R., Figueiredo, T. C., et al. (2018). Viability of *Campylobacter* spp. in frozen and chilled broiler carcasses according to real-time PCR with propidium monoazide pretreatment. *Poult. Sci.* 97, 1706–1711. doi: 10.3382/ps/pey020

Cha, W., Mosci, R., Wengert, S. L., Singh, P., Newton, D. W., Salimnnia, H., et al. (2016). Antimicrobial susceptibility profiles of human *Campylobacter jejuni* isolates and association with phylogenetic lineages. *Front. Microbiol.* 7, 1–12. doi: 10.3389/fmicb.2016.00589

Chapela, M. J., Maestu, A. G., and Cabado, A. G. (2015). Detection of foodborne pathogens by qPCR: a practical approach for food industry applications. *Cogent Food Agric.* 1:1013771, 1–19. doi: 10.1080/23311932.2015.1013771

Chen, J., and Park, B. (2016). Recent advancements in nanobioassays and nanobiosensors for foodborne pathogenic bacteria detection. *J. Food Prot.* 79, 1055–1069. doi: 10.4315/0362-028X.JFP-15-516

Clark, C. G., Berry, C., Walker, M., Petkau, A., Barker, D. O. R., Guan, C., et al. (2016). Genomic insights from whole genome sequencing of four clonal outbreak *Campylobacter jejuni* assessed within the global *C. jejuni* population. *BMC Genomics* 17, 1–16. doi: 10.1186/s12864-016-3340-8

Couturier, B. A., Couturier, M. R., Kalp, K. J., and MFisher, M. A. (2013). Detection of non-*jejuni* and -*coli Campylobacter* species from stool specimens with an immunochromatographic antigen detection assay. *J. Clin. Microbiol.* 51, 1935–1937. doi: 10.1128/JCM.03208-12

Dingle, K. E., Colles, F. M., Ure, R., Wagenaar, J. A, Duim, B., Bolton, F. J., et al. (2002). Molecular characterization of *Campylobacter jejuni* clones: a basis for epidemiologic investigation. *Emerg. Infect. Dis.* 8, 949–955. doi: 10.3201/eid0809.02-0122

Eberle, K. N., and Kiess, A. S. (2012). Phenotypic and genotypic methods for typing *Campylobacter jejuni* and *Campylobacter coli* in poultry. *Poult. Sci.* 91, 255–264. doi: 10.3382/ps.2011-01414

Eisenstein, B. I. (1990). The polymerase chain reaction: a new method of using molecular genetics for medical diagnosis. *N. Engl. J. Med.* 322, 178–183. doi: 10.1056/NEJM199001183220307

Flekna, G., Stefanic, P., Wagner, M., Smulders, F. J. M., Mozina, S. S., and Hein, I. (2007). Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises MA/real-time PCR. *Res. Microbiol.* 158, 405–412. doi: 10.1016/j.resmic.2007.02.008

Floch, P., Goret, J., Bessède, E., Lehours, P., and Francis Mégraud, F. (2012). Evaluation of the positive predictive value of a rapid immunochromatographic test to detect *Campylobacter* in stools. *Gut Path.* 4:17, 1–3. doi: 10.1186/1757-4749-4-17

Florez-Cuadrado, D., Ugarte-Ruiz, M., Meric, G., Quesada, A., Porrero, M. C., Pascoe, B., et al. (2017). Genome comparison of erythromycin resistant *Campylobacter* from turkeys identifies hosts and pathways for horizontal spread of *erm*(B) genes. *Front. Microbiol.* 8, 1–8. doi: 10.3389/fmicb.2017.02240

Gharst, G., Oyarzabal, O. A., and Hussain, S. K. (2013). Review of current methodologies to isolate and identify *Campylobacter* spp. from foods. *J. Microbiol. Methods* 95, 84–92. doi: 10.1016/j.mimet.2013.07.014

Ghatak, S., He, Y., Reed, S., Strobaugh, T. Jr., and Irwin, P. (2017). Whole genome sequencing and analysis of *Campylobacter coli* YH502 from retail chicken reveals a plasmid-borne type VI secretion system. *Genomic Data* 11, 128–131. doi: 10.1016/j.gdata.2017.02.005

Giesendorf, B. A., Quint, W. G., Henkens, M. H., Stegeman, H., Huf, F. A., and Hiesters, H. G. (1992). Rapid and sensitive detection of *Campylobacter* spp. in chicken products using the polymerase chain reaction. *Appl. Environ. Microbiol.* 58, 3804–3808.

Gilbert, M. J., Duim, B., van der Graaf-van Bloois, L., Wagenaar, J. A., and Zomer, A. L. (2018). Homolgous recombination between genetically divergent *Campylobacter fetus* lineages supports host-associated speciation. *Genome Biol. Evol.* 10, 716–722. doi: 10.1093/gbe/evy048

Granato, P. A., Chen, L., Holiday, I., Rawling, R. A., Novak-Weekley, S. M., Quinlan, T., et al. (2010). Comparison of Premier CAMPY Enzyme Immunoassay (EIA), ProSpecT *Campylobacter* EIA, and ImmunoCard STAT! CAMPY Tests with culture for laboratory diagnosis of *Campylobacter* enteric infections. *J. Clin. Microbiol.* 48, 4022–4027. doi: 10.1128/JCM.00486-10

Guerry, P., Logan, S. M., and Thornton, S. (1990). Genomic organization and expression of *Campylobacter* flagellin genes. *J. Bacteriol.* 172, 1853–1860. doi: 10.1128/jb.172.4.1853-1860.1990

Hannson, I., Sandberg, M., Habib, I., Lowman, R., and Evgvall, E. O. (2016). Knowledge gaps in control of *Campylobacter* for prevention of campylobacteriosis. *Tansbound Emerg. Dis.* 65, 30–48. doi: 10.1111/tbed.12870

He, Y., Yao, X., Gunther, N. W., Xie, Y., Tu, S. I., and Shi, X. (2010). Simultaneous detection and differentiation of *Campylobacter jejuni*, *C. coli*, and *C. lari* in chickens using a multiplex real-time PCR assay. *Food Anal. Methods* 3, 321–329. doi: 10.1007/s12161-010-9136-6

Heo, S. A., Nannapaneni, R., Johnson, M. G., Park, J. S., and Seo, K. H. (2009). Production and characterization of a monoclonal antibody to *Campylobacter jejuni*. *J. Food Prot.* 72, 870–875. doi: 10.4315/0362-028X-72.4.870

Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993). Kinetic PCR Analysis: Real-time monitoring of DNA amplification reactions. *Nat. Biotechnol.* 11, 1026–1030.

Hill, W. E. (1996). The polymerase chain reaction: applications for the detection of foodborne pathogens. *Crit. Rev. Food Sci. Nutr.* 36, 123–173.

Hofreiter, D., Novik, V., and Galán, J. E. (2008). Metabolic diversity in *Campylobacter jejuni* enhances specific tissue colonization. *Cell Host Microbe* 4, 425–433. doi: 10.1016/j.chom.2008.10.002

Horrocks, S. M., Anderson, R. C., Nisbet, D. J., and Ricke, S. C. (2009). Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* 15, 8–25. doi: 10.1016/j.anaerobe.2008.09.001

Huang, H., Brooks, B. W., Lowman, R., and Carillo, C. D. (2015). *Campylobacter* species in animal, food, and environmental sources, and relevant testing programs in Canada. *Can. J. Microbiol.* 61, 701–721. doi: 10.1139/cjm-2014-0770

Huang, J., Yang, G., Meng, W., Wu, L., Zhu, A., and Jiao, X. (2010). An electrochemical impedimetric immunosensor for label-free detection of *Campylobacter jejuni* in diarrhea patients' stool based on O-carboxymethylchitosan surface modified Fe_3O_4 nanoparticles. *Biosens. Bioelectron.* 25, 1204–1211. doi: 10.1016/j.bios.2009.10.036

Huggett, J. F., Cowen, S., and Foy, C. A. (2015). Considerations for digital PCR as an accurate molecular diagnostic tool. *Clin. Chem.* 61, 79–88. doi: 10.1373/clinchem.2014.221366

Humphrey, T., O'Brien, S., and Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol.* 117, 237–257. doi: 10.1016/j.ijfoodmicro.2007.01.006

Huq, M., Gonis, G., and Istivan, T. (2014). Development and evaluation of a multiplex PCR for the detection of *Campylobacter concisus* and other *Campylobacter* spp. from gastroenteritis cases. *Open J. Med. Microbiol.* 4, 29–37. doi: 10.4236/ojmm.2014.41005

Inglis, G. D., and Kalischuk, L. D. (2003). Use of PCR for direct detection of *Campylobacter* species in bovine species. *Appl. Environ. Microbiol.* 69, 3435–3447. doi: 10.1128/AEM.69.6.3435-3447.2003

Jackson, C. J., Fox, A. J., and Jones, D. M. (1996a). A novel polymerase chain reaction assay for the detection of speciation of thermophilic *Campylobacter* spp. *J. Appl. Microbiol.* 81, 467–473. doi: 10.1111/j.1365-2672.1996.tb03534.x

Jackson, C. J., Fox, A. J., Warening, D. R. A., Hutchinson, D. N., and Jones, D. M. (1996b). The application of genotyping techniques to the epidemiological analysis of *Campylobacter jejuni*. *Epidemiol. Infect.* 117, 233–244.

Janda, A., Bowen, A., Greenspan, N. S., and Casadevall, A. (2016). Ig constant region effects on variable region structure and function. *Front. Microbiol.* 7, 1–10. doi: 10.3389/fmicb.2016.00022

Jansen van Rensburg, M., Swift, C., Cody, A. J., Jenkins, C., and Maiden, M. C. J. (2016). Exploiting bacterial whole-genome sequencing data for evaluation of diagnostic assays: *Campylobacter* species identification as a case study. *J. Clin. Microbiol.* 54, 2882–2890. doi: 10.1128/JCM.01797-16

Joensen, K. G., Kuhn, K. G., Muller, L., Bjorkman, J. T., Torpdahl, M., Engberg, J., et al. (2018). Whole genome sequencing of *Campylobacter jejuni* isolated from Danish routine stool samples reveals surprising degree of clustering. *Clin. Microbiol. Infect.* 24, 201.e5–201.e8. doi: 10.1016/j.cmi.2017.07.026

Josefsen, M. H., Jacobsen, N. R., and Hoorfar, J. (2004). Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of food-borne thermotolerant campylobacters. *Appl. Environ. Microbiol.* 70, 3588–3592. doi: 10.1128/AEM.70.6.3588-3592.2004

Josefsen, M. H., Löfström, C., Hansen, T. B., Chtistensen, L. S., Olsen, J. E., and Hoorfar, J. (2010). Rapid quantitation of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monazide treatment, as a tool for quantitative risk assessment. *Appl. Environ. Microbiol.* 76, 5097–5104. doi: 10.1128/AEM.00411-10

Kamei, K., Kawabata, H., Asakura, M., Samosornsuk, W., Hinenoya, A., Nakagawa, S., et al. (2016). A cytolethal distending toxin gene-based multiplex PCR assay for *Campylobacter jejuni*, *C. fetus*, *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, and *C. lari*. *Jpn. J. Infect. Dis.* 69, 256–258. doi: 10.7883/yoken.JJID.2015.182

Kawase, J., Kurosaki, M., Kawakami, Y., Kashimoto, T., Tsunomori, Y., Sato, K., et al. (2014). Comparison of two methods of bacterial DNA extraction from fecal samples contaminated with *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella Typhimurium*, *Campylobacter jejuni*. *Jpn. J. Infect.* 67, 441–446. doi: 10.7883/yoken.67.441

Kawatsu, K., Kumeda, Y., Taguchi, M., Yamazaki-Matsune, W., Kanki, M., and Inoue, K. (2008). Development and evaluation of immunochromatographic assay for simple and rapid detection of *Campylobacter jejuni* and *Campylobacter coli* in human stool specimens. *J. Clin. Microbiol.* 46, 1226–1231. doi: 10.1128/JCM.02170-07

Kim, S. A., Park, S. H., Lee, S. I., Owens-Hanning, C., and Ricke, S. C. (2017a). Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Sci. Rep.* 7:43354. doi: 10.1038/srep43354

Kim, S. A., Park, S. H., Lee, S. I., and Ricke, S. C. (2017b). Development of a rapid method to quantify *Salmonella* Typhimurium using a combination of MPN with qPCR and a shortened time incubation. *Food Microbiol.* 65, 7–18. doi: 10.1016/j.fm.2017.01.013

Klena, J. D., Parker, C. T., Knibb, K., Ibbitt, J. C., Devane, P. M., Horn, S. T., et al. (2004). Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipd A gene lpxA. *J. Clin. Microbiol.* 42, 5549–5557. doi: 10.1128/JCM.42.12.5549-5557.2004

Kralik, P., and Ricchi, M. (2017). A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Front. Microbiol.* 8, 1–9. doi: 10.3389/fmicb.2017.00108

Krüger, N. J., Buhler, C., Iwoba, A. N., Huber, I., Ellerbroek, L., Appel, B., et al. (2014). "Limits of control" – crucial parameters for a reliable quantification of viable *Campylobacter* by real-time PCR. *PLoS One* 9:e88108. doi: 10.1371/journal.pone.0088108

Lahti, E., Löfdahl, M., Ågren, J., Hansson, I., and Olsson Engvall, E. (2017). Confirmation of a campylobacteriosis outbreak associated with chicken liver pâté using PFGE and WGS. *Zoonoses Public Health* 64, 14–20. doi: 10.1111/zph.12272

Lamoureux, M., Mackay, A., Messier, S., Fliss, I., Blais, B. W., Holley, R. A., et al. (1997). Detection of *Campylobacter jejuni* in food and poultry viscera using immunomagentic separation and microtitre hybridization. *J. Appl. Microbiol.* 83, 641–651. doi: 10.1046/j.1365-2672.1997.00273.x

Lawton, S. J., Weis, A. M., Byrne, B. A., Fritz, H., Taff, C. C., Townsend, A. K., et al. (2018). Comparative analysis of *Campylobacter* isolates from wild birds and chickens using MALDI-TOF MS, biochemical testing, and DNA sequencing. *J. Vet. Diagn. Investig.* 30, 354–361. doi: 10.1177/1040638718762562

Leekitcharoenphon, P., Garcia-Graells, C., Botteldoorn, N., Dierick, K., Kempf, I., Olkkola, S., et al. (2018). Comparative genomics of quinolone-resistant and susceptible *Campylobacter jejuni* of poultry origin from major poultry producing European countries (GENCAMP). *EFSA Supporting Publications* 15:1398E.

Leskinen, S. D., and Lim, D. V. (2008). Rapid ultrafiltration concentration and biosensor detection of enterococci from large volumes of Florida recreational water. *Appl. Environ. Microbiol.* 7415, 4792–4798. doi: 10.1128/AEM.00052-08

Linton, D., Owen, R. J., and Stanley, J. (1996). Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res. Microbiol.* 147, 707–718. doi: 10.1016/S0923-2508(97)85118-2

Linton, D., Lawson, A. J., Owen, R. J., and Stanley, J. P. C. R. (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J. Clin. Microbiol.* 35, 2568–2572.

Llarena, A. K., Taboada, E., and Rossi, M. (2017). Whole-genome sequencing in epidemiology of *Campylobacter jejuni* infections. *J. Clin. Microbiol.* 55, 1269–1275. doi: 10.1128/JCM.00017-17

Logan, J. M. J., Edwards, K. J., Saunders, N. A., and Stanley, J. (2001). Rapid identification of *Campylobacter* spp. by melting peak analysis of biprobes

in real-time PCR. *J. Clin. Microbiol.* 39, 2227–2232. doi: 10.1128/JCM.39.6.2227-2232.2001

Loge, F. J., Thompson, D. E., and Call, D. R. (2002). PCR detection of specific pathogens in water: a risk-based analysis. *Environ. Sci. Technol.* 3612, 2754–2759. doi: 10.1021/es015777m

Lund, M., Nordentoft, S., Pedersen, K., and Madsen, M. (2004). Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. *J. Clin. Microbiol.* 42, 5125–5132. doi: 10.1128/JCM.42.11.5125-5132.2004

Lüth, S., Kleta, S., and Dahouk, S. A. (2018). Whole genome sequencing as a typing tool for foodborne pathogens like *Listeria monocytogenes*—the way towards global harmonization and data exchange. *Trends Food Technol. Technol.* 73, 67–75. doi: 10.1016/j.tifs.2018.01.008

Maciorowski, K. G., Herrera, P., Jones, F. T., Pillai, S. D., and Ricke, S. C. (2006). Cultural and immunological detection methods for detection of *Salmonella* spp. in animal feeds: a review. *Veterinary Res. Comm.* 30, 127–137. doi: 10.1007/s11259-006-3221-8

Mackay, I. M. (2004). Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* 10, 190–212. doi: 10.1111/j.1198-743X.2004.00722.x

Mäesaar, M., Meremäe, K., Ivanova, M., and Roasto, M. (2018). Antimicrobial resistance and multilocus sequence types of *Campylobacter jejuni* isolated from Baltic broiler chicken meat and Estonian human patients. *Poult. Sci.* 97, 3645–3651. doi: 10.3382/ps/pey219

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

Manfreda, G., and De Cesare, A. (2005). *Campylobacter* and *Salmonella* in poultry and poultry products: hows and whys of molecular typing. *World's Poultry Sci. J.* 61, 185–197. doi: 10.1079/WPS200448

Masdor, N. A., Altintas, Z., and Tothill, I. E. (2017). Surface plasmon resonance immunosensor for the detection of *Campylobacter jejuni*. *Chem. Aust.* 5, 1–15. doi: 10.3390/chemosensors5020016

Masdor, N. A., Altintas, Z., and Tothill, I. E. (2016). Sensitive detection of *Campylobacter jejuni* using nanoparticles enhanced QCM sensor. *Biosens. Bioelectron.* 78, 328–336. doi: 10.1016/j.bios.2015.11.033

Mehla, K., and Ramana, J. (2017). Surface proteome mining for identification of potential vaccine candidates against *Campylobacter jejuni*: an in silico approach. *Funct. Integr. Genomics* 17, 27–37. doi: 10.1007/s10142-016-0530-z

Metzker, M. L. (2010). Sequencing technologies–the next generation. *Nature Revs.* 11, 31–46. doi: 10.1038/nrg2626

Miller, W. G., Parker, C. T., Heath, S., and Lastovica, A. J. (2007). Identification of genomic differences between *Campylobacter jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* at the *nap* locus leads to the development of a *C. jejuni* subspeciation multiplex PCR method. *BMC Microbiol.* 7, 1–8. doi: 10.1186/1471-2180-7-11

Myers, A. L., Jackson, M. A., and Selvarangan, R. (2011). False-positive results of *Campylobacter* rapid antigen testing. *Pediatr. Infect. Dis. J.* 30:542. doi: 10.1097/INF.0b013e31821524db

Nachamkin, I., and Hart, A. M. (1986). Common and specific epitopes of *Campylobacter* flagellin recognized by monoclonal antibodies. *Appl. Environ. Microbiol.* 53, 438–440.

Neal-McKinney, J. M., Liu, K. C., Jinneman, K. C., Wu, W.-H., and Rice, D. H. (2018). Whole genome sequencing and multiplex qPCR methods to identify *Campylobacter jejuni* encoding *cst-II* or *cst-III* sialyltransferase. *Front. Microbiol.* 9, 1–8. doi: 10.3389/fmicb.2018.00408

Nogva, H. K., Bergh, A., Holck, A., and Rudi, K. (2000). Application of the 5′-nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 66, 4029–4036. doi: 10.1128/AEM.66.9.4029-4036.2000

Nzuma, R. M., Liu, F., and Grant, I. R. (2018). Generation and characterization of a novel recombinant scFv antibody specific for *Campylobacter jejuni*. *Appl. Microbiol. Biotechnol.* 102, 4873–4885. doi: 10.1007/soo253-018-8949-x

Oakley, B. B., Morales, C. A., Line, J. E., Seal, B. S., and Hiett, K. L. (2012). Application of high-throughput sequencing to measure the performance of commonly used selective cultivation methods for the foodborne pathogen *Campylobacter*. *FEMS Microbiol. Ecol.* 79, 327–336. doi: 10.1111/j.1574-6941.2011.01219.x

O'Connell, P. J., O'Sullivan, C. K., and Guibault, G. G. (2000). Biosensors for food analysis. *Irish J. Agric. Food Res.* 39, 321–329.

O'Kane, P. M., and Connerton, I. F. (2017). Characterisation of aerotolerant forms of a robust chicken colonizing *Campylobacter coli*. *Front. Microbiol.* 8:513. doi: 10.3389/fmicb.2017.00513

On, S. L., Brandt, S. M., Corneluis, A. J., Fusco, V., Quero, G. M., Maćkiw, E., et al. (2013). PCR revisited: a case for revalidation of PCR assays for microorganisms using identification of *Campylobacter* species as an example. *Qual. Assurance Safety Crops Foods* 5, 49–62. doi: 10.3920/QAS2012.0158

On, S. L. W., Holmes, B., and Sackin, M. J. (1996). A probability matrix for the identification of campylobacters, helicobacters and allied taxa. *J. Appl. Bacteriol.* 81, 425–432.

Oyarzabal, O. A., and Battie, C. (2012). "Chapter 13. Immunological methods for the detection of *Campylobacter* spp.–current applications and potential use in biosensors" in *Trends in immunolabelled and related techniques*. ed. Abuelzein, E. (Rijeka, Croatia: InTech), 203–226.

Oyofo, B. A., Thornton, S. A., Burr, D. H., Trust, T. J., Pavlovskis, O. R., and Guerry, P. (1992). Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by using polymerase chain reaction. *J. Clin. Microbiol.* 30, 2613–2619.

Pacholewicz, E., Swart, A., Lipman, L. J. A., Wagenaar, J. A., Havelaar, A. H., and Duim, B. (2013). Propidium monazide does not fully inhibit the detection of dead *Campylobacter* on broiler chicken carcasses by qPCR. *J. Microbiol. Methods* 95, 32–38. doi: 10.1016/j.mimet.2013.06.003

Papic, B., Pate, M., Henigman, U., Zajc, U., Gruntar, I., Biazzo, M., et al. (2017). New approaches on qunatifiaction of *Campylobacter jejuni* in poultry samples: the use of digital PCR and real-time PCR against the ISO standard plate count method. *Front. Microbiol.* 8, 1–13. doi: 10.3389/fmicb.2017.00331

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., et al. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food Microbiol.* 38, 250–262. doi: 10.1016/j.fm.2013.10.002

Park, S. H., Hanning, I., Jarquin, R., Moore, P., Donoghue, D. J., Donoghoue, A. M., et al. (2011). Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. *FEMS Microbiol. Letters* 316, 7–15. doi: 10.1111/j.1574-6968.2010.02188.x

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Pendleton, S., Hanning, I., Biswas, D., and Ricke, S. C. (2013). Evaluation of whole-genome sequencing as a genotyping tool for *Campylobacter jejuni* in comparison with pulsed-field gel electrophoresis and *flaA* typing. *Poult. Sci.* 92, 573–580. doi: 10.3382/ps.2012-02695

Pennisi, E. (2017). Pocket-sized sequencers start to pay off big. *Science* 356, 572–573. doi: 10.1126/science.356.6338.572

Pennisi, E. (2016). Pocket DNA sequencers make real-time diagnostics a reality–Advances in accuracy of nanopore sequencing help pave the way for on-the-spot DNA tests. *Science* 351, 800–801. doi: 10.1126/science.351.6275.800

Persson, S., and Olsen, K. E. P. (2005). Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples. *J. Med. Microbiol.* 54, 1043–1047. doi: 10.1099/jmm.0.46203-0

Pontiroli, A., Travis, E. R., Sweeney, F. P., Porter, D., Gaze, W. H., Mason, S., et al. (2011). Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. *PLoS One* 6:e17916. doi: 10.1371/journal.pone.0017916

Preiner, J., Kodera, N., Tang, J., Ebner, A., Brameshuber, M., Blaas, D., et al. (2014). IgGs are made for walking on bacterial and viral surfaces. *Nature Comm.* 5:4394. doi: 10.1038/ncomms5394

Qian, H., Pang, E., Du, Q., Chang, J., Dong, J., Toh, S. L., et al. (2008). Production of a monoclonal antibody specific for the major outer membrane protein of *Campylobacter jejuni* and characterization of the epitope. *Appl. Environ. Microbiol.* 74, 833–839. doi: 10.1128/AEM.01559-07

Raja, S., Rao, A., Babu, N., and Thamizhannal (2016). Detection of emerging food pathogens in chicken meat using multiplex polymerase chain reaction. *J. Agric. Sci.* 8, 217–225. doi: 10.5539/jas.v8n9p217

Rasmussen, H. N., Olsen, J. E., Jørgensen, K., and Rasmussen, O. F. (1996). Detection of *Campylobacter jejuni* and *Camp. coli* in chicken faecal samples by PCR. *Lett. Appl. Microbiol.* 23, 363–366. doi: 10.1111/j.1472-765X.1996.tb00209.x

Regnath, T., and Ignatius, R. (2014). Accurate detection of *Campylobacter* spp. antigens by immunochromatography and enzyme immunoassay in routine microbiological laboratory. *European J. Microbiol. Immunol.* 4, 156–158. doi: 10.1556/EUJMI-D-14-00018

Reis, L. P., Meneze, L. D. M., Lima, G. K., de Souza Santos, E. L., Dorneles, E. M. S., de Assis, D. C. S., et al. (2018). Detection of *Campylobacter* spp. in chilled and frozen broiler carcasses comparing immunoassay, PCR and real time PCR methods. *Ciênc. Rural, Santa Maria* 48:e20161034. doi: 10.1371/journal.pone.0208488

Reshetilov, A. N. (2005). Microbial, enzymatic, and immune biosensors for ecological monitoring and control of biotechnological processes. *Appl. Biochem. Microbiol.* 41, 442–449. doi: 10.1007/s10438-005-0079-4

Revez, J., Llarena, A.-K., Schott, T., Kuusi, M., Hakkinen, M., Kivisto, R., et al. (2014). Genome analysis of *Campylobacter jejuni* strains isolated from a waterborne outbreak. *BMC Genomics* 15, 1–8. www.biomedcentral.com/1471-2164/15/768. doi: 10.1186/1471-2164-15-768

Rokney, A., Valinsky, L., Moran-Gilad, J., Vranckx, K., Agmon, V., and Weinberger, M. (2018). Genomic epidemiology of *Campylobacter jejuni* transmission in Isreal. *Front. Microbiol.* 9:2432. doi: 10.3389/fmicb.2018.02432

Rodrigues, R. C., Haddad, N., Chevret, D., Cappelier, J. M., and Tresse, O. (2016). Comparsion of proteomics profiles of *Campylobacter jejuni* strain Bf under microaerobic and aerobic conditions. *Front. Microbiol.* 7, 1–12, doi: 10.1002/cpt.1328

Ronholm, J. (2018). Editorial: game changer–next generation sequencing and its impact on food microbiology. *Front. Microbiol.* 9, 1–3. doi: 10.3389/fmicb.2018.00363

Roop II, R. M., Smibert, R. M., Johnson, J. L., and Krieg, N. R. (1984). Differential characteristics of catalase–positive campylobacters correlated with DNA homology groups. *Can. J. Microbiol.* 30, 938–951. doi: 10.1139/m84-147

Rothrock Jr., M., Hiett, K. L., Kiepper, B. H., Ingram, K., and Hinton, A. (2013). Commercial poultry processing water samples using droplet digital PCR. *Adv. Microbiol.* 3, 403–411.

Rothrock Jr., M. J., Hiett, K. L., Kiepper, B. H., Ingram, K., and Hinton, A. (2015). Quantification of zoonotic bacterial pathogens within commercial poultry processing water samples using droplet digital PCR. *Adv. Microbiol.* 3, 403–411. doi: 10.4236/aim.2013.35055

Rudi, K., Moen, B., Drømtorp, S. M., and Holck, A. L. (2005). Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Appl. Environ. Microbiol.* 71, 1018–1024. doi: 10.1128/AEM.71.2.1018-1024.2005

Rudi, K., Høidal, H. K., Katla, T., Johansen, B. K., Nordal, J., and Jakobsen, K. S. (2004). Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. *Appl. Environ. Microbiol.* 70, 790–797. doi: 10.1128/AEM.70.2.790-797.2004

Sacher, J. C., Yee, E., Szymanski, C. M., and Miller, W. G. (2018). Complete genome sequences of three *Campylobacter jejuni* phage-propagating strains. *Genome Announc.* 6, e00514–e00518. doi: 10.1128/genomeA.00514-18

Sails, A. D., Bolton, F. J., Fox, A. J., Wareing, D. R., and Greenway, D. L. (2002). Detection of *Campylobacter jejuni* and *Campylobacter coli* in environmental waters by PCR enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* 68, 1319–1324. doi: 10.1128/AEM.68.3.1319-1324.2002

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

Seinige, D., Krischek, C., Klein, G., and Kehrenberg, C. (2014). Comparative analysis and limitations of ethidium monoazide and propidium treatments for the differentiation of viable and nonviable *Campylobacter* cells. *Appl. Environ. Microbiol.* 80, 2186–2192. doi: 10.1128/AEM.03962-13

Sekse, C., Holst-Jensen, A., Dobrindt, U., Johannessen, G. S., Li, W., Spilsberg, B., et al. (2017). High throughput sequencing for detection of foodborne pathogens. *Front. Microbiol.* 8, 1–26. doi: 10.3389/fmicb.2017.02029

Sheppard, S. K., and Maiden, M. C. J. (2015). The evolution of *Campylobacter jejuni* and *Campylobacter coli*. *Cold Spring Harb. Perspect. Biol.* 7:a018119, 1–13. www.cshperspectives.org. doi: 10.1101/cshperspect.a018119

Steele, M., Gyles, C., Chan, V. L., and Odumeru, J. (2002). Monoclonal antibodies specific for hippurate hydrolase of *Campylobacter jejuni*. *J. Clin. Microbiol.* 40, 1080–1082. doi: 10.1128/jcm.40.3.1080-1082.2002

Taboada, E. N., Graham, M. R., Carriço, J. A., and Van Domselaar, G. (2017). Food safety in the age of next generation sequencing, bioinformatics, and open data access. *Front. Microbiol.* 8, 1–10. doi: 10.3389/fmicb.2017.00909

Taboada, E. N., Clark, C. G., Sproston, E. L., and Carrillo, C. D. (2013). Current methods for molecular typing of *Campylobacter* species. *J. Microbiol. Methods* 95, 24–31. doi: 10.1016/j.mimet.2013.07.007

Taveirne, M. E., Dunham, D. T., Perault, A., Beauchamp, J. M., Huynh, S., Parker, C. T., et al. (2017). Complete annotated genome sequences of three *Campylobacter jejuni* strains isolated from naturally colonized farm-raised chickens. *Genome Announc.* 5, e01407–e01416. doi: 10.1128/genomeA.01407-16

Thépault, A., Rose, V., Quesne, S., Poezevara, T., Béven, V., Hirchaud, E., et al. (2018). Ruminant and chicken: important sources of campylobacteriosis in France despite a variation of source attribution in 2009 and 2015. *Sci. Rep.* 8:9305. doi: 10.1038/s41598-018-27558-z

Tholozan, J. L., Cappelier, J. M., Tissier, J. P., Delattre, G., and Federighi, M. (1999). Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Appl. Environ. Microbiol.* 65, 1110–1116.

Toplak, N., Kovač, M., Piskernik, S., Možina, S. S., and Jeršek, B. (2012). Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. *J. Appl. Microbiol.* 112, 752–764. doi: 10.1111/j.1365-2672.2012.05235.x

Turonova, H., Haddad, N., Hernould, M., Chevret, D., Paziarova, J., and Tresse, O. (2017). Profiling of *Campylobacter jejuni* proteome in exponential and stationary phase of growth. *Front. Microbiol.* 8, 1–12. doi: 10.3389/fmicb.2017.00913

Ugarte-Ruiz, M., Wassenaar, T. M., Gómez-Barrero, S., Porrero, M. C., Navarro-Gonzalez, N., and Dominguez, L. (2013). The effect of different isolation protocols on detection and molecular characterization of *Campylobacter* from poultry. *Lett. Appl. Microbiol.* 57, 427–435. doi: 10.1111/lam.12130

Uyttendaele, M., Schukkink, R., van Gemen, B., and Debevere, J. (1995). Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). *Int. J. Food Microbiol.* 61, 1341–1347.

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

Villamizar-Rodríguez, G., Fernández, J., Marín, L., Muñiz, J., González, I., and Lombó, F. (2015). Multiplex detection of nine food-borne pathogens by mPCR and capillary electrophoresis after using a universal pre-enrichment medium. *Front. Microbiol.* 6, 1–16. doi: 10.3389/fmicb.2015.01194

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., et al. (2002). Colony multiplex PCR assay for identification and differenctiation of *Campylobacter jejuni*, *C. coli*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J. Clin. Microbiol.* 40, 4744–4747. doi: 10.1128/JCM.40.12.4744-4747.2002

Wassenaar, T. M., and Newell, D. G. (2000). Genotyping of *Campylobacter* spp. *Appl. Environ. Microbiol.* 66, 1–9. doi: 10.1128/AEM.66.1.1-9.2000

Wassenaar, T. M., Geilhausen, B., and Newell, D. G. (1998). Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. *Appl. Environ. Microbiol.* 64, 1816–1821.

Wei, D., Oyarzabal, O. A., Huang, T.-S., Balasubramanian, S., Sista, S., and Simonian, A. L. (2007). Development of a surface plasmon resonance biosensor for the identification of *Campylobacter jejuni*. *J. Microbiol. Methods* 69, 78–85. doi: 10.1016/j.mimet.2006.12.002

Whitehouse, C. A., Young, S., Li, C., Hsu, C.-H., and Martin, G. (2018). Use of whole-genome sequencing for *Campylobacter* surveillance from NARMS retail poultry in the United States in 2015. *Food Microbiol.* 73, 122–128. doi: 10.1016/j.fm.2018.01.018

Willner, I. (2002). Biomaterials for sensors, fuel cells, and circuitry. *Science* 298, 2407–2408. doi: 10.1126/science.298.5602.2407

Wilson, D. L., Rathinam, V. A. K., Qi, W., Wick, L. M., Landgraf, J., Bell, J. A., et al. (2010). Genetic diversity in *Campylobacter jejuni* is associated with differential colonization of broiler chickens and C57BL/6J IL10-deficient mice. *Microbiology* 156, 2046–2057. doi: 10.1099/mic.0.035717-0

Wittwer, C. T., Herrmann, M. G., Gundry, C. N., and Elenitoba-Johnson, K. S. J. (2001). Realtime multiplex PCR assays. *Methods* 25, 430–442. doi: 10.1006/meth.1265

Yamazaki-Matsune, W., Taguchi, M., Seto, K., Kawahara, R., Kawatsu, K., Kumeda, Y., et al. (2007). Development of a multiplex PCR assay for identification of *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis*. *J. Med. Microbiol.* 56, 1467–1473. doi: 10.1099/jmm.0.47363-0

Yang, C., Jiang, Y., Huang, K., Zhu, C., and Yin, Y. (2003). Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS Immunol. Med. Microbiol.* 38, 265–271. doi: 10.1016/S0928-8244(03)00168-8

Yang, H., Li, Y., and Johnson, M. (2006). Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and chicken skin during poultry scalding and chilling. *J. Food Protect.* 64, 770–776.

Yolken, R. H. (1982). Enzyme immunoassays for the detection of infectious antigens in body fluids: current limitations and future prospects. *Rev. Infect. Dis.* 4, 35–68.

Yolken, R. H. (1988). Nucleic acids or immunoglobulins: which are the molecular probes of the future? *Mol. Cell. Probes* 2, 87–96. doi: 10.1016/0890-8508(88)90030-8

Zeng, D., Chen, Z., Jiang, Y., Xue, F., and Li, B. (2016). Advances and challenges in viability detection of foodborne pathogens. *Front. Microbiol.* 7, 1–12. doi: 10.3389/fmicb.2016.01833

Zhao, C., Ge, B., Villena, J. D., Sudler, R., Yeh, E., Zhao, S., et al. (2001). Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D.C., area. *Appl. Envrion. Microbiol.* 67, 5431–5436. doi: 10.1128/AEM.67.12.5431-5436.2001

Zhao, S., Tyson, G. H., Chen, Y., Li, C., Mukherjee, S., Young, S., et al. (2016). Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in *Campylobacter* spp. *Appl. Environ. Microbiol.* 82, 459–466. doi: 10.1128/AEM.02873-15

Ziprin, R. L., Droleskey, R. E., Hume, M. E., and Harvey, R. B. (2003). Failure of viable nonculturable *Campylobacter jejuni* to colonize the cecum of newly hatched Leghorn chicks. *Avian Dis.* 47, 753–758. doi: 10.1637/7015

Conflict of Interest Statement: The WC and HP are employed by Diamond V (Cedar Rapids, IA, USA).

The remaining 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 SN and handling Editor declared their shared affiliation.

frontiers in Microbiology

REVIEW
published: 31 January 2019
doi: 10.3389/fmicb.2019.00091

Potential for Prebiotics as Feed Additives to Limit Foodborne *Campylobacter* Establishment in the Poultry Gastrointestinal Tract

*Sun Ae Kim[1], Min Ji Jang[1], Seo Young Kim[1], Yichao Yang[2], Hilary O. Pavlidis[3] and Steven C. Ricke[4]**

[1] Department of Food Science and Engineering, Ewha Womans University, Seoul, South Korea, [2] Department of Poultry Science, University of Arkansas, Fayetteville, AR, United States, [3] Diamond V, Cedar Rapids, IA, United States, [4] Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, AR, United States

OPEN ACCESS

Edited by:
Andrea Gomez-Zavaglia, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Argentina

Reviewed by:
Ana Florencia Moretti, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Argentina
Robert J. Moore, RMIT University, Australia

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

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

Received: *18 October 2018*
Accepted: *16 January 2019*
Published: *31 January 2019*

Citation:
Kim SA, Jang MJ, Kim SY, Yang Y, Pavlidis HO and Ricke SC (2019) Potential for Prebiotics as Feed Additives to Limit Foodborne Campylobacter Establishment in the Poultry Gastrointestinal Tract. Front. Microbiol. 10:91. doi: 10.3389/fmicb.2019.00091

Campylobacter as an inhabitant of the poultry gastrointestinal tract has proven to be difficult to reduce with most feed additives. In-feed antibiotics have been taken out of poultry diets due to the negative reactions of consumers along with concerns regarding the generation of antibiotic resistant bacteria. Consequently, interest in alternative feed supplements to antibiotics has grown. One of these alternatives, prebiotics, has been examined as a potential animal and poultry feed additive. Prebiotics are non-digestible ingredients by host enzymes that enhance growth of indigenous gastrointestinal bacteria that elicit metabolic characteristics considered beneficial to the host and depending on the type of metabolite, antagonistic to establishment of pathogens. There are several carbohydrate polymers that qualify as prebiotics and have been fed to poultry. These include mannan-oligosaccharides and fructooligosaccharides as the most common ones marketed commercially that have been used as feed supplements in poultry. More recently, several other non-digestible oligosaccharides have also been identified as possessing prebiotic properties when implemented as feed supplements. While there is evidence that prebiotics may be effective in poultry and limit establishment of foodborne pathogens such as *Salmonella* in the gastrointestinal tract, less is known about their impact on *Campylobacter*. This review will focus on the potential of prebiotics to limit establishment of *Campylobacter* in the poultry gastrointestinal tract and future research directions.

Keywords: *Campylobacter*, poultry, prebiotics, gastrointestinal tract, synbiotics

INTRODUCTION

Campylobacter is a prevalent foodborne pathogen in poultry such as chicken and turkey. It causes foodborne disease in humans (campylobacteriosis) due to consumption of contaminated poultry products, thus constituting a major public health issue (Sahin et al., 2002; Newell and Fearnley, 2003). Control of *Campylobacter* in poultry to improve microbiological safety is a primary concern for consumers and government food safety agencies (Lin, 2009). Traditionally, antibiotics

have been widely utilized for improving growth performance in poultry; however, the word 'antibiotic' provokes a negative reaction from consumers and using antibiotics could also lead to the potential generation of antibiotic resistant pathogenic bacteria, thus the routine supplementation of antibiotics into poultry feeds has become less prevalent over time (Edens, 2003; Jones and Ricke, 2003; Ferket, 2004; Dibner and Richards, 2005; Griggs and Jacob, 2005).

As a result of the shift away from antibiotic supplementation there has been a tremendous growth in research and implementation of effective alternative control methods using a wide array of approaches including hygiene and biosecurity farming practices, drinking water treatments, chemical feed additives, bacteriophage application, vaccination, passive immunization, competitive exclusion cultures, host genetic selection strategies, bacteriophage therapy, and bacteriocin application (Tsubokura et al., 1997; Mead, 2000; Newell and Wagenaar, 2000; Boyd et al., 2005; Carrillo et al., 2005; Cole et al., 2006; Wagenaar et al., 2006; de Zoete et al., 2007; Li et al., 2008; Lin, 2009; Buckley et al., 2010; Scupham et al., 2010; Skånseng et al., 2010; Svetoch and Stern, 2010; Van Gerwe et al., 2010a; Hermans et al., 2011a,b; Layton et al., 2011; Sibanda et al., 2018). Given the ability of *Campylobacter* to readily establish in the poultry gastrointestinal tract (GIT) of poultry (Indikova et al., 2015), an obvious target for limiting its proliferation are feed additives that serve as mitigation agents when introduced to the GIT of poultry. These would include inhibitory agents such as botanicals, organic acids and bacteriophage and colonization preventative biologicals such as prebiotics and probiotics. In practice, prevention of *Campylobacter* colonization by probiotics and prebiotics may prove more difficult than initially perceived since *Campylobacter* appear to be extensively interconnected with the indigenous microbiota of the poultry GIT (Indikova et al., 2015). While both approaches have been considered as potential control measures, the focus in this review will be on prebiotics as a means to alter or shift the composition of the already established poultry GIT microbiota and the resulting impact on *Campylobacter* populations.

Prebiotics have also been considered as one of the effective methods to increase the beneficial bacteria within the microbiota in the GIT of various food animal species, including chickens, as well as limit foodborne pathogens in the GIT (Flickinger et al., 2003; Patterson and Burkholder, 2003; Callaway and Ricke, 2011; Hermans et al., 2011b; Hutkins et al., 2016; Gibson et al., 2017; Ricke, 2018; Santovito et al., 2018). Prebiotics also appear to be generally effective in poultry and reduce colonization of foodborne pathogens such as *Salmonella* in the GIT of poultry (Ricke, 2015; Roto et al., 2015; Micciche et al., 2018). However, much less is known on the effectiveness of prebiotics to limit *Campylobacter* establishment in poultry. The specific aim of the present review is to provide an overview of *Campylobacter* in the poultry GIT along with the definition of prebiotics and their subsequent effects on the GIT microbiota. This will be accompanied by a discussion on the recent findings on the impact of prebiotics as feed supplements on *Campylobacter* in the poultry GIT, and offer potential directions for future research.

Campylobacter IN THE POULTRY GIT

Much of the focus on controlling *Campylobacter* spp. is on decreasing contamination on meat and skin of poultry (Kudirkienë et al., 2013; Rosenquist et al., 2013) while relatively limited information is available detailing the *Campylobacter* prevalence or populations in preharvest poultry production and the mitigation efforts associated with management on the farm (Sibanda et al., 2018). For feed amendments such as prebiotics to be successful it is important to identify the sites in the poultry GIT where *Campylobacter* colonization is most likely to occur. Representative studies reporting *Campylobacter* prevalence and their population levels in the poultry GIT are listed in **Table 1**. Since *Campylobacter* colonization usually occurs in the lower GIT particularly in the ceca (Beery et al., 1988; Jeffrey et al., 2001; Fernandez et al., 2000), historically most of the research efforts have been focused on characterization and mitigation strategies associated with *Campylobacter* and the lower GIT of poultry. However, there is growing evidence that the upper compartments of the poultry GIT, particularly the crop, may also play an important role.

Byrd et al. (1998) investigated the prevalence of *Campylobacter* in crops (n = 359) and ceca (n = 240) of market-age broiler chickens using Campy-Ceflex plates after Bolton broth enrichment and reported that the percentages of *Campylobacter*-positive in crops (224/359: 62.4%) were higher than that of the ceca (9/240: 3.8%). They concluded that *Campylobacter* in the crop may be a critical control point to minimize entry of *Campylobacter* into the poultry processing plant and to reduce their contamination in broilers. The higher incidence in the crop is somewhat surprising compared to the results from other studies. The authors suggested that the higher incidence in the crop may be related to the prolonged feed withdrawal required for broiler production in the United States prior to being transported to the processing plant. In addition, it must be noted that these results represent the percentage of birds positive for *Campylobacter* and not necessarily the total numbers of *Campylobacter* in the crop and ceca.

Musgrove et al. (2001) studied *Campylobacter* in the ceca and crops of 32 New York-dressed broilers (n = 128, 32 birds with four repetitions) collected from commercial processing plants with and without enrichment. The 128 ceca (100%) and 122 (95.3%) crops were contaminated with *Campylobacter* when they were identified by direct plating on Campy-Cefex agar plates. After enrichment, detection rate of *Campylobacter* in crops was increased (122/128: 99.2%) while enrichment resulted in significantly fewer positive samples from the ceca (81/128: 63%). The authors suggested that the reason for the decrease of 36.7% in the detection rate of *Campylobacter* in ceca was due to being subjected to enrichment compared to direct plating. This may be attributed to the fact that there are more bacterial species in the ceca than in the crop with higher numbers of total bacteria and thus *Campylobacter* species grow more slowly than other bacterial species along with poor competition ability in its intestinal niche (Musgrove et al., 2001). The contamination level in the ceca (6.8 log CFU/g) was twice as much as crops (3.6 log CFU/g) (Musgrove et al., 2001).

TABLE 1 | *Campylobacter* in the poultry gastrointestinal tract.

Poultry	Sample	Sample number	Prevalence of *Campylobacter*	*Campylobacter* spp. counts (log CFU/g)	Reference
Market-age broiler chickens	Ceca	240	9 samples positive (3.8%)	–	Byrd et al., 1998
	Crop	359	224 samples positive (62.4%)	–	
New York-dressed broiler	Ceca	128 (32 birds * 4 repetition)	128 samples positive (100%) with direct plating 81 sample positive (63%) after enrichment	6.8	Musgrove et al., 2001
	Crop	128 (32 birds * 4 repetition)	122 samples positive (95.3%) with direct plating 127 samples positive (99.2%) after enrichment	3.7	
Market-weight turkey	Ceca	84	2 samples *C. jejuni* positive (2.1%)	–	Wesley et al., 2005
			96 samples *C. coli* positive (100%)	–	
	Crop	96	11 samples *C. jejuni* positive (13.1%)	–	
			61 samples *C. coli* positive (72.6%)	–	
Turkey during slaughtering	Ceca at evisceration step	30 (collected on July)	30 samples positive (100%)	6.0	Bily et al., 2010
		30 (collected on Sep)	22 samples positive (73.3%)	2.1	
		30 (collected on Oct)	30 samples positive (100%)	7.2	
		30 (collected on Nov)	30 samples positive (100%)	6.0	
Chicken	Ceca	24	–	8.5	Van Gerwe et al., 2010b
	Crop	23	–	4.8	
Chicken	Gizzard	4 male	–	2.5	Yusrizal and Chen, 2003
		4 female	–	2.5	
	Small Intestine	4 male	–	3.6	
		4 female	–	3.8	
	Large intestine	4 male	–	5.3	
		4 female	–	5.4	
	Cecal intestine	4 male	–	5.3	
		4 female	–	4.4	

Prevalence of *C. jejuni* and *C. coli* on market-weight turkeys was studied by Wesley et al. (2005). A total of 84 crops and 96 ceca were tested (enrichment in blood-free enrichment broth, plating on Campy-Cefex agar, and confirmation of presumptive colonies with a multiplex PCR) to determine whether *C. jejuni* and *C. coli* were positive for each sample. Two ceca samples (2.1%) were *C. jejuni* positive and 96 ceca samples (100%) were *C. coli* positive. In the case of crops, *Campylobacter jejuni* and *coli* was detected in 11 (13.1%) and 61 (72.6%) samples, respectively. All ceca had *C. jejuni* or *C. coli* while 12 crops (14.3%) did not yield both *C. jejuni* and *C. coli* (Wesley et al., 2005).

Bily et al. (2010) evaluated *Campylobacter* spp. carriage in cecal contents of turkeys both quantitatively and qualitatively, during and following the slaughtering process. For quantitative analysis, samples were directly plated on Karmali plates. For qualitative analysis, samples were enriched in Preston broth and then enriched samples were streaked onto a Virion medium with agar and blood. Four turkey flocks were sampled in four different months (July, September, October, and November) and 30 ceca were collected at post-evisceration from each flock. Most of the ceca were positive for *Campylobacter* with all ceca collected in July, October, and November being *Campylobacter* positive (100%) and their contamination levels were 6.0, 7.2, and 6.0 log CFU/g, respectively. Ceca samples collected in September indicated the lowest *Campylobacter* prevalence and contamination level; the detection rate was 73.3% and average population was 2.1 log CFU/g (Bily et al., 2010).

Van Gerwe et al. (2010b) compared *Campylobacter* counts in ceca ($n = 24$) and crops ($n = 23$) of broilers of 31 days of age with a direct plating method using modified charcoal cefoperazone deoxycholate agar plates and detected an average 8.5 and 4.8 log CFU/g in ceca and crop, respectively (Van Gerwe et al., 2010b). When Yusrizal and Chen (2003) investigated the effects of inulin obtained from chicory roots on *Campylobacter* they enumerated *Campylobacter* in various organs including gizzard, small intestine, large intestine, cecal intestine and feces of the broiler chickens. *Campylobacter* populations in GIT from control chickens without inulin treatment revealed contamination levels in lower GIT that were higher than the upper GIT while *Campylobacter* populations in the large intestine (5.3 and 5.4 log CFU/g for male and female, respectively) and cecal intestine (5.3 and 4.4 log CFU/g for male and female, respectively) were relatively higher than the gizzard (2.5 and 2.5 log CFU/g) and small intestine (3.6 and 3.8 log CFU/g) (Yusrizal and Chen, 2003).

Most studies investigating *Campylobacter* prevalence in GIT have included ceca as the primary GIT organ of focus and the

majority of these studies reported that poultry ceca had a greater percentage of *Campylobacter* (at least 73.3% positive) (Musgrove et al., 2001; Wesley et al., 2005; Bily et al., 2010) except for one study that resulted in only 3.8% *Campylobacter* positive birds (Byrd et al., 1998). The prevalence of *Campylobacter* was also generally high in poultry crops (Byrd et al., 1998; Musgrove et al., 2001). According to the study performed by Wesley et al. (2005), both ceca and crops of turkey were predominantly contaminated with *C. coli* rather than *C. jejuni* (Wesley et al., 2005). In terms of *Campylobacter* populations, their counts were generally higher in ceca than crop. In most studies more than 5 log CFU/g *Campylobacter* in ceca occurred and these population levels were generally greater when compared with populations enumerated from crops (Musgrove et al., 2001; Yusrizal and Chen, 2003; Bily et al., 2010; Van Gerwe et al., 2010b). These results suggest that the cecum appears to be a preferred habitat for *Campylobacter* but the proportion of *Campylobacter* versus total GIT microbial populations in each respective GIT compartment would need to be determined to confirm this. Consequently, even if the ceca is a primary target for the GIT, mitigation strategies directed toward other GIT compartments may be important and should be considered for mitigation as well, particularly if the lower GIT *Campylobacter* colonization is influenced by the appearance of *Campylobacter* in the upper GIT.

PREBIOTICS – GENERAL CONCEPTS

Prebiotics were initially defined in 1995 as 'non-digestible food ingredients that have a beneficial effect on the host by selectively stimulating already existing bacterial species's growth and/or activity in the colon, therefore attempt to improve host health' (Gibson and Roberfroid, 1995). Prebiotics can reach the lower intestine and become accessible for GIT indigenous microbiota and subsequent stimulation of growth of specific bacterial groups considered "beneficial" leading to the production of short chain fatty acids and other fermentation products (Al-Sheraji et al., 2013). As defined by Roberfroid (2007) ideal prebiotics should (1) withstand exposure to gastric acid, hydrolysis by mammalian enzymes, and gastrointestinal absorption; (2) be fermented by intestinal microbiota; and (3) selectively stimulate the growth and/or activity of colon bacteria that are helpful to the host. Instead of only focusing on colonic bacteria, Gibson et al. (2004) generalized the definition of prebiotics which 'are selectively fermented ingredient that represent particular changes in the gastrointestinal microflora's composition and/or activity that confers benefits on host wellbeing and health.'

Research efforts to understand and interpret interactions between microbiota of the intestine and prebiotic substrates increased dramatically with the advent of community-wide sequencing, thus becoming essential to achieve consensus regarding the most appropriate definition of a prebiotics among the scientific community (Hutkins et al., 2016). The definition of prebiotics has been expanded to include non-carbohydrate substances and to deliver health benefits to host body sites other than the GIT (Gibson et al., 2017). With increased knowledge of interactions between the GIT microbiota and prebiotics, the classification of prebiotics has shifted to include a wide range of non-digestible oligosaccharides with varying carbon chain length and not being digestible by the host (Ricke, 2018). This reframing of prebiotic sources in turn influences approaches for identifying prebiotic candidates for use in poultry, their respective benefits on poultry health and the potential for limiting *Campylobacter* colonization in the avian GIT.

PREBIOTICS TO IMPROVE GIT HEALTH OF POULTRY

Various prebiotics have been applied to poultry feed with mannan-oligosaccharides, inulin and its hydrolysate (fructooligosaccharides), and xylooligosaccharides being some of the more common prebiotic sources that have been examined for inclusion in poultry diets. Most of the research focus has been on the impact these various prebiotics have on reducing pathogen colonization in poultry. In some cases effort has been made to identify whether certain groups of GIT bacteria considered beneficial to the bird are increased by the presence of prebiotics in the diet. The impact on beneficial GIT bacteria along with the production of specific fermentation products has been suggested as having potential positive impacts on the bird host.

Mannan-oligosaccharides, derived from the yeast cell wall, are comprised of mannose, glucan, proteins, and phosphate radicals that are linked by β-1,4 glycosidic bonds (Klis et al., 2002). The fact that poultry do not have enzymes for metabolizing mannan-oligosaccharides ensures that they reach the GIT without any host derived enzymatic digestion (Pourabedin and Zhao, 2015). Using mannan-oligosaccharides helps to reduce pathogenic bacteria by interference with attachment via the type-1 fimbriae found on many Gram-negative bacteria (e.g., *Escherichia coli* and *Salmonella*) (Ferket, 2004). Those particular bacteria and mannose-based oligosaccharides can bind together through the lectin, therefore mannan-oligosaccharides can reduce attachment of the corresponding pathogen to the gut (Oyofo et al., 1989; Spring et al., 2000; Ferket, 2004). Supplementation of mannan-oligosaccharides in feed diets modulated cecal microbial content of broilers by markedly reducing the number of *Clostridium perfringens,* which is of great significance as a poultry pathogen (Jamroz et al., 2004; Yang et al., 2008), and selecting for the growth of beneficial bacteria for host health such as *Bifidobacteria* spp. and *Lactobacillus* spp. which are generally viewed as GIT bacteria capable of eliciting positive impacts on the host (Baurhoo et al., 2007).

Inulin is a fructan connected by β (2-1) glycosidic bond extracted from chicory (*Cichorium intybus*) (Phelps, 1965; Niness, 1999). Supplementation of inulin in poultry feed is believed to modulate intestinal microbiota resulting in the proliferation of beneficial bacteria including *Lactobacillus* and *Bifidobacteria* while simultaneously inhibiting pathogenic bacteria such as *Escherichia coli*, *C. perfringens*, or *Staphylococcus aureus* (Nabizadeh, 2012; Lopes et al., 2013; Buclaw, 2016). Microbiota populations in the cecal contents of chickens were altered by addition of inulin in feed, with increasing numbers of *Bifidobacteria* and decreasing the populations of

E. coli (Nabizadeh, 2012). Some bifidobacterial species can suppress Gram-positive and Gram-negative pathogens including *Salmonella*, *Campylobacter*, and *E. coli* (Gibson and Wang, 1994; Samanta et al., 2012). The activities of enzymes derived from gut microbiota of rats and humans were affected by the pH level (as the pH level increased, Beta – glucosidase activity was diminished) (Mallett et al., 1989) and the pH level of the cecal contents notably decreased when 1% inulin was added to feed for broiler chickens (Nabizadeh, 2012).

Arabinoxylans are present in cereal fibers and mainly consist of two pentose sugars- arabinose and xylose (James et al., 2003). Hydrolytic degradation of the heteropolymer arabinoxylans generates arabinose-substituted xylooligosaccharides and non-substituted xylooligosaccharides (Broekaert et al., 2011). Xylooligosaccharides are xylopyranoise units which are linked by β-1,4 linkages (Carvalho et al., 2013). Broilers possess only limited enzymatic capabilities to hydrolyze the linkage between xylose thus xylooligosaccharides can arrive relatively intact to the cecum and the lower intestinal tract (Pourabedin and Zhao, 2015). Supplementation of feed with xylooligosaccharides (2 g xylooligosaccharides/kg diet) increased the proportion of *Lactobacillus* in the cecum (Pourabedin et al., 2015). In the ceca of broilers supplemented by xylooligosaccharides in feeds, significantly higher abundance of *Lactobacillus crispatus* and *Anaerostipes butyraticus* was observed with xylooligosaccharides supplementation and higher gene copies of butyryl-CoA:acetate-CoA transferase, which is an enzyme in a butyrate production pathway in the gut, were also detected (Duncan et al., 2004; De Maesschalck et al., 2015). Butyrate is considered beneficial to gastrointestinal function (De Maesschalck et al., 2015) and it can also improve growth performance of animals and modulate the microbiota composition and metabolic activity in the intestine (Guilloteau et al., 2010; Canani et al., 2011).

PREBIOTIC SUPPLEMENTATION TO LIMIT *Campylobacter* IN CONVENTIONALLY RAISED POULTRY

While prebiotics appear to be generally effective to limit establishment of foodborne pathogens in the GIT and improve overall GIT health, less is known about their impact directly on *Campylobacter* levels in the avian GIT. Several studies have examined the influence of prebiotics as feed additives on *Campylobacter* populations in GIT of poultry (Fernandez et al., 2000; Yusrizal and Chen, 2003; Baurhoo et al., 2009; Arsi et al., 2015; Guyard-Nicodeme et al., 2015; Rezaei et al., 2015; Park et al., 2017a). The majority of the *in vivo* studies reported reductions on *Campylobacter* counts or relative abundance in cecal contents and other intestinal sections of the chicken GIT (Fernandez et al., 2000; Yusrizal and Chen, 2003; Baurhoo et al., 2009; Arsi et al., 2015; Guyard-Nicodeme et al., 2015) while a few studies did not observe significant changes in *Campylobacter* populations and relative abundance when prebiotics were supplemented in an attempt to limit *Campylobacter* compared to control birds not fed prebiotics (Rezaei et al., 2015; Park et al., 2017a). **Table 2** lists some of the studies examining prebiotics as feed additives on *Campylobacter* counts in poultry GIT *in vivo*.

Adding 1.0% inulin (Raftifeed®IPF) which was obtained from chicory roots extraction with hot water resulted in significant reduction of *Campylobacter* counts in large intestine contents from 42 day old female broilers; 3.8 log CFU/g of *Campylobacter* by inulin supplement versus 5.4 log CFU/g in control birds (Yusrizal and Chen, 2003). Although there were no significant differences, *Campylobacter* populations in the large intestine contents from male broilers fed inulin supplements were lower than control birds (4.4 log CFU/g versus 5.3 log CFU/g) (Yusrizal and Chen, 2003). Birds given feed containing 1.0% oligofructose (Raftifeed®OPS) (a partial enzymatic hydrolysate of chicory inulin) exhibited significant reductions in *Campylobacter* counts in the large intestinal contents of 42-day old female and male broilers; 3.69 CFU/g (oligofructose) versus 5.3 log CFU/g (control) in male broilers and 4.1 log CFU/g (oligofructose) versus 5.4 log CFU/g (control) in female broilers (Yusrizal and Chen, 2003). Oligofructose also decreased *Campylobacter* counts in the cecal contents of 42-day old male broilers (3.3 log CFU/g vs. 5.3 log CFU/g). However, there were no significant changes in *Campylobacter* populations in the fecal, gizzard, and small intestine contents from birds fed both inulin and oligofructose (Yusrizal and Chen, 2003).

Baurhoo et al. (2009) investigated the effects of diets supplemented with mannan-oligosaccharide or antibiotics on *Campylobacter* colonization of broiler chicken ceca. They tested an antibiotic free diet (control), a diet with commonly used antibiotics (virginiamycin and bacitracin), and a diet containing mannan-oligosaccharide administered at different concentrations (0.2 and 0.5%). They enumerated *Campylobacter* populations in cecal contents from birds at 14, 24, and 34 days of age using *Campylobacter* agar base with lysed horse blood, Preston *Campylobacter* selective supplement, and *Campylobacter* growth supplement (Baurhoo et al., 2009). The addition of mannan-oligosaccharide to the feed resulted in a significant decrease of *Campylobacter* counts in birds fed 0.2% supplemented feed while there were no significant changes at day 34 in birds fed 0.5% supplemented feed when compared to control birds (Baurhoo et al., 2009). This lack of a dosage response is consistent with the conclusion of the authors that there were generally no additional GIT health benefits when mannan-oligosaccharide levels were increased from 0.2 to 0.5%. It is not clear mechanistically why additional mannan-oligosaccharide would not further decrease *Campylobacter* levels. However, increased *Bifidobacteria* were only detected in the ceca of 0.5% supplemented birds versus 0.2% fed birds at day 24 and not day 34, while cecal lactobacilli were not statistically different between 0.2 and 0.5% supplemented birds on either day 24 or 34. The authors noted that mannan-oligosaccharides are believed to competitively inhibit GIT colonization of pathogens such as *Campylobacter* by binding to their mannose specific type 1 fimbriae but it should be noted that they did not see a statistical difference in litter concentrations of *Campylobacter* for any of the days sampled (days 14, 24, and 34) for either 0.2 or 0.5% mannan-oligosaccharides. Further studies involving more intermediate increments of mannan-oligosaccharides will

TABLE 2 | Impact of prebiotics as feed additive on *Campylobacter* counts in poultry gastrointestinal tract.

Prebiotic treatment	Tested GIT	*Campylobacter* test method	Changes in *Campylobacter* counts/relative abundance	Reference
Feed with 0.1% xylanase (Avizyme-1300)	Cecal, sall intestine, and large intestine	Direct plating	*Campylobacter* counts were reduced by xylanase supplemented diet in cecal, small intestine, and large intestine	Fernandez et al., 2000
Feed with 1.0% inulin (Raftifeed®IPF) Feed with 1.0% oligofructose (Raftifeed®OPS)	Fecal, gizzard, small intestine, large intestine, and cecal intestine	Direct plating	*Campylobacter* counts were reduced by inulin or oligofructose supplemented diet in the large and cecal intestine No significant changes in *Campylobacter* counts in fecal microflora, gizzard contents, small intestine compared to control	Yusrizal and Chen, 2003
Feed with 0.2% mannan-oligosaccharides Feed with 0.5% mannan-oligosaccharides	Cecal intestine	Direct plating	*Campylobacter* counts were reduced by 0.2% mannan-oligosaccharide supplemented diet in cecal intestine No significant reductions in 0.5% mannan-oligosaccharide	Baurhoo et al. (2009)
Feed with 0.2% Biolex®MB40 Feed with 0.2% Leiber®ExCel	Cecal intestine	Direct plating	*Campylobacter* counts were reduced by both prebiotics supplemented diet in cecal intestine	Park et al., 2014
Probiotics (isolate 1, 2, or 3) + prebiotics (0.125, 0.25, or 0.5% fructooligosaccharide or 0.04, 0.08, or 0.16% manna-oligosaccharides)	Cecal intestine	Direct plating	*Campylobacter* counts were not reduced by fructooligosaccharide or manna-oligosaccharide only *Campylobacter* counts were reduced by combination of probiotics and prebiotics in cecal intestine	Arsi et al., 2015
Feed with 0.125% prebiotic-like product (Original XPC™)	Cecal intestine	Direct plating	*Campylobacter* counts were reduced by prebiotic-like product supplemented diet in cecal intestine	Guyard-Nicodeme et al., 2015
Feed supplemented with 0.5 and 1% oligosaccharides extract from palm kernel expeller (OligoPKE)	Cecal intestine	Quantitative real time PCR	No significant changes in *Campylobacter* counts in cecal contents by oligosaccharides compared to control	Rezaei et al., 2015
Feed with 0.2% β-glucan and mannan-oligosaccharides (Biolex®MB40)	Cecal intestine	Next generation sequencing with Illumina MiSeq	No significant changes in the relative abundance of *Campylobacter* compared in cecal contents by β-glucan and mannan-oligosaccharides compared to control Significant lower *Campylobacter* abundance in prebiotic treated group compared to antibiotic treated group	Park et al., 2017a
Feed with 0.1% plum fibers Feed with 0.1% fructooligosaccharides Feed with 0.2% galactooligosaccharides	Cecal intestine	Direct plating	No significant changes in *Campylobacter* counts in cecal contents by prebiotics compared to control	Park et al., 2017b

be needed to delineate whether dosage responses in birds are possible with this prebiotic.

There have also been studies where no impact on *Campylobacter* occurred in chicken cecal contents when prebiotics or prebiotic-like compounds were included (Rezaei et al., 2015; Park et al., 2017a,b). Rezaei et al. (2015) tested three dietary treatments including basal diet as control and basal diet supplemented with 0.5 and 1% oligosaccharides (extract from palm kernel expeller, OligoPKE) and enumerated *Campylobacter* counts in cecal contents at day 21 and 35 broiler chickens with quantitative real time PCR; resulting in no significant changes in *Campylobacter* enumerated populations following prebiotic addition.

Park et al. (2017a) applied next generation sequencing technology based on an Illumina MiSeq platform to investigate microbiological compositions in the cecal contents of 1, 2, 4, and 6 weeks old chickens fed either a basal diet, a diet with antibiotic (consisting bacitracin methylene disalicylate and bacitracin, BMD50), or a diet with a yeast-based prebiotic (consisting 1,3-1,6-β-D-glucan and mannan-oligosaccharides derived from the cell walls of *Saccharomyces cerevisiae*, Biolex®MB40; Leiber GmbH, Hafenstrase, Germany). When compared to the control group fed the basal diet, the treatment group fed with prebiotic exhibited similar *Campylobacter* levels. However, a significantly lower *Campylobacter* abundance was observed compared to treatment group fed with antibiotics at 4 weeks of age. *Lactobacillus* abundance was significantly lower in the antibiotic treated group compared to the control and prebiotic fed groups. Successfully predicting the potential for success or failure of a particular prebiotic to mitigate foodborne pathogens such as *Campylobacter* will require further in-depth delineation of the mechanisms associated with prebiotics and the GIT microbiota. This will no doubt require not only next generation sequencing to identify specific taxa but the metabolic activities of the resident GIT microbial populations in the presence of particular prebiotics and

how changes may impact *Campylobacter* either directly or indirectly.

PREBIOTIC SUPPLEMENTATION TO LIMIT *Campylobacter* IN NON-CONVENTIONALLY RAISED POULTRY

Most of the research on prebiotics and poultry have been conducted with birds raised under conventional commercial housing conditions or at least experimental environments that attempted to simulate these types of conditions (Park et al., 2013; Ricke, 2015). Less work has been done with poultry raised under free range or pasture flock settings. However, the choices in feed additives are considered more restrictive for birds raised under these antibiotic – free conditions even though there is probably more of a need for feed additives to reduce exposure of these birds to a wide range of pathogens (Park et al., 2013). As this industry expands there is a clear need for development of feed additives that are not only acceptable but amendable to routine supplementation. Prebiotics, depending on their source and how they are generated, could certainly be a possibility.

Park et al. (2014) tested the effects of two commercial prebiotics, Biolex® MB40 and Lieber® ExCel derived from brewer's yeast cell walls, when added to feed provided to pasture raised naked neck broilers. *Campylobacter* counts recovered from the cecal contents were assessed using Campy-Cefex plates. The populations of *Campylobacter* in the control group were 6.49 log CFU/100 mg and both treatment groups exhibited significantly lower counts (6.07 log CFU/100 mg for both groups) than control birds (Park et al., 2014). While statistically significant it remains unclear whether these biologically slight differences in cecal *Campylobacter* would impact *Campylobacter* levels of the processed birds or be relevant for incidence of human campylobacterosis. Given the relationship between the GIT microbiota and *Campylobacter*, denatured gradient gel electrophoresis (DGGE) was also employed in this study to detect changes in the cecal microbiota in birds fed the different treatments. Banding patterns of DGGE recovered from the birds were generally similar among all treatments but certain specific bands were specifically identified with a particular group and banding intensities also differed in groups shared among the treatment groups. Isolation and sequencing of specific bands of interest using an ABI 3100 capillary analyzing system (Applied Biosystems) revealed *Bacteroides salanitronis* occurring in all groups and *Barnesiella viscericola* and *Firmicutes* identified from bands associated with both treatments. Interestingly based on band sequencing three *Campylobacter* species were identified, namely, *C. jejuni*, *C. coli*, and *C. lari*.

Clearly, as Park et al. (2014) noted, DGGE-based methods suffer from several limitations that handicap comprehensive interpretation. More in-depth assessment of the pasture flock cecal microbiome response has since become possible with the introduction of next generation sequencing of 16S rRNA genes. Park et al. (2017b) evaluated the effects of prebiotics when they were supplemented to feed such as 0.1% plum fibers, 0.1% fructooligosaccharides, and 0.2% galactooligosaccharides to broiler chickens. *Campylobacter* levels in cecal contents were enumerated with a plating method using Campy-Line agar and the cecal microbiome was also evaluated with next generation sequencing using an Illumina HiSeq platform. With the direct plating method, there were no significance differences among all groups (control and three treatment groups) (Park et al., 2017b). Using synthetic learning in microbial ecology (SLiME) analysis, they found that feed supplements modulated the diversity of microbiota and operational taxonomic units identified as belonging to the genus *Alistipes* and *Lactobacillus intestinalis* were indicated as a potential predictive feature for *Campylobacter* populations (>10% and >25% increase mean squared error, respectively) suggesting a potential metabolic interaction (Park et al., 2017b). However, as Park et al. (2017b) point out, age of the bird was also highly related to the pattern of appearance for both *Campylobacter* and these two non-*Campylobacter* bacteria and consequently some of this apparent relatedness could be coincidental. Some of this may become more clear as the resolution of bioinformatic tools continues to improve to the point that specific factors can be delineated as to their respective contributions.

PREBIOTICS COMBINED WITH OTHER FEED ADDITIVES

While there has been extensive research focused on prebiotics administered as stand alone feed amendments, efforts have been undertaken to evaluate them in the presence of other GIT modulators. An obvious combination would be to combine them with specific probiotic cultures of bacteria that could use the corresponding prebiotic as a substrate. The resulting synbiotics are the combination of prebiotics and probiotics that in theory would select and help to maintain probiotic sustainability in the GIT (Collins and Gibson, 1999). At the beginning of the 20th century Elie Metchnikoff, commonly considered a pioneer of modern probiotics, initially observed enhancement of health and longevity in humans upon the regular consumption of lactic acid bacteria and consequently hypothesized that lactic acid bacteria could replace or diminish the number of putrefactive bacteria in the gut (Anukam and Reid, 2007). The concept of probiotics was introduced in 1965 as 'substances secreted by single microorganism which stimulates others growth' (Lilly and Stillwell, 1965). The term 'probiotics' gained general acceptance and its definition was further refined; Fuller (1989) defined probiotics as follows: 'a live microbioal feed supplement beneficially affects the host by improving its intestinal microbiota' (Fuller, 1989). *Lactobacilli* spp., *Bifidobacteria* spp., and Gram-positive cocci including *Enterococcus faecium*, *Streptococci diaacetylactis* are common examples of probiotics (Collins and Gibson, 1999). Ideal probiotics should (1) not have toxic substances or be pathogens; (2) resist gastric acid and bile from the liver thus survive in the intestinal tract; (3) attach themselves to epithelial tissue; (4) produce inhibitory compounds against pathogens;

(5) stimulate the immune system; and (6) alter microbial activities (Fuller, 1989; Gibson and Fuller, 2000; Rolfe, 2000; Simmering and Blaut, 2001; Patterson and Burkholder, 2003). However, the effect of using probiotics could be transient because of the difficulty to achieve continuous adherence and/or retention in the GIT (Goldin and Gorbach, 1984; Jonsson, 1986). Given the considerable complexity and large numbers of indigenous bacteria already predominating the GIT prior to application of probiotics, it is not surprising that continuous feeding may be necessary to prolong their efficacy (Fuller, 1989). Likewise, the extensive shifting in the composition and subsequent diversity of the cecal microbiota as birds age could play a key role in the respective efficacy of different prebiotics and pathogen colonization (Park et al., 2017c; Ricke, 2018).

The impact of the combination of probiotics and prebiotics (synbiotics) on *Campylobacter* counts was investigated by Arsi et al. (2015). Feed additives included strains isolated from healthy chickens (isolate 1: *Bacillus* spp., isolate 2: *Lactobacillus salivarius* subsp. *salivarius*, and isolate 3: *Lactobacillus salivarius* subsp. *salicinius*) and/or a prebiotic with different concentrations (0.125, 0.25, or 0.5% fructooligosaccharide or 0.04, 0.08, or 0.16% mannan-oligosaccharide) (Arsi et al., 2015). *Campylobacter* populations were enumerated with direct plating on Campy-Line agar. Individual treatments of either prebiotic or probiotic did not result in detectable reductions of *Campylobacter* population in cecal contents. However, their combination resulted in a considerable level of reduction; isolate 3 + 0.04% mannan-oligosaccharide in feed exhibited a 3.3 log reduction compared to control (Arsi et al., 2015). The authors presumed that this may be attributed to the promotion of the growth of probiotics by mannan-oligosaccharide thus resulting in the concomitant reduction of *Campylobacter* in the cecal contents.

Non-probiotic feed additives have also been combined with prebiotics in poultry studies. For example, Guyard-Nicodeme et al. (2015) evaluated efficacy of various commercially available feed additives containing organic acid, short chain fatty acids, monoglycerides, plant extracts, prebiotic-like compounds, and probiotics (Lacto-butyrin, Biotronic®Top3 Campylostat, Admix®Precision, Excential Butycoat, Power Protexion®, Excential Alliin Plus, Anta®Phyt, Calsporin®, Ecobiol®, PoultryStar®, and Original XPC™). Chicks were fed either a control diet or diets supplemented with feed additives (total of 13 groups) and *Campylobacter* populations were enumerated in ceca of broilers at 14, 35, and 42 days of age by direct plating on modified Charcoal cefoperazone deoxycholate agar (Guyard-Nicodeme et al., 2015). Prebiotic-like compounds (Original XPC™) consisting of post-fermentation growth medium residues, residual yeast cells, and yeast cell wall fragments (mannan-oligosaccharides and β-glucans) yielded no significant reductions of *Campylobacter* at 14 and 35 days of age compared to control while a 3.2 log reduction were obtained in birds at 42 days of age. This level of reduction was greatest among the 12 tested feed additive with 2.1 and 1.7 log reductions being observed in the presence of the Admix®Precision and Calsporin®treatments, respectively, while the other 9 feed additives exhibited negligible bactericidal effects in birds at 42 days of age (Guyard-Nicodeme et al., 2015). Before comprehensive overall conclusions can be drawn from such results further studies will need to be conducted to more quantitatively separate individual feed amendment treatments impacts on bird GIT health, performance and pathogen levels and link these to mechanism(s) associated with specific additives. This will require a number of independent trials to be conducted over a wide range of management practices along with in-depth profiling of GIT responses using molecular methods to characterize the GIT microbial populations and detailed GIT lumen metabolite and host GIT tissue responses.

Given that some prebiotic sources are not only indigestible but can occur as complex polymers, combining them with active feed grade enzymes has merit for increasing efficacy (Ricke, 2018). Consequently, combining enzymes with prebiotics has been suggested as a means for enhancing the endogenous enzyme production in birds, aid digestion of fiber components, render nutrients for easier digestion, reduce the effects of antinutritional factors, and raise efficacy in feed formulation (Ferket, 1993). Limited studies have been conducted to examine the impact of such combinations and *Campylobacter* occurrence. Fernandez et al. (2000) assessed the *C. jejuni* population levels in the small intestine, cecum, and large intestines of broiler chickens fed a wheat diet supplemented with a xylanase (control: wheat and maize-based feeds). They used a marker strain of *C. jejuni* with resistance to nalidixic acid and *Campylobacter* populations in the intestine were determined using a direct plating method on *Campylobacter* blood-free selective agar with ceforperazone selective supplement and nalidixic acid. Minimal differences in *C. jejuni* were obtained in the small and large intestines of chicken fed the 0.1% xylanase-supplemented diet (1.4 and 2.3 log CFU, respectively) compared to controls (wheat-based: 1.7 and 2.4 log CFU; maize-based: 1.5 and 2.4 log CFU, respectively). However, there were statistically less *C. jejuni* in the ceca of birds fed the wheat-xylanase combination (2.3 log CFU, respectively) compared to birds fed wheat and maize-based diets (wheat-based: 2.8, log CFU; maize-based: 2.6 log CFU, respectively) without xylanase which corresponded to a significant decrease in jejunum viscosity in the enzyme supplemented birds (Fernandez et al., 2000). How, biological relevant these relatively small reductions would be to achieve consistent overall *Campylobacter* reduction in commercial operations remains to be determined.

FUTURE DIRECTIONS FOR PREBIOTIC APPLICATION TO *Campylobacter* CONTROL IN CHICKENS

While it appears that the majority of the poultry studies conducted thus far on *Campylobacter* colonization and response to prebiotics indicate some level of reduction, the results remain variable. Some of this may be related to differences in methodologies used to detect and quantitate *Campylobacter* recovered from the poultry GIT. Typically, *Campylobacter* counts are enumerated by direct plating on selective medium from collected intestinal contents and in some cases culture independent methods such as quantitative PCR and next

generation sequencing technology are also employed (**Table 2**). However, culture-based methods may not always accurately reflect actual *Campylobacter* populations. For example, Kim et al. (2017) sequenced pooled colonies recovered from Campy-Cefex selective media which had been the selective media recommended by USDA at the time for *Campylobacter* isolation from chicken carcass rinsates using an Illumina MiSeq flatform. Based on the 16S rDNA microbiome sequencing the *Campylobacter* selective media apparently supported growth of a mixed background bacterial population, some of which were identified as *Campylobacter* but others were also be recovered such as *Clostridiaceae*, *Paenibacillus*, *Lactobacillus*, *Bacillaceae*, *Acinetobacter*, *Enterobacteriaceae*, *Bacillus*, *Planococcaceae*, *Clostridium*, *Enterococcus*, and *Sporanaerobacter* (Kim et al., 2017). Intuitively it would be anticipated that choice of selective media could be influenced and potentially biased for recovering *Campylobacter* from the highly diverse poultry GIT. The growth of these non-*Campylobacter* background bacteria could cause lower accuracy of the media for enumerating *Campylobacter* and in turn, assessing the impact of feed additives such as prebiotics. Therefore, other culture independent methods such as qPCR could be an effective alternative method to culture based methods with plating on selective media to overcome some of the issues associated with culture-based methods.

Moreover, technical progress in the advent of high-throughput sequencing technology and the corresponding bioinformatic tools will lead to obtaining comprehensive information of the microecology of a variety of samples including the avian GIT (Land et al., 2015; De Filippis et al., 2018). Since prebiotics are well known to have an impact on the gut microbiota and pathogenic bacteria leading to detectable shifts in gut microbial populations, a series of research approaches involving high-throughput sequencing technologies and bioinformatics to evaluate changes on microbiological compositions in the GIT by prebiotics could be invaluable for achieving a better understanding of the mechanism(s) associated with prebiotics on limiting pathogens including *Campylobacter* (Cummings and Macfarlane, 2002; Edens, 2003; Yang et al., 2009; Hajati and Rezaei, 2010).

CONCLUSION

In the present review, *Campylobacter* in the GIT was briefly discussed, followed by prebiotics and their general effects on the intestine and finally recent research studies aimed at providing empirical data on prebiotic efficacy to reduce *Campylobacter* counts/abundance in poultry intestine. Based on the limited research discussed here, addition of prebiotics to the poultry feed generally appeared to result in detectable decreases in *Campylobacter* populations in the different regions of poultry GIT; indicating that the potential ability of prebiotics as feed supplements to limit *Campylobacter* in the poultry GIT does exist. Whether these colonization reductions in the chicken GIT specifically associated with feeding prebiotics are sufficient to result in reduction of human campylobacterosis cases remains to be determined. Only limited research data is available on *Campylobacter* reduction in poultry by prebiotics in large scale commercial trials currently. In conjunction with devising more focused empirical research strategies on the efficacy of prebiotics, examination under typical poultry husbandry conditions is also needed to develop practical recommendations for their use as feed supplements. Along these lines, other important factors such as bird performance and organoleptic meat quality should also be considered along with *Campylobacter* reduction.

Mitigating *Campylobacter* in poultry will continue to be an important focus for the poultry industry. Opportunities exist for accomplishing some control at the pre-harvest poultry production level. However, this clearly requires gaining a better understanding of the interaction between the poultry GIT microbial community and the resident *Campylobacter* species that co-exist. Prebiotics could be a promising strategy to control *Campylobacter* in poultry but definitive recommendations will require in-depth characterization of the poultry GIT microecology and the role the host plays in influencing that ecology. This will require more of an "omics" approach that encompasses not only microbiota sequencing but metabololic profiling as well host GIT tissue metabolic enzyme and immune responses. In addition, understanding *Campylobacter* behavior in the avian GIT tract at the molecular level may be critical as well given its close association with indigenous GIT microbiota. Combining the information generated from the various approaches should help to optimize prebiotic choice and application for practical mitigation of *Campylobacter* in the avian GIT.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The efforts associated with writing this review were supported by the Ewha Womans University Research Grant of 2018.

REFERENCES

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., and Hassan, F. A. (2013). Prebiotics as functional foods: a review. *J. Funct. Foods* 5, 1542–1553. doi: 10.1108/00346650710749062

Anukam, K. C., and Reid, G. (2007). "Probiotics: 100 years (1907-2007) after elie metchnikoff's observation," in *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, Vol. 1, ed. A. Méndez-Vilas (Badajoz: Formatex), 466–474.

Arsi, K., Donoghue, A., Woo-Ming, A., Blore, P., and Donoghue, D. (2015). The efficacy of selected probiotic and prebiotic combinations in reducing *Campylobacter* colonization in broiler chickens. *J. Appl. Poult. Res.* 24, 327–334. doi: 10.3382/japr/pfv032

Baurhoo, B., Ferket, P., and Zhao, X. (2009). Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. *Poult. Sci.* 88, 2262–2272. doi: 10.3382/ps.2008-00562

Baurhoo, B., Phillip, L., and Ruiz-Feria, C. A. (2007). Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poult. Sci.* 86, 1070–1078. doi: 10.1093/ps/86.6.1070

Beery, J., Hugdahl, M., and Doyle, M. (1988). Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54, 2365–2370.

Bily, L., Petton, J., Lalande, F., Rouxel, S., Denis, M., Chemaly, M., et al. (2010). Quantitative and qualitative evaluation of *Campylobacter* spp. contamination of turkey cecal contents and carcasses during and following the slaughtering process. *J. Food Prot.* 73, 1212–1218. doi: 10.4315/0362-028X-73.7.1212

Boyd, Y., Herbert, E. G., Marston, K. L., Jones, M. A., and Barrow, P. A. (2005). Host genes affect intestinal colonisation of newly hatched chickens by *Campylobacter jejuni*. *Immunogenetics* 57, 248–253. doi: 10.1007/s00251-005-0790-6

Broekaert, W. F., Courtin, C. M., Verbeke, K., Van de Wiele, T., Verstraete, W., and Delcour, J. A. (2011). Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. *Crit. Rev. Food Sci. Nutr.* 51, 178–194. doi: 10.1080/10408390903044768

Buckley, A. M., Wang, J., Hudson, D. L., Grant, A. J., Jones, M. A., Maskell, D. J., et al. (2010). Evaluation of live-attenuated *Salmonella* vaccines expressing *Campylobacter* antigens for control of *C. jejuni* in poultry. *Vaccine* 28, 1094–1105. doi: 10.1016/j.vaccine.2009.10.018

Buclaw, M. (2016). The use of inulin in poultry feeding: a review. *J. Anim. Physiol. Anim. Nutr.* 100, 1015–1022. doi: 10.1111/jpn.12484

Byrd, J., Corrier, D., Hume, M., Bailey, R., Stanker, L., and Hargis, B. (1998). Incidence of *Campylobacter* in crops of preharvest market-age broiler chickens. *Poult. Sci.* 77, 1303–1305. doi: 10.1093/ps/77.9.1303

Callaway, T. R., and Ricke, S. C. (2011). *Direct-fed Microbials and Prebiotics for Animals: Science and Mechanisms of Action*. New York, NY: Springer Science.

Canani, R. B., Di Costanzo, M., Leone, L., Pedata, M., Meli, R., and Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* 17:1519. doi: 10.3748/wjg.v17.i12

Carrillo, C. L., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl. Environ. Microbiol.* 71, 6554–6563. doi: 10.1128/AEM.71.11.6554-6563.2005

Carvalho, A. F. A., de Oliva Neto, P., Da Silva, D. F., and Pastore, G. M. (2013). Xylo-oligosaccharides from lignocellulosic materials: chemical structure, health benefits and production by chemical and enzymatic hydrolysis. *Food Res. Int.* 51, 75–85. doi: 10.1016/j.foodres.2012.11.021

Cole, K., Farnell, M., Donoghue, A., Stern, N., Svetoch, E., Eruslanov, B., et al. (2006). Bacteriocins reduce *Campylobacter* colonization and alter gut morphology in turkey poults. *Poult. Sci.* 85, 1570–1575. doi: 10.1093/ps/85.9.1570

Collins, M. D., and Gibson, G. R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* 69, 1052S–1057S. doi: 10.1093/ajcn/69.5.1052s

Cummings, J., and Macfarlane, G. (2002). Gastrointestinal effects of prebiotics. *Br. J. Nutr.* 87, S145–S151. doi: 10.1079/BJN/2002530

De Filippis, F., Parente, E., and Ercolini, D. (2018). Recent past, present, and future of the food microbiome. *Annu. Rev. Food Sci. Technol.* 9, 589–608. doi: 10.1146/annurev-food-030117-012312

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., et al. (2015). Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. *Appl. Environ. Microbiol.* 81, 5880–5888. doi: 10.1128/AEM.01616-15

de Zoete, M. R., van Putten, J. P., and Wagenaar, J. A. (2007). Vaccination of chickens against *Campylobacter*. *Vaccine* 25, 5548–5557. doi: 10.1016/j.vaccine.2006.12.002

Dibner, J. J., and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult. Sci.* 84, 634–643. doi: 10.1093/ps/84.4.634

Duncan, S. H., Louis, P., and Flint, H. J. (2004). Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl. Environ. Microbiol.* 70, 5810–5817. doi: 10.1128/AEM.70.10.5810-5817.2004

Edens, F. (2003). An alternative for antibiotic use in poultry: probiotics. *Rev. Bras. Cienc. Avic.* 5, 75–97. doi: 10.1590/S1516-635X2003000200001

Ferket, P. R. (1993). Practical use of feed enzymes for turkeys and broilers. *J. Appl. Poult. Res.* 2, 75–81. doi: 10.1093/japr/2.1.75

Ferket, P. R. (2004). "Alternatives to antibiotics in poultry production: responses, practical experience and recommendations," in *Proceedings of the Alltech's 20th Annual Symposium on Nursing Faculty Practice. Nutritional Biotechnology in the Feed and Food Industries*, Lexington, KY.

Fernandez, F., Sharma, R., Hinton, M., and Bedford, M. (2000). Diet influences the colonisation of *Campylobacter jejuni* and distribution of mucin carbohydrates in the chick intestinal tract. *Cell. Mol. Life Sci.* 57, 1793–1801. doi: 10.1007/PL00000659

Flickinger, E. A., Loo, J. V., and Fahey, G. C. (2003). Nutritional responses to the presence of inulin and oligofructose in the diets of domesticated animals: a review. *Crit. Rev. Food Sci. Nutr.* 43, 19–60. doi: 10.1080/10408690390826446

Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365–378. doi: 10.1111/j.1365-2672.1989.tb05105.x

Gibson, G., and Wang, X. (1994). Bifidogenic properties of different types of fructo-oligosaccharides. *Food Microbiol.* 11, 491–498. doi: 10.1006/fmic.1994.1055

Gibson, G. R., and Fuller, R. (2000). Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. *J. Nutr.* 130, 391S–395S. doi: 10.1093/jn/130.2.391S

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14:491. doi: 10.1038/nrgastro.2017.75

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., and Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 17, 259–275. doi: 10.1079/NRR200479

Gibson, G. R., and Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125, 1401–1412. doi: 10.1093/jn/125.6.1401

Goldin, B. R., and Gorbach, S. L. (1984). The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *Am. J. Clin. Nutr.* 39, 756–761. doi: 10.1093/ajcn/39.5.756

Griggs, J., and Jacob, J. (2005). Alternatives to antibiotics for organic poultry production. *J. Appl. Poult. Res.* 14, 750–756. doi: 10.1093/japr/14.4.750

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., and Van Immerseel, F. (2010). From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr. Res. Rev.* 23, 366–384. doi: 10.1017/S0954422410000247

Guyard-Nicodeme, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., et al. (2015). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult. Sci.* 95, 298–305. doi: 10.3382/ps/pev303

Hajati, H., and Rezaei, M. (2010). The application of prebiotics in poultry production. *Int. J. Poult. Sci.* 9, 298–304. doi: 10.3923/ijps.2010.298.304

Hermans, D., Martel, A., Van Deun, K., Van Immerseel, F., Heyndrickx, M., Haesebrouck, F., et al. (2011a). The cinnamon-oil ingredient trans-cinnamaldehyde fails to target *Campylobacter jejuni* strain KC 40 in the broiler chicken cecum despite marked in vitro activity. *J. Food Prot.* 74, 1729–1734. doi: 10.4315/0362-028X.JFP-10-487

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., et al. (2011b). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol.* 152, 219–228. doi: 10.1016/j.vetmic.2011.03.010

Hutkins, R. W., Krumbeck, J. A., Bindels, L. B., Cani, P. D., Fahey, G. Jr., Goh, Y. J., et al. (2016). Prebiotics: why definitions matter. *Curr. Opin. Biotechnol.* 37, 1–7. doi: 10.1016/j.copbio.2015.09.001

Indikova, I., Humphrey, T. J., and Hilbert, F. (2015). Survival with a helping hand: *Campylobacter* and microbiota. *Front. Microbiol.* 6:1266. doi: 10.3389/fmicb.2015.01266

James, S., Muir, J., Curtis, S., and Gibson, P. (2003). Dietary fibre: a roughage guide. *Intern. Med. J.* 33, 291–296. doi: 10.1046/j.1445-5994.2003.00404.x

Jamroz, D., Wiliczkiewicz, A., Orda, J., Wertelecki, T., and Skorupinska, J. (2004). Response of broiler chickens to the diets supplemented with feeding antibiotic or mannanoligosaccharides. *Electron. J. Polish Agri. Uni.* 7, 1–6.

Jeffrey, J., Tonooka, K., and Lozanot, J. (2001). Prevalence of *Campylobacter* spp. from skin, crop, and intestine of commercial broiler chicken carcasses at processing. *Poult. Sci.* 80, 1390–1392. doi: 10.1093/ps/80.9.1390

Jones, F. T., and Ricke, S. C. (2003). Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poult. Sci.* 82, 613–617. doi: 10.1093/ps/82.4.613

Jonsson, E. (1986). Persistence of *Lactobacillus* strain in the gut of sucking piglets and its influence on performance and health. *Swed. J. Agric. Res.* 16, 43–47.

Kim, S. A., Park, S. H., Lee, S. I., Owens, C. M., and Ricke, S. C. (2017). Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Sci. Rep.* 7:43354. doi: 10.1038/srep43354

Klis, F. M., Mol, P., Hellingwerf, K., and Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 26, 239–256. doi: 10.1111/j.1574-6976.2002.tb00613.x

Kudirkienė, E., Bunevičienė, J., Šernienė, L., Ramonaitė, S., Olsen, J. E., and Malakauskas, M. (2013). Importance of the producer on retail broiler meat product contamination with *Campylobacter* spp. *J. Sci. Food Agric.* 93, 2293–2298. doi: 10.1002/jsfa.6042

Land, M., Hauser, L., Jun, S.-R., Nookaew, I., Leuze, M. R., Ahn, T.-H., et al. (2015). Insights from 20 years of bacterial genome sequencing. *Funct. Integr. Genomics* 15, 141–161. doi: 10.1007/s10142-015-0433-4

Layton, S., Morgan, M., Cole, K., Kwon, Y., Donoghue, D., Hargis, B., et al. (2011). Evaluation of *Salmonella*-vectored *Campylobacter* peptide epitopes for reduction of *Campylobacter jejuni* in broiler chickens. *Clin. Vaccine Immunol.* 18, 449–454. doi: 10.1128/CVI.00379-10

Li, Y.-P., Ingmer, H., Madsen, M., and Bang, D. D. (2008). Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes. *BMC Microbiol.* 8:107. doi: 10.1186/1471-2180-8-107

Lilly, D. M., and Stillwell, R. H. (1965). Probiotics: growth-promoting factors produced by microorganisms. *Science* 147, 747–748. doi: 10.1126/science.147.3659.747

Lin, J. (2009). Novel approaches for *Campylobacter* control in poultry. *Foodborne Pathog. Dis.* 6, 755–765. doi: 10.1089/fpd.2008.0247

Lopes, M., Roll, V., Leite, F., Dai Prá, M., Xavier, E., Heres, T., et al. (2013). Quicklime treatment and stirring of different poultry litter substrates for reducing pathogenic bacteria counts. *Poult. Sci.* 92, 638–644. doi: 10.3382/ps.2012-02700

Mallett, A., Bearne, C., and Rowland, I. (1989). The influence of incubation pH on the activity of rat and human gut flora enzymes. *J. Appl. Bacteriol.* 66, 433–437. doi: 10.1111/j.1365-2672.1989.tb05112.x

Mead, G. (2000). Prospects for 'competitive exclusion' treatment to control *Salmonella*s and other foodborne pathogens in poultry. *Vet. J.* 159, 111–123. doi: 10.1053/tvjl.1999.0423

Micciche, A. C., Foley, S. L., Pavlidis, H. O., McIntyre, D. R., and Ricke, S. C. (2018). A review of prebiotics against *Salmonella* in poultry: current and future potential for microbiome research applications. *Front. Vet. Sci.* 5:191. doi: 10.3389/fvets.2018.00191

Musgrove, M., Berrang, M., Byrd, J., Stern, N., and Cox, N. (2001). Detection of *Campylobacter* spp. in ceca and crops with and without enrichment. *Poult. Sci.* 80, 825–828. doi: 10.1093/ps/80.6.825

Nabizadeh, A. (2012). The effect of inulin on broiler chicken intestinal microflora, gut morphology, and performance. *J. Anim. Feed Sci.* 21, 725–734. doi: 10.22358/jafs/66144/2012

Newell, D., and Fearnley, C. (2003). Sources of *Campylobacter* colonization in broiler chickens. *Appl. Environ. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Newell, D., and Wagenaar, J. (2000). "Poultry infections and their control at the farm level," in *Campylobacter*, 2nd Edn, eds I. Nachamkin and M. Blaser (Washington, DC: American Society for Microbiology), 497–509.

Niness, K. R. (1999). Inulin and oligofructose: what are they? *J. Nutr.* 129, 1402S–1406S. doi: 10.1093/jn/129.7.1402S

Oyofo, B. A., DeLoach, J. R., Corrier, D. E., Norman, J. O., Ziprin, R. L., and Mollenhauer, H. H. (1989). Effect of carbohydrates on *Salmonella typhimurium* colonization in broiler chickens. *Avian Dis.* 33, 531–534. doi: 10.2307/1591117

Park, S., Gibson, K., Almeida, G., and Ricke, S. C. (2014). Assessment of gastrointestinal microflora in pasture raised chickens fed two commercial prebiotics. *J. Prob. Health* 2:122. doi: 10.4172/2329-8901.1000122

Park, S., Hanning, I., Perrota, A., Bench, B., Alm, E., and Ricke, S. C. (2013). Modifying the gastrointestinal ecology in alternatively raised poultry and the potential for molecular and metabolomic assessment. *Poult. Sci.* 92, 546–561. doi: 10.3382/ps.2012-02734

Park, S. H., Kim, S. A., Lee, S. I., Rubinelli, P. M., Roto, S. M., Pavlidis, H. O., et al. (2017a). Original XPCTM effect on *Salmonella typhimurium* and cecal microbiota from three different ages of broiler chickens when incubated in an anaerobic in vitro culture system. *Front. Microbiol.* 8:1070. doi: 10.3389/fmicb.2017.01070

Park, S. H., Lee, S. I., Kim, S. A., Christensen, K., and Ricke, S. C. (2017b). Comparison of antibiotic supplementation versus a yeast-based prebiotic on the cecal microbiome of commercial broilers. *PLoS One* 12:e0182805. doi: 10.1371/journal.pone.0182805

Park, S. H., Perrotta, A., Hanning, I., Diaz-Sanchez, S., Pendleton, S., Alm, E., et al. (2017c). Pasture flock chicken cecal microbiome responses to prebiotics and plum fiber feed amendments. *Poult. Sci.* 96, 1820–1830. doi: 10.3382/ps/pew441

Patterson, J., and Burkholder, K. (2003). Application of prebiotics and probiotics in poultry production. *Poult. Sci.* 82, 627–631. doi: 10.1093/ps/82.4.627

Phelps, C. F. (1965). The physical properties of inulin solutions. *Biochem. J.* 95, 41–47. doi: 10.1042/bj0950041

Pourabedin, M., Guan, L., and Zhao, X. (2015). Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. *Microbiome* 3:15. doi: 10.1186/s40168-015-0079-4

Pourabedin, M., and Zhao, X. (2015). Prebiotics and gut microbiota in chickens. *FEMS Microbiol. Lett.* 362:fnv122. doi: 10.1093/femsle/fnv122

Rezaei, S., Faseleh Jahromi, M., Liang, J. B., Zulkifli, I., Farjam, A. S., Laudadio, V., et al. (2015). Effect of oligosaccharides extract from palm kernel expeller on growth performance, gut microbiota and immune response in broiler chickens. *Poult. Sci.* 94, 2414–2420. doi: 10.3382/ps/pev216

Ricke, S. C. (2015). Potential of fructooligosaccharide prebiotics in alternative and nonconventional poultry production systems. *Poult. Sci.* 94, 1411–1418. doi: 10.3382/ps/pev049

Ricke, S. C. (2018). Impact of prebiotics on poultry production and food safety. *Yale J. Biol. Med.* 91, 151–159.

Roberfroid, M. (2007). Prebiotics: the concept revisited. *J. Nutr.* 137, 830S–837S. doi: 10.1093/jn/137.3.830S

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 130, 396S–402S. doi: 10.1093/jn/130.2.396S

Rosenquist, H., Boysen, L., Krogh, A. L., Jensen, A. N., and Nauta, M. (2013). *Campylobacter* contamination and the relative risk of illness from organic broiler meat in comparison with conventional broiler meat. *Int. J. Food Microbiol.* 162, 226–230. doi: 10.1016/j.ijfoodmicro.2013.01.022

Roto, S. M., Rubinelli, P. M., and Ricke, S. C. (2015). An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. *Front. Vet. Sci.* 2:28. doi: 10.3389/fvets.2015.00028

Sahin, O., Morishita, T. Y., and Zhang, Q. (2002). *Campylobacter* colonization in poultry: sources of infection and modes of transmission. *Anim. Health Res. Rev.* 3, 95–105. doi: 10.1079/AHRR200244

Samanta, A., Senani, S., Kolte, A. P., Sridhar, M., Bhatta, R., and Jayapal, N. (2012). Effect of prebiotic on digestibility of total mixed ration. *Indian Vet. J.* 89, 41.

Santovito, E., Greco, D., Logrieco, A. F., and Avantaggiato, G. (2018). Eubiotics for food security at farm level: yeast cell wall products and their antimicrobial potential against pathogenic bacteria. *Foodborne Pathog. Dis.* 15, 531–537. doi: 10.1089/fpd.2018.2430

Scupham, A., Jones, J., Rettedal, E., and Weber, T. (2010). Antibiotic manipulation of intestinal microbiota to identify microbes associated with *Campylobacter jejuni* exclusion in poultry. *Appl. Environ. Microbiol.* 76, 8026–8032. doi: 10.1128/AEM.00678-10

Sibanda, N., McKenna, A., Richmond, A., Ricke, S. C., Callaway, T., Stratakos, A. C., et al. (2018). A review of the effect of management practices on *Campylobacter* prevalence in poultry farms. *Front. Microbiol.* 9:2002. doi: 10.3389/fmicb.2018.02002

Simmering, R., and Blaut, M. (2001). Pro- and prebiotics-the tasty guardian angels? *Appl. Microbiol. Biotechnol.* 55, 19–28. doi: 10.1007/s002530000512

Skånseng, B., Kaldhusdal, M., Moen, B., Gjevre, A. G., Johannessen, G., Sekelja, M., et al. (2010). Prevention of intestinal *Campylobacter jejuni* colonization in broilers by combinations of in-feed organic acids. *J. Appl. Microbiol.* 109, 1265–1273. doi: 10.1111/j.1365-2672.2010.04766.x

Spring, P., Wenk, C., Dawson, K. A., and Newman, K. E. (2000). The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. *Poult. Sci.* 79, 205–211. doi: 10.1093/ps/79.2.205

Svetoch, E., and Stern, N. (2010). Bacteriocins to control *Campylobacter* spp. in poultry—a review. *Poult. Sci.* 89, 1763–1768. doi: 10.3382/ps.2010-00659

Tsubokura, K., Berndtson, E., Bogstedt, A., Kaijser, B., Kim, M., Ozeki, M., et al. (1997). Oral administration of antibodies as prophylaxis and therapy in *Campylobacter jejuni*-infected chickens. *Clin. Exp. Immunol.* 108, 451–455. doi: 10.1046/j.1365-2249.1997.3901288.x

Van Gerwe, T., Bouma, A., Klinkenberg, D., Wagenaar, J. A., Jacobs-Reitsma, W. F., and Stegeman, A. (2010a). Medium chain fatty acid feed supplementation reduces the probability of *Campylobacter jejuni* colonization in broilers. *Vet. Microbiol.* 143, 314–318. doi: 10.1016/j.vetmic.2009.11.029

Van Gerwe, T., Bouma, A., Wagenaar, J. A., Jacobs-Reitsma, W. F., and Stegeman, A. (2010b). Comparison of *Campylobacter* levels in crops and ceca of broilers at slaughter. *Avian Dis.* 54, 1072–1074.

Wagenaar, J., Mevius, D., and Havelaar, A. (2006). *Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis. *Rev. Sci. Tech. Ser. Sci. Hum.* 25, 581–594. doi: 10.20506/rst.25.2.1680

Wesley, I. V., Muraoka, W. T., Trampel, D. W., and Hurd, H. S. (2005). Effect of preslaughter events on prevalence of *Campylobacter jejuni* and *Campylobacter coli* in market-weight turkeys. *Appl. Environ. Microbiol.* 71, 2824–2831. doi: 10.1128/AEM.71.6.2824-2831.2005

Yang, Y., Iji, P., and Choct, M. (2009). Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. *Worlds Poult. Sci. J.* 65, 97–114. doi: 10.1017/S0043933909000087

Yang, Y., Iji, P., Kocher, A., Mikkelsen, L., and Choct, M. (2008). Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility and gut development of broilers given different cereal-based diets. *J. Anim. Physiol. Anim. Nutr.* 92, 650–659. doi: 10.1111/j.1439-0396.2007.00761.x

Yusrizal and Chen, T. (2003). Effect of adding chicory fructans in feed on fecal and intestinal microflora and excreta volatile ammonia. *Int. J. Poult. Sci.* 2, 188–194. doi: 10.3923/ijps.2003.188.194

Conflict of Interest Statement: HP is employed by the company Diamond V.

The remaining 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: 12 March 2019
doi: 10.3389/fmicb.2019.00476

Phage Biocontrol of *Campylobacter jejuni* in Chickens Does Not Produce Collateral Effects on the Gut Microbiota

*Philip J. Richards, Phillippa L. Connerton and Ian F. Connerton**

Division of Food Sciences, School of Biosciences, University of Nottingham, Loughborough, United Kingdom

Bacteriophage biocontrol to reduce *Campylobacter jejuni* levels in chickens can reduce human exposure and disease acquired through the consumption of contaminated poultry products. Investigating changes in the chicken microbiota during phage treatment has not previously been undertaken but is crucial to understanding the system-wide effects of such treatments to establish a sustainable application. A phage cocktail containing two virulent *Campylobacter* phages was used to treat broiler chickens colonized with *C. jejuni* HPC5. *Campylobacter* counts from cecal contents were significantly reduced throughout the experimental period but were most effective 2 days post-treatment showing a reduction of 2.4 $\log_{10}$ CFU g^{-1} relative to mock-treated *Campylobacter* colonized controls. The administered phages replicated *in vivo* to establish stable populations. Bacteriophage predation of *C. jejuni* was not found to affect the microbiota structure but selectively reduced the relative abundance of *C. jejuni* without affecting other bacteria.

Keywords: bacteriophage, campylobacter, microbiota, chicken, biocontrol

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Catherine Maylin Loc-Carrillo,
The University of Utah, United States
Stephen Tobias Abedon,
The Ohio State University,
United States

***Correspondence:**
Ian F. Connerton
ian.connerton@nottingham.ac.uk

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

Received: *26 November 2018*
Accepted: *25 February 2019*
Published: *12 March 2019*

Citation:
Richards PJ, Connerton PL and Connerton IF (2019) Phage Biocontrol of Campylobacter jejuni in Chickens Does Not Produce Collateral Effects on the Gut Microbiota. Front. Microbiol. 10:476. doi: 10.3389/fmicb.2019.00476

INTRODUCTION

Campylobacter ssp. cause foodborne illness worldwide (Kaakoush et al., 2015) and represent the most commonly reported zoonotic pathogens in the European Union with 246,307 confirmed cases of gastrointestinal illness in 2016 (European Food Safety Authority [EFSA], 2017). *Campylobacter jejuni* is the most common species causing human disease (83.6%), but the burden of disease caused by *Campylobacter coli* is also significant (8.5%) (European Food Safety Authority [EFSA], 2017). Both species readily colonize the poultry gut, where the impact on flock health and production parameters varies with husbandry practice and the colonizing organism (Gormley et al., 2014; Humphrey et al., 2015). Estimates of broiler chicken flock prevalence vary between nations with 0.6 to 13.1% in the Nordic countries up to 74.2–80% in other European countries (European Food Safety Authority [EFSA], 2010a). Source attribution studies have predicted that up to 80% of human illness is as a result of exposure to campylobacter arising from poultry sources (European Food Safety Authority [EFSA], 2010b). A recent source attribution study referenced at the point of exposure confirmed chicken meat as the most important source of *Campylobacter* enteric disease, with an estimated 65–69% of human campylobacteriosis cases (Ravel et al., 2017). Poultry meat contaminated with digesta during slaughter/processing therefore represents a significant risk to public health (Osimani et al., 2017).

Strict on-farm biosecurity measures to mitigate the *Campylobacter* colonization of poultry have been implemented in many countries, but these alone do not eliminate campylobacter from poultry. Additional to these efforts, intervention strategies have been developed to reduce the *Campylobacter* colonization levels of poultry, which have the potential to reduce human exposure if the reductions can be realized on poultry meat (Rosenquist et al., 2003; Newell et al., 2011). The use of *Campylobacter*-specific bacteriophages (commonly referred to as phages) to reduce the intestinal carriage of *Campylobacter* by broiler chickens is one such intervention that has shown promise in controlled trials (Loc Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009) and in broiler house applications (Kittler et al., 2013). These studies have been conducted with phage applications of one or more phage to achieve reductions in the cecal counts of *Campylobacter* colonized chickens of approximately 2 $\log_{10}$ CFU g^{-1} (Carvalho et al., 2010; Connerton et al., 2011; Hammerl et al., 2014).

The use of multiple types of phage targeting different receptors in the form of phage cocktails offers the prospect of expanding the host range of the preparation whilst limiting the potential to develop resistance to all components of the cocktail (Chan et al., 2013). This has been explored experimentally using *Campylobacter*-specific phage cocktails containing either four (Fischer et al., 2013) or two phages (Hammerl et al., 2014). In both cases phages were selected from both Group II and Group III as classified by Sails et al. (1998). The classification was originally based on total genome size and morphology but it is now known that the two groups share little DNA similarity and generally have different host specificities (Javed et al., 2014; Jäckel et al., 2017). Group II and III *Campylobacter* bacteriophages are taxonomically classified as members of the *Myoviridae* subfamily *Eucampyvirinae*, which do not possess the genes required to form stable lysogens and therefore exhibit a lytic or virulent life cycle similar to most Myoviruses (Javed et al., 2014). *Campylobacter* phage isolated using *C. jejuni* and *C. coli* hosts may show some cross-species specificity within strains of *C. jejuni* and *C. coli* species but have not been demonstrated to infect other species in the *Campylobacter* genus, for example *C. lari*, *C. upsaliensis*, *C. fetus*, *C. Sputorum,* or *C. intestinalis* (Loc Carrillo et al., 2007). Neither have they been found to infect *Helicobacter pylori*, *Arcobacter butzleri*, *Citrobacter freundii*, *Salmonella enteritica* serovar Enteritidis, *Escherichia coli,* and *Pseudomonas aeruginosa* (Loc Carrillo et al., 2007). Therefore it may be anticipated that components of a *Campylobacter* phage cocktail would retain their specificity and not give rise to dysbiosis – a detrimental microbiota constitution that often arises post broad-spectrum antibiotic therapies (Dethlefsen et al., 2008). However, a recent study has suggested that exposure of the rat gut microbiome to a cocktail of commercial phage preparations active against *Salmonella enterica*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *E. coli* results in dysbiosis with increased inflammation and gut permeability (Tetz et al., 2017). Bacteriophage mediated cell lysis has the potential to release lipopolysaccharides (endotoxin) from Gram-negative bacterial species that are potent inducers of proinflammatory cytokines in animals and in humans (Medzhitov, 2007). Exposure to a phage cocktail targeting multiple Gram-negative species may well elicit such a response, although it is reported that targeted virulent *E. coli* bacteriophages release less endotoxin than β-lactam antibiotics (Dufour et al., 2017), and that the phage tail adhesin protein Gp12 can bind free lipopolysaccharide to counteract the inflammatory effect (Miernikiewicz et al., 2016). Typically the lipopolysaccharides of Gram-negative bacteria are composed of an outer membrane anchored lipid A substituted with a core polysaccharide structure attached to a set of repeating O-chain subunits. However, *C. jejuni* synthesizes a core oligosaccharide without the repeating O-chain, which is referred to as lipooligosaccharide (Karlyshev et al., 2005).

In this study we demonstrate that *Campylobacter* phage affect the target host bacteria without provoking intestinal dysbiosis, when phages are administered to broiler chickens at therapeutic doses.

METHODOLOGY

Bacterial Strains and Growth Media

Campylobacter were isolated and enumerated by direct plating on modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) selective medium (Oxoid, Basingstoke, United Kingdom) with addition of cefoperazone and amphotericin B selective supplement (Oxoid) using standard techniques. *C. jejuni* HPC5 was isolated from the cecal content of a commercial broiler chicken in the United Kingdom (Loc Carrillo et al., 2005; NCBI accession CP032316). The universal bacteriophage host strain *C. jejuni* PT14 was used to propagate *Campylobacter* bacteriophages (Brathwaite et al., 2013; NCBI accession CP003871). *C. coli* NCTC 12668 was used to discriminate group II and III bacteriophages (Frost et al., 1999). *C. jejuni* HPC5 for the inoculation of broiler chickens and campylobacter for the production of bacterial lawns were cultured on horse blood agar (Blood agar base No 2; Oxoid) with addition of plus 5% (v/v) defibrinated horse blood, (TCS, Buckingham, United Kingdom) under microaerobic conditions (5% O_2, 5% H_2, 10% CO_2, 80% N_2, produced by the evacuation and replacement technique) at 42°C for 24 h.

Bacteriophages and Propagation

Campylobacter-specific bacteriophages CP20 and CP30A were isolated from commercial broiler chicken excreta collected in the United Kingdom in 2001. Bacteriophages were isolated by making a 10% suspension of excreta in SM buffer (50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 8 mM $MgSO_4.7H_2O$, and 0.01% gelatin; Sigma Aldrich, Gillingham, United Kingdom). This was incubated at 4°C with agitation, for 24 h followed by centrifugation at 13,000 × *g* for 5 min to remove bacteria. The resulting supernatant was filtered through a 0.2 μm filter (Minisart, Sartorius, Goettingen, Germany) to remove any remaining bacteria.

When screening for the presence or absence of bacteriophage, 10 μl filtrate aliquots were applied to bacterial lawns of *C. jejuni* PT14 prepared using the soft agar overlay method

as previously described (Connerton et al., 2004). Bacteriophage were propagated by complete plate lysis and recovered in SM buffer before filtration (0.2 μm filter) and concentration by centrifugation at 37,000 × *g*.

CP20 (NCBI nucleotide accession MK408758) corresponds to a group II *Campylobacter* bacteriophage and CP30A (NCBI nucleotide accession JX569801) a group III based on genome sizes, capsid morphologies determined by transmission electron microscopy and genomic DNA sequences (Scott et al., 2007a; Siringan et al., 2011, 2014; Javed et al., 2014; Brathwaite et al., 2015).

Experimental Birds

Commercial *Campylobacter*-free male Ross 308 broiler chicks were obtained as hatchlings (PD Hook, Oxfordshire, United Kingdom). Birds were housed in a controlled environment in individual pens under strict conditions of biosecurity. Temperatures were as outlined in the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Birds were provided with commercial broiler diets (starter, grower, and finisher) and water *ad libitum* for the duration. The birds were weighed and randomly assigned to 3 groups at 14 days of age. These were: Control group consisting of *Campylobacter*-free sentinel birds, Group Cj_phg, consisting of *Campylobacter* infected birds to be administered with phage and Group Cj, consisting of *Campylobacter* infected birds to be administered with placebo instead of phage (mock treatment). Cloacal swabs were taken on day 14 and tested for *Salmonella* by direct plating on Xylose-Lysine desoxycholoate agar (XLD) agar (Oxoid) and for *Campylobacter* by direct plating on mCCDA agar. Excreta samples, from the same day, were tested for *Campylobacter* phage (see below) and for *Salmonella* by enrichment in Rappaport-Vassiliadis soya peptone broth (Oxoid) then plating on Xylose-Lysine desoxycholoate agar (XLD) agar (Oxoid). *C. jejuni* HPC5 has previously been demonstrated to establish intestinal colonization of Ross 308 broiler chickens at 20 days of age within 48 h of oral gavage, and to maintain colonization levels without significant differences in cecal counts over 15 days (Loc Carrillo et al., 2005; Connerton et al., 2018). Four days post oral gavage was selected for phage administration to ensure intestinal colonization, and that any variation in the cecal counts would be evident over the period of the experiment. Birds from Cj and Cj_phg groups were colonized with *C. jejuni* HPC5 at 20 days of age. Each bird received 7 $\log_{10}$ CFU *C. jejuni* in 1 ml of PBS (phosphate buffered saline) by oral gavage. Phages were administered to Group Cj_phg as a single dose of $\log_{10}$ 7 PFU of CP20 and CP30A combined in 1 ml of 30% $CaCO_3$ (antacid) by oral gavage at 24 days of age. Group Cj were administered with 1 ml of 30% $CaCO_3$ as a placebo at 24 days of age. Five birds were sacrificed at 24 h intervals from 25 to 29 days of age following administration of phage or placebo. The ceca, ileum, and colon of the birds were first separated by ligature and then removed by sterile dissection. The luminal contents were collected for *Campylobacter* and bacteriophage isolation as described below and aliquots stored at −80°C for DNA extraction.

Enumeration of Campylobacters

Serial dilutions of digesta were made in maximum recovery diluent (MRD; Oxoid) and enumerated using the Miles and Misera technique on mCCDA agar with 2% (w/v) additional agar to reduce swarming. Plates were incubated under microaerobic conditions at 42°C for 48 h before typical *Campylobacter* colonies were counted.

Enumeration of Bacteriophages

Bacteriophages were recovered by making a 10% suspension of chicken digesta in SM buffer as described above. To enumerate bacteriophage CP20 and CP30A in the phage treated groups, independent lawns of *C. jejuni* HPC5 and *C. coli* NCTC 12668 were prepared. The *C. coli* strain NCTC 12668 was used as a second host because it was sensitive to CP20 but not CP30A, which allowed discrimination of the two phages administered. The CP30A titers were obtained by subtraction of the CP20 titer on *C. coli* 12668, from the total phage count on the *C. jejuni* HPC5 host, which was sensitive to both phages. Serial dilutions of intestinal contents were prepared in SM buffer and applied to these lawns as 10 μl spots in triplicate. The plates were then incubated for 24 h at 42°C under microaerobic conditions.

Acquisition of Bacteriophage Resistance

In order to establish the frequency of resistance to bacteriophages post-intervention, single colonies were lifted from the primary isolation plates onto which cecal content from all phage-treated and mock-treated control birds had been inoculated. Three colonies per cecal sample were picked for each phage-treated bird and subcultured on horse blood agar plates. Bacterial lawns were prepared from successful subcultures and CP20 and CP30A phage were applied at a range of dilutions from 1 to 3 $\log_{10}$ PFU to establish if resistance had been acquired with respect to the efficiency of plating.

DNA Isolation

DNA was isolated from both ileal and cecal content using the Mobio PowerSoil kit (now QIAGEN Ltd., Manchester, United Kingdom). The method used is as described in the Human Microbiome Project SOP for processing of stool Specimens (see Manual of Procedures for Human Microbiome v12, section 7.7 onward[1].

Microbiome Analysis

The V4 regions of the bacterial 16S rRNA genes were PCR amplified using the primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGAC TACHVGGGTWTCTAAT 3′) (Caporaso et al., 2011). Amplicons were then sequenced on the Illumina MiSeq platform using 2 × 250 bp cycles. The 16S rRNA gene sequences were quality filtered and clustered into operational taxonomic units (OTUs) in Mothur (Schloss et al., 2009) using the Schloss lab. MiSeq SOP[2] (Kozich et al., 2013). Batch files of Mothur commands used

[1]http://hmpdacc.org/resources/tools_protocols.php

[2]https://www.mothur.org/wiki/MiSeq_SOP, (accessed 2018-10-05).

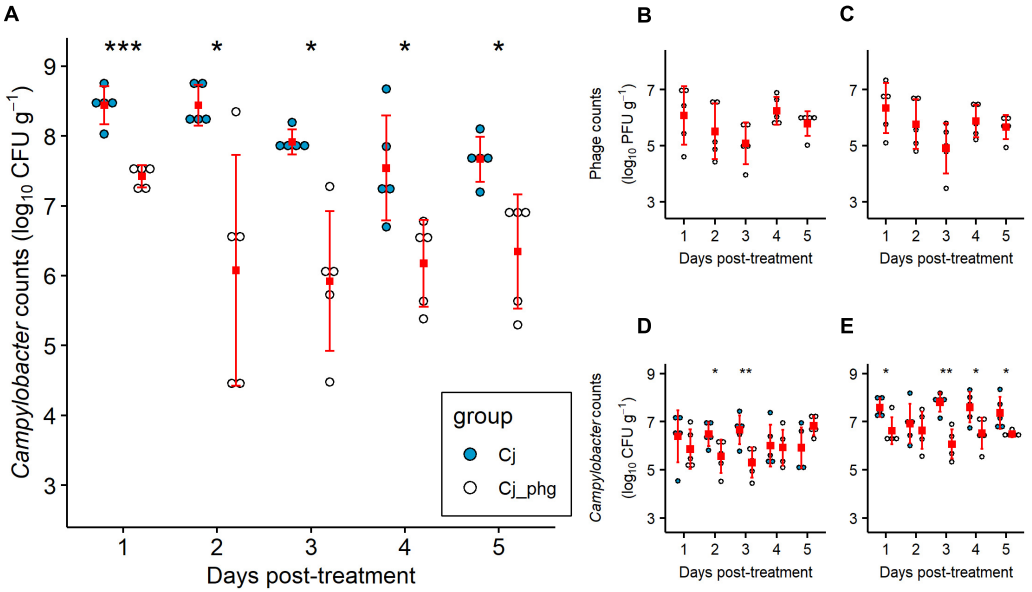

FIGURE 1 | *Campylobacter jejuni* levels are reduced by phage predation post-treatment. Independently housed Ross 308 broilers were given 7 $\log_{10}$ CFU *C. jejuni* by oral gavage at 20 days-old. At 24 days-old birds were orally administered a mixture of two phage at 7 $\log_{10}$ PFU each or a placebo of carrier alone. Five chickens from each group were sacrificed on each day from 25 to 29 days-old, from which *C. jejuni* were enumerated from the intestinal contents of the ceca **(A)**, ileum **(D)**, and colon **(E)**. Titers of phage CP20 **(B)** and CP30A **(C)** were determined from cecal content. Filled squares indicate means. Error bars indicate standard deviation. Asterix indicate statistical significance: *, $p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

in this study are available at: https://github.com/PJRichards. Post-processing rarefaction curves were plotted to assess sampling effort (**Supplementary Figures S1–S3**). Based on these observations the Day 1 (1dpt) ileum communities and two further ileum communities (Group Cj: Day 2 replicate 2; Group Cj_phg: day 4 replicate 4) were judged as having insufficient depth, and were therefore excluded from the analysis. No template controls were included in the analysis.

Raw sequence data are deposited in the NCBI database within the Bioproject PRJNA506577 under the SRA study SRP170194.

Ethics Statement

All experimental animal work was performed in accordance with United Kingdom and EU law. This study was approved by the Local Ethics Committee of the University of Nottingham and performed under Home Office license.

Statistical Analysis

The article was written in R 3.5.1 (R Core Team, 2018) and Rmarkdown 1.10.8 (Allaire et al., 2018; Xie et al., 2018) using Rstudio 1.1.456 (RStudio Team, 2015). R code used to make the figures/tables presented here are available at: https://github.com/PJRichards/Richards_phage_microbiota. **Figure 3** was drawn in R using code adapted from Torondel et al. (2016) and kindly made available at: http://userweb.eng.gla.ac.uk/umer.ijaz. OTUs discriminatory between communities were identified using LEfSE (Segata et al., 2011). Commonality of 16S rDNA sequences of OTUs discriminative of phage-treated (Group Cj_phg) and untreated birds (Group Cj) with other trials performed in our laboratory were determined using stand-alone ncbi-blast-2.7.1+ (Zhang et al., 2000)[3].

RESULTS

Dual *Campylobacter* Bacteriophage Treatment of Reduces Levels of *Campylobacter jejuni* Colonization

Campylobacter were enumerated using standard culture methods from intestinal luminal contents collected from the ileum, ceca, and colon of all birds. High levels of *Campylobacter* were recovered from all digesta collected from *C. jejuni* colonized birds (Group Cj) throughout the 5-day period of the experiment (mean $\log_{10}$ CFU g^{-1}: ileum 6.289, ceca 8.000, colon 7.452 for $n = 25$). A control group comprising of a cohort of non-colonized sentinel birds confirmed the effectiveness of the biosecurity measures adopted as they remained free of *Campylobacter* and phage contamination.

Co-administration of a single phage dose containing CP20 (7 $\log_{10}$ PFU) and CP30A (7 $\log_{10}$ PFU) 4 days after *Campylobacter* exposure at 24 days of age, significantly reduced *C. jejuni* numbers in the ceca of phage-treated birds (Group Cj_phg) compared to mock-treated birds throughout the period of the experiment ($p \leq 0.032$; **Figure 1A**). The phages were most effective 2 days post-treatment (dpt), resulting in a reduction in *C. jejuni* numbers of 2.365 $\log_{10}$ CFU g^{-1} in Group Cj_phg compared to *C. jejuni* colonized controls in Group Cj.

[3]https://ftp.ncbi.nlm.nih.gov/blast/executables/LATEST/

After this time, the numbers of *C. jejuni* in Group Cj_phg birds increased but remained significantly lower than the levels observed from mock-treated birds (Group Cj) by 1.321 $\log_{10}$ CFU g^{-1} after 5 days.

Phage treatment was most effective in the cecum, the organ that represents the greatest biomass of digesta and reservoir of *C. jejuni*. However, treatment was also effective in reducing *C. jejuni* in the ileum (p = 0.044 and 0.008 at 2 and 3 dpt, respectively) and the colon at all days excluding 2 dpt ($p \leq 0.041$; **Figure 1E**). The reductions in *Campylobacter* counts were not as great as those observed for the ceca, with a maximum reduction in the ileum of 1.359 $\log_{10}$ CFU g^{-1} at 3 dpt and a maximum reduction in the colon of 1.740 $\log_{10}$ CFU g^{-1} also at 3 dpt (**Figures 1D,E**). Bacteriophages were enumerated over the 5 day trial period and were detected in the cecal contents of all 5 birds treated in Group Cj_phg from 24 h after administration (**Figures 1B,C**). The CP20 phage titre recovered from cecal contents of the treated birds remained stable over time (mean 5.738 $\log_{10}$ PFU g^{-1}; *SD* 0.460; **Figure 1B**), confirming that the phage were replicating *in vivo*. Similarly, the mean CP30A titre was 5.708 $\log_{10}$ PFU g^{-1} (*SD* 0.517; **Figure 1C**) confirming the two phages co-exist without competitive exclusion. The levels of phages recovered from ileum and colon contents also remained stable over the course of the experiment (**Supplementary Figure S4**).

Bacteriophage Resistance Post Treatment

The overall levels of phage resistance (isolates resistant to one or both phage) in *C. jejuni* HPC5 isolates in Group Cj_phg was approximately 10% (n = 7/67) of the strains recovered post-treatment. Of these, three (4.5%) were resistant to both CP20 and CP30A phages whilst one (1.5 %) was resistant to CP20 but not CP30A and three (4.5%) were resistant to CP30A only. No phage resistance was detected in the *C. jejuni* recovered from birds that had not received phage (n = 32).

Bacteriophage Predation of *Campylobacter jejuni* Does Not Affect Microbiota Structure

The α-diversity (inverse Simpson index) of the cecal or ileal microbiota of bacteriophage-treated birds (Group Cj_phg) were not significantly different to those from mock-treated birds (Group Cj) ($p \geq 0.095$ and ≥ 0.841, respectively; **Figures 2A,C**). There was no difference in the richness (Chao) of the cecal

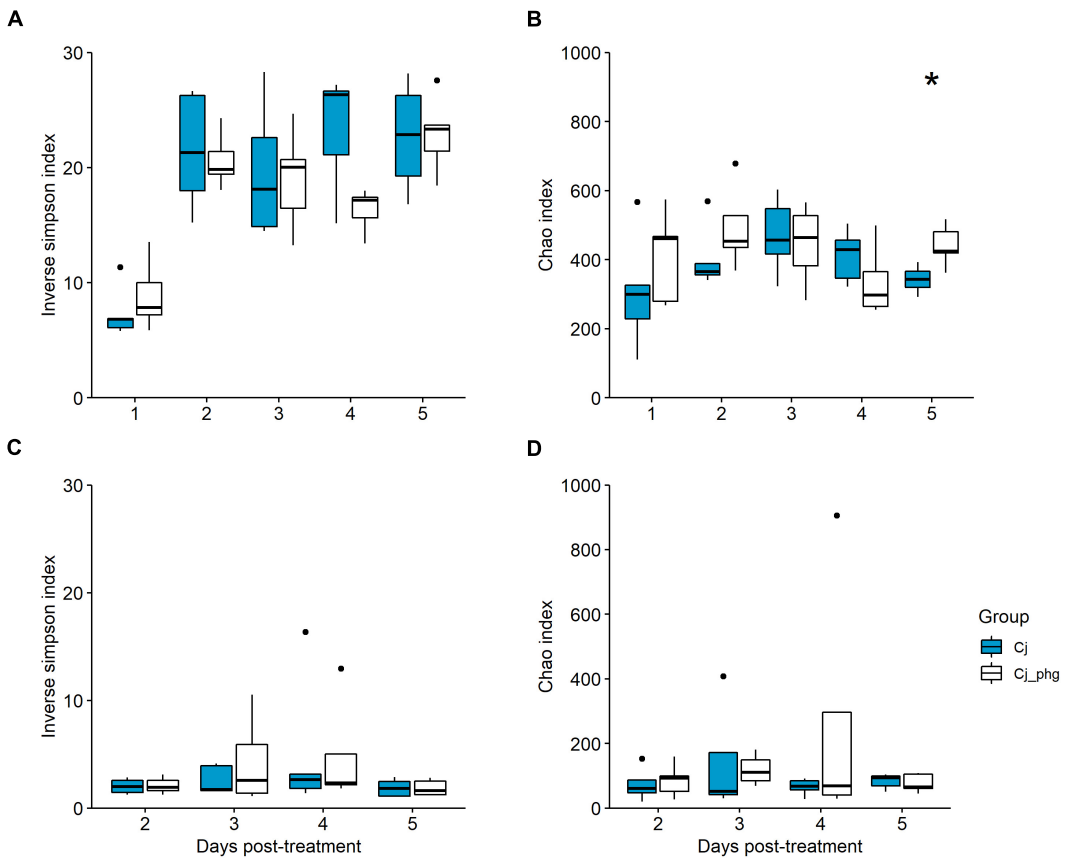

FIGURE 2 | Bacterial diversity within the gastrointestinal tract is not affected by phage treatment. Box-and-whisker plot describing bacterial 16S rDNA gene content surveys of cecal lumen contents from phage-treated (Cj_phg) or mock-untreated (Cj) groups in terms of α-diversity (inverse Simpson's index; **(A)** and richness (Chao index; **(B)**. Corresponding α-diversity and richness for ileal lumen contents are shown in panels **(C,D)**, respectively. Asterix indicate statistical significance: *$p \leq 0.05$.

microbiota between 1 and 4 dpt ($p \geq 0.151$) or the ileum microbiota at any time ($p \geq 0.548$; **Figures 2B,D**). The Chao-richness of the cecal microbiota of bacteriophage-treated birds (Group Cj_phg) was significantly different from mock-treated birds (Group Cj) by 5 dpt ($p = 0.032$; **Figure 2B**).

Exposure to *Campylobacter*-Phage Selectively Reduces Proportions of *C. jejuni* Without Affecting the Wider Microbiota

Throughout the experiment the predominant bacterial phylum present in the ileal lumen was the Firmicutes with a median relative abundance (RA) of 83.091% (**Supplementary Figure S5A**). There were no differences in relative abundance between age-matched bacteriophage-treated (Group Cj_phg) and mock-treated birds (Group Cj) in this phylum ($p \geq 0.063$, Wilcoxon test; **Supplementary Figure S5A**). The next most abundant phylum are the Proteobacteria where a difference at 4 dpt was observed ($p = 0.016$), but not on any other day ($p \geq 0.730$, Wilcoxon test; **Supplementary Figure S5A**). Phyla-level composition of the cecal microbiotas of both groups were likewise dominated by Firmicutes (median 90.620% RA), and to a much lesser extent Proteobacteria (4.747% RA) (**Supplementary Figure S5B**). However, there was no difference in the relative abundance of Firmicutes or Proteobacteria between phage-treated (Group Cj_phg) and mock-treated birds (Group Cj) at any time post-treatment ($p \geq 0.178$, *t*-test; and $p \geq 0.151$, Wilcoxon test; respectively).

At the OTU level, phage treatment did not affect the β-diversity (Bray Curtis distance) between communities of ileal lumen bacteria from age-matched phage-treated (Group Cj_phg) and mock-treated birds (Group Cj) at any time point ($p = 0.106$; AMOVA; **Supplementary Figure S6A**). The 11 most abundant OTUs in the ileum lumen are shown in **Figure 3A**. After the OTUs were filtered to include only those ≥1% of total reads, the only discriminative OTU between phage-treated and mock-treated birds identified using LEfSE (Segata et al., 2011) was OTU0013 [*Campylobacter* (100)] at 3 dpt ($p = 0.009$).

The 11 most abundant OTUs in the cecal lumen are shown in **Figure 3B**. For populations of cecal bacteria the β-diversity was not different at 1 to 4 dpt ($p \geq 0.088$, **Supplementary Figure S6B**). However, bacterial populations could be distinguished at 5 dpt ($p = 0.021$). At 5 dpt OTU0009 [Clostridiales_unclassified (100)] was the sole discriminative OTU between the treatments ($p = 0.027$; LEfSE; **Supplementary Figure S7D**), which was present in significantly greater proportions in the mock-treated birds (Group Cj) relative to phage-treated birds (Group Cj_phg) (mean % RA = 1.211 and 6.668, respectively) (**Figure 3A**). Previous work from our laboratory identified an OTU with 100% DNA sequence

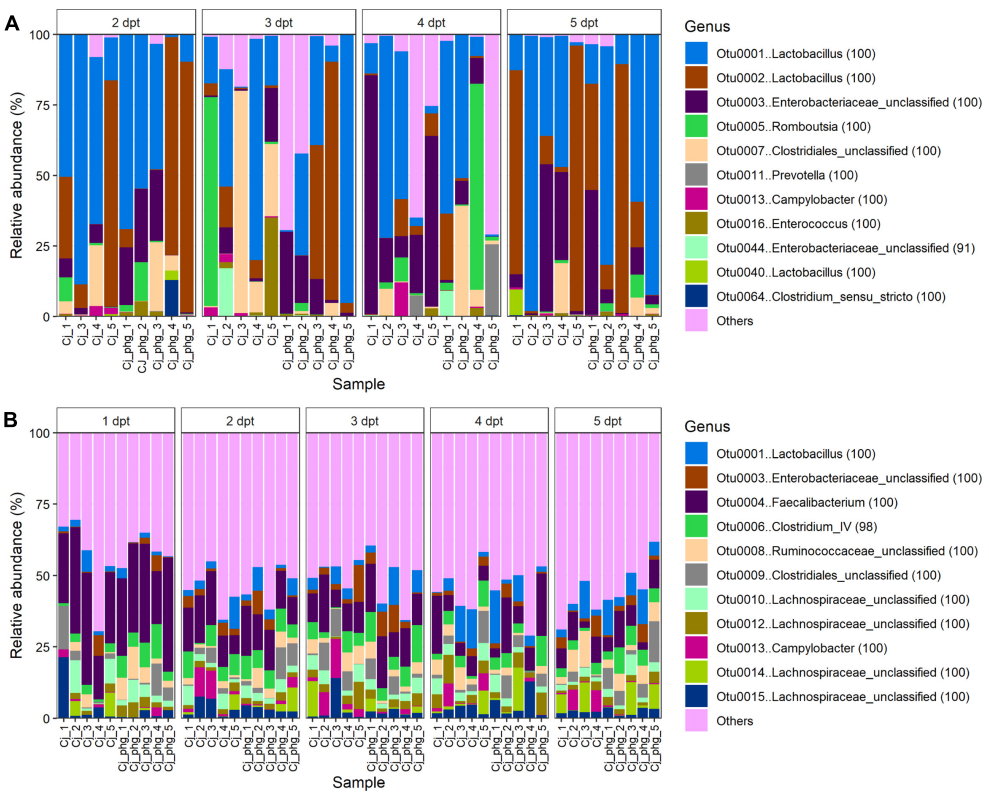

FIGURE 3 | Bacteriophage treatment reproducibly only affects *C. jejuni* proportions. Stacked bar charts showing bacterial community compositions from the ileum **(A)** and ceca **(B)** of all birds. For clarity only the 11 most abundant OTUs are described with all other OTUs summarized as "other."

identity to OTU0009 as being associated with *Campylobacter*-colonized birds [see OTU0073 in Connerton et al. (2018)]. Discriminative analysis of the cecal communities associated a greater abundance of *Camplylobacter* (OTU0013) with the mock-treated birds (Group Cj) at 3 and 4 dpi ($p < 0.047$; **Supplementary Figures S7B,C,** respectively). A positive association was observed between phage-treated (Cj_phg) birds at 1 dpt and OTUs 0006 and 0021, representing Clostridium IV (98) and Clostridiales_unclassified (100), respectively ($p = 0.047$; **Supplementary Figure S7A** and **Figure 3B**).

At 5 dpt the bacterial communities of the ceca or ileum of *Campylobacter*-free sentinel control birds could not be distinguished from mock-treated *Campylobacter*-colonized birds (Group Cj, $p > 0.05$; **Supplementary Figures S6A,B**), but cecal populations of bacteria in the phage-treated birds (Group Cj_phg) could be distinguished from non-colonized control birds ($p = 0.014$). Correspondingly, the OTUs that are associated with phage-treated treated birds (Group Cj_phg) are OTU0006 Clostridium IV (98) and OTU0009 Clostridiales_unclassified (100), which were also identified as discriminating phage-treated treated birds from mock-treated birds at 1 and 5 dpt, respectively (**Supplementary Figure S8B**). Although there was no difference in the β-diversity of non-colonized and mock-treated colonized birds in these cohorts, as expected OTU0013, representing *Campylobacter* (100), showed association with colonized birds, but conversely OTU0031, *Bifidobacterium* (100), showed association with the non-colonized control birds (**Supplementary Figure S8A**). This observation is also consistent with our previous study which demonstrates the differential association of an OTU with 100% DNA sequence identity with non-colonized birds compared to those 2 days post-colonization with *C. jejuni* at 20 days of age (NCBI database SRA study SRP133552; Connerton et al., 2018).

Interestingly, the low diversity of the ileal bacterial communities, relative to the cecal microbiota (see **Figures 2A,B**), is revealed in the observation that the top 11 most abundant OTUs across all ileal communities constitute 90.754% of reads, whereas the top 11 most abundant OTUs across all cecal communities account for a lower quotient of 48.670% reads (**Figures 3A,B**). We note that ileal samples showed some similarity to no template controls at phyla level (**Supplementary Table 1**), and that there was notable correspondence in the γ-proteobacteria [*Escherichia*/*Shigella* (100)] among the top 11 most abundant OTUs from ileal and cecal samples and the most abundant kitome/contaminant OTUs (**Figures 3A,B**; **Supplementary Table 2**).

DISCUSSION

The results described here provide further evidence of the efficacy of phage treatment to reduce the *Campylobacter* colonization of chickens. It has been widely suggested that an advantage of phage biocontrol over antibiotic use or other broad-spectrum types of therapy against pathogens that inhabit the intestinal tract, is the specificity of the bacteriophage selected for a particular host (Sulakvelidze and Barrow, 2004; Sulakvelidze and Kutter, 2004). This specificity is presumed to avoid the possibility of causing dysbiosis but recently this assumption has been challenged using a multi-phage treatment to affect collateral changes in the composition of the microbiota of rats with decreases in the abundance of *Blautia*, *Catenibacterium*, *Lactobacillus,* and *Faecalibacterium* species, and increases in the abundance of *Butyrivibrio*, *Oscillospira,* and *Ruminococcus* (Tetz et al., 2017). This is in contrast to directed studies using simulated gut microbial consortia (duodenum and ileum) containing a specific *Escherichia coli* as a bacteriophage target, where the impact of phage therapy was compared with ciprofloxacin treatment (Cieplak et al., 2018). Bacteriophage and antibiotic therapies were equally as effective in reducing the target *Escherichia coli* population by 2 to 3 $\log_{10}$ CFU ml^{-1} but notably the bacteriophage treatment had no measurable impact on non-target bacteria.

Although *Campylobacter* phages selected for the biocontrol of campylobacter in chickens appear to be confined to replication in *C. jejuni* and *C. coli* as hosts (Loc Carrillo et al., 2007), this discrimination had not previously been verified from the intestinal microbiota of *Campylobacter* colonized chickens. Frequency and abundance estimates of campylobacters and phages recovered from the ceca of commercial broiler chickens support the contention that production birds are often exposed to phage, and that phage presence coincides with a reduction in the mean *Campylobacter* cecal counts by approximately 1.8 $\log_{10}$ CFU g^{-1} (Atterbury et al., 2005). However, this study also demonstrated that phage which replicate on *Campylobacter* could be recovered in the absence of culture detectable *Campylobacter* host bacteria. The study left an open question as to whether the presence of phage under certain circumstances can drive *Campylobacter* populations below the culture detection limit (<2 $\log_{10}$ CFU g^{-1}) or that phage infecting campylobacters could also replicate on alternative host bacteria present in the microbiota of chickens. Further motivation for the current study was to establish whether phage therapy under these circumstances constitutes a minimal targeted intervention that utilizes biocontrol agents that are not detrimental to the intestinal microbiota of farmed chickens, and to which consumers are already exposed.

Phage therapy of *C. jejuni* colonized chickens produced significant reductions in intestinal *C. jejuni* counts compared to mock-treated controls over 5 days. However, the introduction of the phages did not affect the structures of the cecal or ileal microbiotas of the birds based on calculations of α-diversity (inverse Simpson index). The richness (Chao) of the microbiota remained similarly indistinguishable until 4 dpt. At the phyla level no difference in the abundance of the major components were observed for the cecal microbiotas representing the greatest biomass, and at only one time point was any difference observed post-phage treatment for the ileal microbiotas (4 dpt). Analysis of differences in the relative abundance between phage-treated and mock-treated ileal and cecal community OTUs highlights significant differences in OTU0013 that represents the phage therapy target, *C. jejuni*.

Additional to this, the cecal community member OTU0009 (Clostridiales_unclassified) showed significantly greater proportions in the mock-treated birds relative to phage-treated birds, as had previously been identified on the basis of the association of a DNA sequence identical OTU with *Campylobacter*-colonized birds (Connerton et al., 2018). The consistent association could be indicative of a key reliance for the corresponding clostridial organism(s) on high levels of *Campylobacter* colonization of the chicken gut. These observations further the idea that campylobacters can act as a hydrogen sink to improve the growth and competitive standing of specific clostridia (Kaakoush et al., 2015). The *Bifidobacterium* OTU0031 showed association with non-colonized sentinel birds, which is in contrast to 16S rRNA qPCR data reported by Thibodeau et al. (2015) that showed an increase in the molecular detection of *Bifidobacterium* sp. upon *C. jejuni* colonization. The authors noted that *Bifidobacterium* had previously been reported to hinder *C. jejuni* colonization (Ding et al., 2005; Santini et al., 2010), and that subtle effects may occur during *C. jejuni* colonization of chickens.

We observed the emergence of phage resistant *C. jejuni* post-phage treatment in this study as reported previously (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Fischer et al., 2013; Hammerl et al., 2014). We did not recover any phage resistant *C. jejuni* from the non-phage treated chickens despite the propensity of *C. jejuni* to undergo phase variation in genes leading to phage resistance (Aidley et al., 2017), and suggesting the observed phage escape mutation frequency of 10% in this experiment was a consequence of selection due to phage predation. The observed phage resistance frequency is within those reported previously of 1–14% (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Carvalho et al., 2010; Fischer et al., 2013; Hammerl et al., 2014). The phage treatment of chickens to reduce *Campylobacter* colonization has been demonstrated to be most effective over a 2–3 day period post-treatment (Loc Carrillo et al., 2005; El-Shibiny et al., 2009), since thereafter *Campylobacter* populations begin to recover. The continued impact of *Campylobacter* phage predation on the wider intestinal microbiota could not be assessed within the treatment timeframe examined in the current experiment (Connerton et al., 2018). However, when the time to slaughter after phage therapy was extended, the cecal *Campylobacter* levels were not reported to achieve the levels observed in non-treated controls (Fischer et al., 2013). This is likely due to reduced competitive fitness of the resistant types as described previously (Loc Carrillo et al., 2005; Scott et al., 2007a,b), and supports the supposition of Wagenaar et al. (2005) that the release of virulent *Campylobacter* phages into the environment would not constitute any greater risk.

Bacteriophage CP20 is a group II phage based on genome size and DNA sequence similarities. Group II phage are generally flagellotropic that require the host to be motile with a functional flagellar (Coward et al., 2006; Scott et al., 2007a,b; Baldvinsson et al., 2014; Lis and Connerton, 2016; Liang and Connerton, 2018). CP30A is a group III phage that in common with this phage group exhibits dependence on capsular polysaccharide structures (Sørensen et al., 2011; Lis and Connerton, 2016). The post-phage treatment *C. jejuni* isolates we identified as resistant to both phage classes will be of interest to examine with respect to their ability recolonize chickens and the nature of the mutation. Although we note they remain a minority population within the chicken gut even in the presence of bacteriophage controlling the wild type *C. jejuni* populations. These bacteria are likely at a competitive disadvantage to the wild type.

We have rigorously examined the microbiota of *Campylobacter* colonized chickens treated with either phages or a placebo to provide strong evidence for the lack of any collateral effect on the gut microbiome.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI, NCBI Bioproject PRJNA506577 under the SRA study SRP170194.

AUTHOR CONTRIBUTIONS

IC designed the experiments. PR, PC, and IC executed the experiments, analyzed the data, and prepared the manuscript.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council (Grant No. BB/I024682/1), United Kingdom.

ACKNOWLEDGMENTS

The technical assistance of Nicola Cummings is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00476/full#supplementary-material

FIGURE S1 | Rarefaction curves indicating coverage of *Campylobacter*-colonized (experimental) cecal bacterial communities. 16S rDNA bacterial communities from: **(A)** 1 day post-treatment (dpt); **(B)** 2 dpt; **(C)** 3 dpt; **(D)** 4 dpt; **(E)** 5 dpt. Cohort: **(i)** Group Cj **(ii)** Group Cj_phg.

FIGURE S2 | Rarefaction curves indicating coverage of *Campylobacter*-free non-colonized (control) cecal bacterial communities. 16S rDNA bacterial communities from *Campylobacter*-free non-colonized control birds at 29 days-old, equivalent to 5 days post-treatment.

FIGURE S3 | Rarefaction curves indicating coverage of ileal bacterial communities. 16S rDNA bacterial community from: **(A)** 2 day post-treatment (dpt); **(B)** 3 dpt; **(C)** 4 dpt; **(D)** 5 dpt. The non-phage treated cohort (Group Cj) are shown in panels Ai to Di, and the phage treated cohort (Group Cj_phg) in panels Aii to Dii.

FIGURE S4 | *Campylobacter* phage titers in the ileum and colon post-treatment. Phage tiers were determined for CP20 **(A)** and CP30A phage **(B)** from ileal content and colon content **(C)**, CP20; **(D)**, CP30A). Filled squares indicate mean. Error bars indicate standard deviation.

FIGURE S5 | Stacked barchart showing phyla-level microbiota composition for ileal and cecal communities. OTUs were filtered so that only Phyla present in more than one ileal or cecal community are included in the analysis. Figures in parenthesis in the key indicate bootstrap values for that taxonomic assignment generated in Mothur. All taxa shown in the key were detected in the ileum lumen including those of low abundance **(A)**. However, note that only Actinobacteria, Bacteria_unclassified, Bacteroidetes, Firmicutes, and Proteobacteria phyla were detected in cecal communities **(B)**.

FIGURE S6 | Relationship between communties of gut bacteria from phage-treated and mock-treated birds. PCoA plot of Bray-Curtis distance between **A**) ileal (R^2 = 0.41; **A,B**) cecal communities (R^2 = 0.75; **B**).

FIGURE S7 | Cecal bacterial taxa responsive to *Campylobacter*-phage treatment. OTUs were filtered to only include those representing at least 1% of the total reads before all communities were randomly subsampled an equal depth for unbiased comparison. Discriminatory OTUs were then described between age-matched groups using LEfSE. LDA refers to Linear Discriminant Analysis. 1 dpt **(A)**, 3 dpt **(B)**, 4 dpt **(C)**, 5 dpt **(D)**. There were no differential OTUs at 2 dp.

FIGURE S8 | Gut bacterial taxa responsive to *Campylobacter*-colonization at 5 days post-treatment. OTUs were filtered as described for **Supplementary Figure S7** and discriminatory OTUs identified using LEfSE. LDA refers to Linear Discriminant Analysis. Control-Group Cj comparison **(A)**, Control-Group Cj_phg comparison **(B)**.

TABLE S1 | Description of phyla associated with no template controls. Due to potential for low biomass and dominant contaminant profile from ileum samples the kitome microbiota profile is included. Table text in parenthesis indicates Mothur taxonomy bootstrap values. Samples: kitome for all samples, excluding 1 dpt (kit Ai and kit Aii); kitome for 1 dpt (kit B); sequencing negative control for all samples, excluding 1 dpt (seq Ai and seq Aii); sequencing negative control for 1 dpt (seq B).

TABLE S2 | Description of top 10 OTU associated with no template controls. Table text in parenthesis indicates Mothur taxonomy bootstrap values.

REFERENCES

Aidley, J., Sørensen, M. C. H., Bayliss, C. D., and Brøndsted, L. (2017). Phage exposure causes dynamic shifts in the expression states of specific phase-variable genes of *Campylobacter jejuni*. *Microbiology* 163, 911–919. doi: 10.1099/mic.0.000470

Allaire, J., Xie, Y., McPherson, J., Luraschi, J., Ushey, K., Atkins, A., et al. (2018). *Rmarkdown: Dynamic Documents for R*. Available at: https://rmarkdown.rstudio.com.

Atterbury, R. J., Dillon, E., Swift, C., Connerton, P. L., Frost, J., Dodd, C. E., et al. (2005). Correlation of *Campylobacter* bacteriophage with reduced presence of hosts in broiler chicken ceca. *Appl. Environ. Microbiol.* 71, 4885–4887. doi: 10.1128/AEM.71.8.4885-4887.2005

Baldvinsson, S. B., Sørensen, M. C. H., Vegge, C. S., Clokie, M. R., and Brøndsted, L. (2014). *Campylobacter jejuni* motility is required for infection by the flagellotropic bacteriophage F341. *Appl. Environ. Microbiol.* 80, 7096–7106. doi: 10.1128/AEM.02057-14

Brathwaite, K. J., Siringan, P., Connerton, P. L., and Connerton, I. F. (2015). Host adaption to the bacteriophage carrier state of *Campylobacter jejuni*. *Res. Microbiol.* 166, 504–515. doi: 10.1016/j.resmic.2015.05.003

Brathwaite, K. J., Siringan, P., Moreton, J., Wilson, R., and Connerton, I. F. (2013). Complete genome sequence of universal bacteriophage host strain *Campylobacter jejuni* subsp. *jejuni* PT14. *Genome Announc.* 1:e00969-13. doi: 10.1128/genomeA.00969-13

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Carvalho, C. M., Gannon, B. W., Halfhide, D. E., Santos, S. B., Hayes, C. M., Roe, J. M., et al. (2010). The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiol.* 10:232. doi: 10.1186/1471-2180-10-232

Chan, B. K., Abedon, S. T., and Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiol.* 8, 769–783. doi: 10.2217/fmb.13.47

Cieplak, T., Soffer, N., Sulakvelidze, A., and Nielsen, D. S. (2018). A bacteriophage cocktail targeting *Escherichia coli* reduces *E. coli* in simulated gut conditions, while preserving a non-targeted representative commensal normal microbiota. *Gut Microbes* 9, 391–399. doi: 10.1080/19490976.2018.1447291

Connerton, P., Carrillo, C. L., Swift, C., Dillon, E., Scott, A., Rees, C., et al. (2004). Longitudinal study of *Campylobacter jejuni* bacteriophages and their hosts from broiler chickens. *Appl. Environ. Microbiol.* 70, 3877–3883. doi: 10.1128/AEM.70.7.3877-3883.2004

Connerton, P., Timms, A., and Connerton, I. (2011). *Campylobacter* bacteriophages and bacteriophage therapy. *J. Appl. Microbiol.* 111, 255–265. doi: 10.1111/j.1365-2672.2011.05012.x

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'Kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome* 6:88. doi: 10.1186/s40168-018-0477-5

Coward, C., Grant, A. J., Swift, C., Philp, J., Towler, R., Heydarian, M., et al. (2006). Phase-variable surface structures are required for infection of *Campylobacter jejuni* by bacteriophages. *Appl. Environ. Microbiol.* 72, 4638–4647. doi: 10.1128/AEM.00184-06

Dethlefsen, L., Huse, S., Sogin, M. L., and Relman, D. A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol.* 6:e280. doi: 10.1371/journal.pbio.0060280

Ding, W., Wang, H., and Griffiths, M. W. (2005). Probiotics down-regulate flaA σ^{28} promoter in *Campylobacter jejuni*. *J. Food Protect.* 68, 2295–2300. doi: 10.4315/0362-028X-68.11.2295

Dufour, N., Delattre, R., Ricard, J.-D., and Debarbieux, L. (2017). The lysis of pathogenic *escherichia coli* by bacteriophages releases less endotoxin than by β-lactams. *Clin. Infect. Dis.* 64, 1582–1588. doi: 10.1093/cid/cix184

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P., and Connerton, I. (2009). Application of a group II campylobacter bacteriophage to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *J. Food Protect.* 72, 733–740. doi: 10.4315/0362-028X-72.4.733

European Food Safety Authority [EFSA] (2010a). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008 - part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA J.* 8:1503. doi: 10.2903/j.efsa.2010.1503

European Food Safety Authority [EFSA] (2010b). Scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA J.* 8:1437.

European Food Safety Authority [EFSA]. (2017). The european union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* 15:e05077.

Fischer, S., Kittler, S., Klein, G., and Glünder, G. (2013). Impact of a single phage and a phage cocktail application in broilers on reduction of *Campylobacter jejuni* and development of resistance. *PLoS One* 8:e78543. doi: 10.1371/journal.pone.0078543

Frost, J. A., Kramer, J. M., and Gillanders, S. A. (1999). Phage typing of *Campylobacter jejuni* and *Campylobacter coli* and its use as an adjunct to serotyping. *Epidemiol. Infect.* 123, 47–55. doi: 10.1017/S095026889900254X

Gormley, F. J., Bailey, R. A., Watson, K. A., McAdam, J., Avendaño, S., Stanley, W. A., et al. (2014). *Campylobacter* colonisation and proliferation in the broiler chicken under natural field challenge is not affected by bird growth rate or breed. *Appl. Environ. Microbiol.* 80, 6733–6738. doi: 10.1128/AEM.02162-14

Hammerl, J. A., Jäckel, C., Alter, T., Janzcyk, P., Stingl, K., Knüver, M. T., et al. (2014). Reduction of *Campylobacter jejuni* in broiler chicken by successive application of group II and group III phages. *PLoS One* 9:e114785. doi: 10.1371/journal.pone.0114785

Humphrey, S., Lacharme-Lora, L., Chaloner, G., Gibbs, K., Humphrey, T., Williams, N., et al. (2015). Heterogeneity in the infection biology of *Campylobacter jejuni* isolates in three infection models reveals an invasive

and virulent phenotype in a st21 isolate from poultry. *PLoS One* 10:e0141182. doi: 10.1371/journal.pone.0141182

Jäckel, C., Hammerl, J. A., Rau, J., and Hertwig, S. (2017). A multiplex real-time pcr for the detection and differentiation of *Campylobacter* phages. *PLoS One* 12:e0190240. doi: 10.1371/journal.pone.0190240

Javed, M. A., Ackermann, H.-W., Azeredo, J., Carvalho, C. M., Connerton, I., Evoy, S., et al. (2014). A suggested classification for two groups of *Campylobacter* myoviruses. *Arch. Virol.* 159, 181–190. doi: 10.1007/s00705-013-1788-2

Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi: 10.1128/CMR.00006-15

Karlyshev, A. V., Ketley, J. M., and Wren, B. W. (2005). The *Campylobacter jejuni* glycome. *FEMS Microbiol. Rev.* 29, 377–390.

Kittler, S., Fischer, S., Abdulmawjood, A., Glünder, G., and Klein, G. (2013). Effect of bacteriophage application on *Campylobacter jejuni* loads in commercial broiler flocks. *Appl. Environ. Microbiol.* 79, 7525–7533. doi: 10.1128/AEM.02703-13

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-1013

Liang, L., and Connerton, I. F. (2018). FlhF (T368A) modulates motility in the bacteriophage carrier state of *Campylobacter jejuni*. *Mol. Microbiol.* 110, 616–633. doi: 10.1111/mmi.14120

Lis, L., and Connerton, I. F. (2016). The minor flagellin of *Campylobacter jejuni* (FlaB) confers defensive properties against bacteriophage infection. *Front. Microbiol.* 7:1908. doi: 10.3389/fmicb.2016.01908

Loc Carrillo, C., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl. Environ. Microbiol.* 71, 6554–6563. doi: 10.1128/AEM.71.11.6554-6563.2005

Loc Carrillo, C. M., Connerton, P. L., Pearson, T., and Connerton, I. F. (2007). Free-range layer chickens as a source of *Campylobacter* bacteriophage. *Antonie Van Leeuwenhoek* 92:275. doi: 10.1007/s10482-007-9156-4

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449:819. doi: 10.1038/nature06246

Miernikiewicz, P., Kłopot, A., Soluch, R., Szkuta, P., Kska, W., Hodyra-Stefaniak, K., et al. (2016). T4 phage tail adhesin gp12 counteracts LPS-induced inflammation *in vivo*. *Front. Microbiol.* 7:1112. doi: 10.3389/fmicb.2016.01112

Newell, D. G., Elvers, K. T., Dopfer, D., Hansson, I., Jones, P., James, S., et al. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. *Appl. Environ. Microbiol.* 77, 8605–8614. doi: 10.1128/AEM.01090-1010

Osimani, A., Aquilanti, L., Pasquini, M., and Clementi, F. (2017). Prevalence and risk factors for thermotolerant species of *Campylobacter* in poultry meat at retail in Europe. *Poult. Sci.* 96, 3382–3391. doi: 10.3382/ps/pex143

R Core Team. (2018). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Ravel, A., Hurst, M., Petrica, N., David, J., Mutschall, S. K., Pintar, K., et al. (2017). Source attribution of human campylobacteriosis at the point of exposure by combining comparative exposure assessment and subtype comparison based on comparative genomic fingerprinting. *PLoS One* 12:e0183790. doi: 10.1371/journal.pone.0183790

Rosenquist, H., Nielsen, N. L., Sommer, H. M., Nørrung, B., and Christensen, B. B. (2003). Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol.* 83, 87–103. doi: 10.1016/S0168-1605(02)00317-3

RStudio Team. (2015). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc.

Sails, A., Wareing, D., Bolton, F., Fox, A., and Curry, A. (1998). Characterisation of 16 *Campylobacter jejuni* and *C. coli* typing bacteriophages. *J. Med. Microbiol.* 47, 123–128. doi: 10.1099/00222615-47-2-123

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Scott, A. E., Timms, A. R., Connerton, P. L., Carrillo, C. L., Radzum, K. A., and Connerton, I. F. (2007a). Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. *PLoS Pathog.* 3:e119. doi: 10.1371/journal.ppat.0030119

Scott, A. E., Timms, A. R., Connerton, P. L., El-Shibiny, A., and Connerton, I. F. (2007b). Bacteriophage influence *Campylobacter jejuni* types populating broiler chickens. *Environ. Microbiol.* 9, 2341–2353.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Siringan, P., Connerton, P. L., Cummings, N. J., and Connerton, I. F. (2014). Alternative bacteriophage life cycles: the carrier state of *Campylobacter jejuni*. *Open Biol.* 4:130200. doi: 10.1098/rsob.130200

Siringan, P., Connerton, P. L., Payne, R. J., and Connerton, I. F. (2011). Bacteriophage-mediated dispersal of *Campylobacter jejuni* biofilms. *Appl. Environ. Microbiol.* 77, 3320–3326. doi: 10.1128/AEM.02704-10

Sørensen, M. C. H., Alphen, L. B., van Harboe, A., Li, J., Christensen, B. B., Szymanski, C. M., et al. (2011). The f336 bacteriophage recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *J. Bacteriol.* 193, 6742–6749. doi: 10.1128/JB.05276-11

Sulakvelidze, A., and Barrow, P. (2004). "Phage therapy in animals and agribusiness," in *Bacteriophages: Biology and Application*, eds E. Kutter and A. Sulakvelidze (Boca Raton, FL: CRC Press), 335–380.

Sulakvelidze, A., and Kutter, E. (2004). "Bacteriophage therapy in humans," in *Bacteriophages: Biology and Application*, eds E. Kutter and A. Sulakvelidze (Boca Raton: CRC Press), 381–436.

Tetz, G. V., Ruggles, K. V., Zhou, H., Heguy, A., Tsirigos, A., and Tetz, V. (2017). Bacteriophages as potential new mammalian pathogens. *Sci. Rep.* 7:7043. doi: 10.1038/s41598-017-07278-6

Thibodeau, A., Fravalo, P., Yergeau, É, Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS One* 10:e0131978. doi: 10.1371/journal.pone.0131978

Torondel, B., Ensink, J. H., Gundogdu, O., Ijaz, U. Z., Parkhill, J., Abdelahi, F., et al. (2016). Assessment of the influence of intrinsic environmental and geographical factors on the bacterial ecology of pit latrines. *Microb. Biotechnol.* 9, 209–223. doi: 10.1111/1751-7915.12334

Wagenaar, J. A., Van Bergen, M. A., Mueller, M. A., Wassenaar, T. M., and Carlton, R. M. (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Vet. Microbiol.* 109, 275–283. doi: 10.1016/j.vetmic.2005.06.002

Xie, Y., Allaire, J., and Grolemund, G. (2018). *R Markdown: The Definitive Guide*. Boca Raton, FL: Chapman. doi: 10.1201/9781138359444

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7, 203–214. doi: 10.1089/10665270050081478

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: 14 May 2019
doi: 10.3389/fmicb.2019.01058

Essential Oils as an Intervention Strategy to Reduce *Campylobacter* in Poultry Production: A Review

*Andrew Micciche[1], Michael J. Rothrock Jr.[2], Yichao Yang[3] and Steven C. Ricke[1]**

[1] Center of Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [2] United States Department of Agriculture, Agricultural Research Service, Athens, GA, United States, [3] Department of Poultry Science, University of Arkansas, Fayetteville, AR, United States

Campylobacter is a major foodborne pathogen and can be acquired through consumption of poultry products. With 1.3 million United States cases a year, the high prevalence of *Campylobacter* within the poultry gastrointestinal tract is a public health concern and thus a target for the development of intervention strategies. Increasing demand for antibiotic-free products has led to the promotion of various alternative pathogen control measures both at the farm and processing level. One such measure includes utilizing essential oils in both pre- and post-harvest settings. Essential oils are derived from plant-based extracts, and there are currently over 300 commercially available compounds. They have been proposed to control *Campylobacter* in the gastrointestinal tract of broilers. When used in concentrations low enough to not influence sensory characteristics, essential oils have also been proposed to decrease bacterial contamination of the poultry product during processing. This review explores the use of essential oils, particularly thymol, carvacrol, and cinnamaldehyde, and their role in reducing *Campylobacter* concentrations both pre- and post-harvest. This review also details the suggested mechanisms of action of essential oils on *Campylobacter*.

Keywords: *Campylobacter*, poultry, essential oils, oregano, thymol, carvacrol, cinnamaldehyde

OPEN ACCESS

Edited by:
Vittorio Capozzi,
University of Foggia, Italy

Reviewed by:
Abhinav Upadhyay,
University of Connecticut, United States
Issmat Kassem,
American University of Beirut, Lebanon

****Correspondence:***
Steven C. Ricke
sricke@uark.edu

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

Received: *30 January 2019*
Accepted: *26 April 2019*
Published: *14 May 2019*

Citation:
Micciche A, Rothrock MJ Jr, Yang Y and Ricke SC (2019) Essential Oils as an Intervention Strategy to Reduce Campylobacter in Poultry Production: A Review. Front. Microbiol. 10:1058. doi: 10.3389/fmicb.2019.01058

INTRODUCTION

Campylobacter is a leading cause of human gastroenteritis in the United States and worldwide (Corcionivoschi et al., 2012; Sibanda et al., 2018). In the United States alone, the Centers for Disease Controls and Prevention (CDC) estimates an annual 1.3 million cases occurring per year (Centers for Disease Control and Prevention [CDC], 2018). From 1999 to 2008, *Campylobacter* was estimated to cause an annual 8,463 hospitalizations and 76 deaths in the United States, along with an annual $1.7 billion financial cost to the United States (Batz et al., 2012). In 2015, of the 4,598 hospitalizations caused by foodborne disease 1,087 were the result of *Campylobacter* (Centers for Disease Control and Prevention [CDC], 2017a). This burden on public health was second only to *Salmonella* when considering bacterial infections (Batz et al., 2012; Scallan et al., 2015). When aggregating the loss of life and health due to illness, Scallan et al. (2015) determined that annually, *Campylobacter* caused 22,500 disability-adjusted life years (DALY). *Campylobacter* was ranked 3rd as the leading impact of public health due to foodborne illness behind non-typhoidal *Salmonella* (32,900 DALY) and Toxoplasma (32,700 DALY). A systematic analysis from 1990 to 2013 identified *Campylobacter* as the fourth leading cause of diarrheal disease behind rotavirus, typhoid fever,

and cryptosporidiosis (Murray et al., 2015). According to the World Health Organization (WHO), diarrheal diseases, viewed as a whole, cause an estimated 550 million illness each year resulting in the annual death of approximately half a million infants under the age of two (World Health Organization [WHO], 2013).

In a population-based study on patients with diarrheal illness induced by *Campylobacter*, over 95% of the 1,316 cases were caused by *Campylobacter jejuni* (Friedman et al., 2004). This species, along with pathogen *Campylobacter coli*, have been found in the gastrointestinal tract (GIT) of poultry (Wang et al., 2002; European Food Safety Authority [EFSA], 2010; Centers for Disease Control and Prevention [CDC], 2017b). Broilers and layer flocks have consistently been shown to contain *Campylobacter* prevalences greater than 70% (Corry and Atabay, 2001; Stern et al., 2001; Ansari-Lari et al., 2011; Sahin et al., 2015). Schets et al. (2017) found that 97% of layer and 93% of broiler flocks tested positive for *Campylobacter*. Across eight flocks in the Netherlands, 55 cecal samples were taken and found that *C. jejuni* was the *Campylobacter* isolate for 100% of cecal broiler samples where in layer hens 52% were *C. coli*, 40% were *C. jejuni* (40%) and 7% were *C. lari*. Identical sequence types were found in the soil, sediment, and surface water which indicates potential contamination of the bird through environmental means. Through contamination of the poultry carcass, *Campylobacter* can cause foodborne illness and has been the etiological agent in outbreaks caused by poultry products (Nauta et al., 2009; Geissler and Powers, 2011). Therefore there is a continued urgency to implement pre- and post-harvest technologies to prevent *Campylobacter* contamination at all stages of poultry production.

With the rise of multidrug-resistant bacteria, consumer preference for antibiotic-free chicken, and government regulations such as the European Union's 2006 ban of antibiotics, alternative antimicrobials have become necessary (Cervantes, 2015; Johnson, 2015; Shin et al., 2015). One alternative to conventionally implemented pre- and post-harvest antimicrobial strategies is the use of essential oils (EOs) (Tiihonen et al., 2010; Amerah et al., 2012; Calo et al., 2015; O'Bryan et al., 2015; Thibodeau et al., 2015; Micciche et al., 2018b). The EOs industry had a United States market share of $6.6 billion in 2016, a 286% increase from the 2004 market share of $2.3 billion (Zviely, 2004; Grand View Research, 2018). They are often used as flavoring agents in food products and perfumes, embalmment, anti-inflammatory and anesthesia remedies (Burt, 2004; Bakkali et al., 2008). Some EOs, such as eugenol, have been reported to have a preventative effect against cancer (Burt, 2004; Tsuneki et al., 2005; Bakkali et al., 2008). Plant-based EOs such as eugenol, thymol, carvacrol, and cinnamaldehyde have been examined and screened for their antimicrobial properties against a number of pathogens including *Campylobacter* (Friedman et al., 2002; Chouliara et al., 2007; Thibodeau et al., 2015; Kelly et al., 2017; Upadhyay et al., 2017). They are also commercially attractive as alternative antimicrobials, because they are considered acceptable for organic and non-conventional applications (Micciche et al., 2018b; National Organic Program, 2018). They may be useful not only in preventing human health-related diseases but in improving the performance of the bird (Alcicek et al., 2004; Hernandez et al., 2004; Diaz-Sanchez et al., 2015; National Organic Program, 2018). As such, the objective of this review was to discuss EOs as an intervention approach for limiting *Campylobacter* contamination in the poultry industry pre- and post-harvest. By investigating the metabolic activity of *Campylobacter*, a greater understanding of the mechanistic effects of EOs may be elucidated for more optimal application approaches in the future.

Campylobacter CHARACTERISTICS AND METABOLISM

Campylobacter belongs to family Campylobacteraceae, which also includes *Arcobacter* and *Helicobacter* (Fitzgerald and Nachamkin, 2011). They are non-sporulating Gram-negative microorganisms, with an s-shape and cell dimensions between 0.5 and 5 μm and ranging in width from 0.2 to 0.9 μm (Vandamme et al., 2006). A single polar flagellum is found in 20 of the 22 species with the exception being the non-motile *C. gracilis* and *C. showae* that possess multiple flagella (Debruyne et al., 2008; Facciolà et al., 2017). *Campylobacter* spp. are microaerophilic and grow optimally in a gas composition of 5% O_2, 10% CO_2, and 85% N_2, pH of 6.5 to 7.57 and from 37 to 42°C (Garénaux et al., 2008; Davis and DiRita, 2017; Facciolà et al., 2017). *Campylobacter* will not grow at temperatures below 30°C, due to an absence of a cold shock protein gene, or with a water activity below 0.987, which is necessary to maintain turgor pressure (Hazeleger et al., 1998; De Cesare et al., 2003; Wallace, 2003; Levin, 2007; Facciolà et al., 2017). Cold shock proteins range from 65 to 75 amino acids serve as nucleic acid chaperones that prevent mRNA secondary structures from forming which enable efficient transcription and translation (Phadtare and Severinov, 2010; Keto-Timonen et al., 2016). Interestingly, despite the lack of a cold shock protein, biofilms of *C. jejuni* have been found to form and persist in 13°C conditions and formed biofilms with the largest surface area compared to 20, 37, and 42°C (Sanders et al., 2008). Attachment of planktonic cells to the biofilms was not significantly impacted by temperature (Sanders et al., 2008).

Compared to other foodborne pathogens occupying the poultry GIT, *Campylobacter* possesses a metabolism that is somewhat different and thus may be a challenge for some intervention strategies. *Campylobacter* spp. do not utilize the traditional glycolysis pathway, instead favoring amino acids such as aspartate, glutamate, serine, and proline for their cellular respiration pathways (Stahl et al., 2012). The traditional glycolytic pathway is incomplete as genes encoding glucokinase and 6-phosphofructokinase are absent (Parkhill et al., 2000). Furthermore, despite a complete tricarboxylic acid cycle (TCA), gluconeogenesis from glucose-6-phosphate to glucose is not observed, and the genes necessary for encoding the proteins associated with the pathway are absent (Parkhill et al., 2000; Velayudhan and Kelly, 2002). Some of the gluconeogenesis and glycolysis genes and respective proteins are present which has led to the hypothesis that *C. jejuni* can catabolize intermediary molecules (Stahl et al., 2012). For instance, Hofreuter et al. (2006)

suggested that the presence of a glycerol-3-phosphate transporter (GlpT) in *C. jejuni* 81176 could be indicative of the breakdown of glycerol-3-phosphate to generate pyruvate and potential further breakdown through the TCA cycle. Additionally, the non-oxidative portion of the pentose pathway shunt (transaldolase, transketolase, ribulose-3-phosphate epimerase, and ribose-5-phosphate isomerase) is suggested to have the ability to metabolize pentose sugars (Line et al., 2010).

Two studies independently identified novel l-fucose pathways with a l-fucose permease that is homologous to the one characterized in *E. coli* (Dang et al., 2010; Muraoka and Zhang, 2010; Stahl et al., 2011). Fucose is a component of eukaryotic glycoproteins and is found in mucin (Allen and Griffiths, 2001; Robbe et al., 2004). *Campylobacter jejuni* utilizes fucose as a chemoattractant for mucin attachment (Hugdahl et al., 1988; Tu et al., 2008; Gangaiah et al., 2010; Kassem et al., 2013). While fucose uptake was phenotypically observed in *C. jejuni* strain NCTC 11168 (isolated from human feces), it was not detected in strains 81116 and 81176 (Muraoka and Zhang, 2010). This is due to the presence of a genomic island cj0480c–cj0490, which has been linked to an increase in virulence (Parker et al., 2006). Stahl et al. (2011) noted that this metabolic pathway improved colonization of the piglet model for human disease indicating its importance in human-associated pathogenesis. Moreover, when the l-fucose pathway genes were mutated, *C. jejuni* was still able to colonize the ceca of chickens but was outcompeted in a co-colonization experiment by the wild-type strain (Muraoka and Zhang, 2010). Despite the presence of this metabolic pathway, *Campylobacter* is largely asaccharolytic and relies primarily on amino acids and organic acids for its energy and carbon needs (Lin et al., 2009; Stahl et al., 2012; Kassem et al., 2013; Kelly et al., 2017).

Amino acids utilized by *Campylobacter* as carbon and energy sources include asparagine, glutamine, serine, aspartate, and proline (Wright et al., 2009). *Campylobacter jejuni* will preferentially utilize serine, aspartate, asparagine, and glutamate (Guccione et al., 2008; Wright et al., 2009; Stahl et al., 2012). Parsons (1984) determined the most common amino acids within the ceca of leghorn hens through ion-exchange chromatography. Serine ranked 4th (92.2 mmol/mol ceca content), aspartate ranked 2nd (109.9 mmol/mol ceca content), and glutamate ranked 1st (137.5 mmol/mol ceca content) as the most concentrated amino acids within the ceca. Asparagine, glutamine, glycine, cysteine, and tryptophan concentrations were not reported. While these concentrations will vary depending on diet, GIT microbial population, and bird type, the data provides insight into why amino acid metabolism of *C. jejuni* could play an essential role in its ecological niche within the poultry GIT (Ravindran and Bryden, 1999; Stahl et al., 2012). Whereas other species must rely on carbohydrate fermentation, the plentiful concentration of amino acids in the poultry ceca, due to protein-rich diets, allows *Campylobacter* to thrive (Józefiak et al., 2004; Vegge et al., 2009; Hermans et al., 2012).

Not all poultry dietary components contribute directly to the nutrition of the bird but may still interact with the GIT microbial population including resident foodborne pathogens. For example, there are primary plant compounds and secondary plant metabolites present in the poultry GIT that may not necessarily serve directly as nutrients but can still nutritionally influence bird performance (Smithard, 2002). While some fiber components, particularly lignin, are generally considered indigestible in the avian GIT, they can still impact GIT microbial composition, alter fermentation patterns, and influence metabolite absorption (Ricke et al., 1982, 2013; Jung and Fahey, 1983; van der Aar et al., 1983; Dunkley et al., 2007; Baurhoo et al., 2008; Sima et al., 2018). These high fiber sources have also been shown to limit the establishment of foodborne pathogens such as *Salmonella* Enteritidis in laying hens when serving as the primary dietary source in laying hens (Ricke, 2003; Woodward et al., 2005; Ricke et al., 2013). Plants also contain phenolic monomers (Jung and Fahey, 1983). Phenolic monomers can not only cross-link carbohydrates with lignin in the plant, decreasing fiber degradation in the GIT, but in their free form can be antimicrobial to aerobic and anaerobic bacteria (Zemek et al., 1979; Jung and Fahey, 1983).

Aromatic compounds can also be modified anaerobically (Tschech and Schink, 1985; Netzer et al., 2016). Often phenolic compounds such as chlorophenol and chlorobenzoate can be utilized as electron sinks, resulting in their reduction and modification within the lower GIT (Häggblom et al., 1993; Frazer, 1994). While *Campylobacter* has been shown to utilize some aromatic compounds such as resorcinol and β or γ-resorcylate, this was only shown in the presence of *Clostridium* spp. (Tschech and Schink, 1985; Evans and Fuchs, 1988). However, other aromatic compounds can be utilized as energy sources by some bacteria (Overhage et al., 2006). MetaCyc identifies five complete aromatic degradation pathways present in *E. coli* including cinnamate, phenylethylamine, nitroaromatic, and phenols (version 22.5; BioCyc; Menlo Park, CA, United States) (Caspi et al., 2015). Chlorobenzene, paraoxon, parathion, and shikimate degradation pathways have been identified in *L. monocytogenes* and degradation of *p*-cymene, along with other aromatic compounds, have been characterized in *Pseudomonas* spp. In *Campylobacter*, only the catechol degradation pathway has been detected. Numerous biodegradation enzymes have been elucidated in *E. coli;* for instance, eugenol has been shown to be degraded to ferulic acid by the *vaoA* gene-encoded enzymes (Díaz et al., 2001; Overhage et al., 2003, 2006). However, only resorcinol, protocatechuate, and phloroglucionol degradation enzymes are known to occur in *Campylobacter* (Evans and Fuchs, 1988; Villemur, 1995). Given the limited metabolic capacity of *Campylobacter* for modification, it would be intuitive that aromatic compounds that exhibit antimicrobial properties could effectively reduce *Campylobacter* populations in poultry production. This is important since aromatic compounds are one of the constituents present in phytobiotics.

PHYTOBIOTICS

Phytobiotics are plant-based compounds or extracts that have been suggested for use in commercial, and possibly organic, poultry production (Windisch and Kroismayr, 2007; Bakkali et al., 2008; Diaz-Sanchez et al., 2015; Micciche et al., 2018b).

Most phytobiotics are Generally Recognized As Safe (GRAS) by the U.S Food and Drug Administration [FDA], 2018 and are less toxic and typically more residual-free compared to synthetic antibiotics (Diaz-Sanchez et al., 2015; U.S Food and Drug Administration [FDA], 2018). Botanicals, a subset of phytobiotics, are leaves, roots, bark, or other parts of a plant, and the terminology is often used interchangeably with phytobiotics (Windisch and Kroismayr, 2006; Mohammadhosseini et al., 2017). Other types of phytobiotics include herbs, which are derived from flowering non-persistent plants, oleoresins which are non-aqueous extracts such as balsam, and EOs (Prior and Cao, 2000). In this review, the focus will primarily be on the activity of EOs, but other phytobiotic compounds such as herbs will also be mentioned.

Essential oils, also known as volatile or ethereal oils, are oily plant-based liquids that possess aromatic properties (Burt, 2004; Hardin et al., 2010). The term "essential oils" was first coined by Paracelsus von Hohenheim in the 16th century (Guenther(ed.), 1948). The term 'essential' relates to the effective element in a medical preparation of the drug and is therefore loosely defined (Oyen and Dung, 1999). Currently, there are over 3,000 known EOs with approximately 300 being commercially relevant (Bakkali et al., 2008; Diaz-Sanchez et al., 2015). They include oils such as turpentine, eugenol, and cinnamaldehyde, and can be derived from other botanical compounds and herbs such as thyme, oregano, rosemary, and lemon (Burt, 2004; Fisher and Phillips, 2008; Diaz-Sanchez et al., 2015). Essential oils are extracted from their corresponding plants by steam distillation, hydrodistillation, or solvent extraction which all can create a concentrate of aromatic and volatile compounds including terpenoids and phenylpropanoids (Nakatsu et al., 2000; Hardin et al., 2010; Raut and Karuppayil, 2014). The concentrations of the 'essential' compounds in EOs vary wildly and are not typically defined (Lee et al., 2004a; Benavides et al., 2012).

NON-ANTIMICROBIAL EFFECTS OF EOs IN POULTRY

Essential oils have a wide range of applications including turpentine for paint mixing or linalool and linalyl acetate that are used as alternative sleep aides (Buchbauer et al., 1991; Burt, 2004). Many are incorporated as ingredients for their palatable tastes and smells in foods and aromatic sprays (Franz et al., 2010). Oregano, thyme, and cinnamon are well-known flavor enhancers (Khan and Abourashed, 2011; Wang et al., 2013). Bergamon is used for its aromatic properties of Earl Gray Tea, while synthetic based citrus oils are an ingredient in soft drinks (Fabricant, 2008; Gonzalez-Molina et al., 2009; Callaway et al., 2011). While 32.9% of EOs are applied in the food and beverage industry, the second largest application of EOs in 2015 was for spa or relaxation purposes (30.84%) (Grand View Research, 2018).

In poultry, EOs have been utilized in preharvest management settings for non-pathogen related benefits (Diaz-Sanchez et al., 2015). Digestibility of poultry feed has been shown to be improved by the addition of EOs (Williams and Losa, 2001). CRINA® (Akzo Nobel, Crina S.A, Switzerland) is a commercial blend of EOs containing thymol, eugenol, and piperine. Broilers fed 50 mg/kg of CRINA®, exhibited improved activity of total amylase, trypsin, and maltase of 40kU/pancreas, 63U/pancreas, and 12.6 μM/g mucosa respectively, compared to the control group activity of 29kU/pancreas, 42U/pancreas, and 10.6 μM/g mucosa, respectively (Jang et al., 2004, 2007). This effect was not observed; however, when 0.1% of lactate was also introduced into the diet (Jang et al., 2004). The use of 200 ppm of a blend of oregano, cinnamon, and pepper improved fecal digestibility of dry matter (Hernandez et al., 2004). Ileal absorption levels of amino acids such as threonine, serine, asparagine, phenylalanine, histidine, and lysine were positively improved by 7.8 and 8.8% by the addition of 150 and 300 ppm of a plant extract containing capsaicin, carvacrol, and cinnamaldehyde (Jamroz et al., 2003).

Amino acid absorption studies have also been performed using rat intestines. A catheter was attached to both ends of a sector of the jejunum of anesthetized rats (Kreydiyyeh et al., 2000). In the treatment groups, the jejuna were preincubated with 1000 ppm cinnamaldehyde or 850 ppm of eugenol or a saline control. Alanine was then fed through the jejuna, and it was observed that after 40 min the jejuna preincubated with cinnamaldehyde and eugenol absorbed 22 to 25 nmol of alanine compared to 60 nmol in the control. This suggests reduced nutrient absorption (Kreydiyyeh et al., 2000; Lee et al., 2004a). However, conclusions drawn from this study may be difficult to apply to production animals due to the high concentrations necessary in the feed to result in similar concentrations in the GIT as utilized in Kreydiyyeh et al. (2000). Lee et al. (2003) observed that 100 ppm of thymol or cinnamaldehyde exhibited no significant effect on pancreatic digestive enzyme activity at day 21 or day 40 within female broilers (Lee et al., 2004a). This study shows that thymol alone is unable to impact pancreatic digestive enzymes within poultry and a combination of multiple EOs, such as in Jang et al. (2004, 2007), may be necessary to impact digestibility responses in broilers. However, it may be specific combinations of EOs that impact digestibility and nutrient absorption.

Improvements in nutrient absorption and digestibility can result in improved growth rates within broilers (Gous, 2010). Feed conversion ratio (FCR), the ratio between feed intake and average weight gain, is one of the more commonly used metrics to determine if a feed additive is beneficial to commercial livestock production (Leenstra, 1986). Several studies have reported no improvement in growth rate or FCR when diets have been supplemented with EOs (Lee et al., 2003; Jang et al., 2004, 2007). However, FCR and body weight gain (BWG) improvements have been observed in other studies that utilized EOs (Denli et al., 2004; Cabuk et al., 2006; Basmacioğlu Malayoğlu et al., 2010). Cabuk et al. (2006) demonstrated that 24 or 48 mg/kg of Heryumix^{TM} significantly improved FCR on days 21 to 1.53 and 1.56 compared to the control at 1.62. On day 42, compared to the control at 1.87, the FCR was also improved to 1.80 and 1.77 with 24 or 48 mg/kg of Heryumix^{TM} (Cabuk et al., 2006). Heryumix^{TM} is composed of oregano, laurel leaf, sage, myrtle, fennel, and citrus peel extracts (Herba Gida Maddeleri; Seferihisar, Turkey). Denli et al. (2004) observed similar improvements in quail when their diet was supplemented with 60 mg/kg of thyme or black seed oil.

This discrepancy may be due to certain variables including bird type, feed composition, and EO type. The intestinal mucosa and GIT microbiome composition of birds vary depending on the species and age of the bird (Zoetendal et al., 2004; Stanley et al., 2014). While the phyla *Bacteroidetes* and *Firmicutes* are dominant within quail and broiler chicken cecal microbiomes, the represented genera are different (Oakley et al., 2014; Liu et al., 2015). Over 117 different genera were identified by Wei et al. (2013) in broiler chicken ceca. The quail microbiome, however, is not as taxonomically rich at the genus level with only 32 genera being detected by Wilkinson et al. (2016). Furthermore, while the top five most dominant genera based on taxa analyses were *Lactobacillus, Rumiunoccocus*, *Clostridium*, *Bacteroides*, *Faecalibacterium* in the chicken cecal microbiome, the quail ceca microbiome was dominated by *Bacteroides, Ruminococcus*, *Faecalibacterium*, *Enterococcus,* and *Clostridium* (Wei et al., 2013; Wilkinson et al., 2016). These microbiome differences along with unknown host-species interactions should be considered when evaluating and comparing the effectiveness of any particular feed amendment across species (Koutsos and Arias, 2006).

Additionally, the feed may play a major role in how EOs improve FCR. As elucidated in Jamroz et al. (2003), EOs can impact the absorption of amino acids and potentially other nutrients in the ileum. As a consequence, the FCR should be improved in birds on typical commercial diets amended with EOs. Furthermore, improved absorption of amino acids could conceivably also allow for the use of non-conventional diets that contain less protein. In-depth studies employing not only commercial bird performance measurements but intestinal pathway and tissue profiling to screen and compare feed composition and EO blend combinations would be necessary to address this hypothesis.

Finally, while terpenoids are the main constituents of EOs, their chemistry can vary widely (Jager, 2010). For instance, carvacrol is a monoterpene alcohol and, in rats, its aliphatic group readily undergo aromatic hydroxylation while its alcohol group undergoes carboxylation (Jahrmann, 2007; Jager, 2010). Thymol on the other hand forms derivatives of benzyl alcohol and 2-phenylpropanol when reduced (Austgulen et al., 1987). When carvacrol was fed to rats, their excreted urine contained more of 2-(3-Hydroxy-4-methylphenyl)propan-2-ol then carvacrol but when thyme was fed to rats it was in the highest concentration out of its five derivates (Austgulen et al., 1987). This suggests, in rats, that carvacrol undergoes chemical interactions and is metabolized more than thymol. This functional difference between EO metabolism, along with the importance of bird type and feed composition, may in part explain the variation in EOs benefit to broiler nutrition.

Although there is variation in EOs benefit to poultry growth, the antioxidant activity of many EOs is well-known (Baratta et al., 1998; Ruberto et al., 2000; Martucci et al., 2015). Rosemary oil, thymol, carvacrol, oregano, ginger, and coriander all possess antioxidant activity (Wei and Shibamoto, 2007). An oxidation deterioration test involving the application of EOs to lard indicated that 0.20% oregano possessed the most antioxidant capacity followed by thyme, dittany, marjoram, spearmint, then lavender (Economou et al., 1991). Economou et al. (1991) also found that combinations of thyme and marjoram and thyme and spearmint EOs also had potential synergistic properties in protecting lard from oxidation. These properties benefit bird health and can be marketable if EOs are used on the finished product (Lee et al., 2004b; Diaz-Sanchez et al., 2015).

Additionally, the sensory characteristics of EOs mean that they can enhance the sensory characteristics of the final products if used in the appropriate concentrations. The addition of 300 mg/kg of oregano, garlic, or an equal combination in the diets was shown to significantly improve the flavor of frozen chicken breasts up to 60 days (Kirkpinar et al., 2014). Birds fed the EOs were processed, and the breasts were stored at −25°C for sensory analysis. Overall flavor evaluated on days 1, 15, and 30 indicated that all EOs treatments scored significantly higher than the control. Overall acceptability scores of breast meat indicated that only garlic resulted in a more palatable final product on days 1, 15, and 30. Overall acceptability scores were based on flavor, appearance, and tenderness. Days 45 and 60 scores were not analyzed statistically due to spoilage. On organic seabass filets, the addition of 0.2% thyme oil improved the sensory characteristics (Kostaki et al., 2009). A panel of seven judges evaluated the filets on nine days within a 21-day storage trial where the filets were held at −30°C. While the control group reached the acceptability limit on taste in 6 days the addition of 0.2% thyme improved the sensory characteristics scores extending shelf life by 2 days. When the filets were placed in modified atmospheric packaging (MAP) (60% CO_2; 30% N_2; 10% O_2), sensory characteristics were improved to allow for a shelf life of 14 days. When thyme oil was added to the MAP, shelf life was extended by 3 days. Despite these potential benefits, their antimicrobial activities might be some of their more important attributes to commercial poultry production.

ANTIMICROBIAL MECHANISMS OF ESSENTIAL OILS

Indirect Antimicrobial Mechanisms

There are indirect characteristics associated with the presence of EOs that may play a role in reducing *Campylobacter* and other pathogen loads on the final poultry meat product. While no definitive mechanism has been elucidated, there have been several potential antimicrobial outcomes that may indirectly impact *Campylobacter*. For instance, the improved ileal absorption of amino acids within broilers, as demonstrated by Jamroz et al. (2003), may limit a required nutrient source for *Campylobacter* in the ceca (Velayudhan et al., 2004). Improvement of the immune response may also impact pathogen concentrations (Diaz-Sanchez et al., 2015). Layer hens exhibited improved antibody titer levels to Newcastle disease and infectious bursal disease when their diets were supplemented with Heryumix^{TM} (Özek et al., 2011). In another study with Heryumix^{TM}, the humoral immune response of layer hens in heat stress was not stimulated (Bozkurt et al., 2012). Basmacioğlu Malayoğlu et al. (2010), noted that broilers fed 0, 250, or 500 mg/kg of oregano exhibited IgG concentrations of 27.42, 30.50, and 39.41 mg/dL, respectively. The IgM concentrations were 7.91, 9.58, and

11.71 mg/dL, respectively (Basmacioğlu Malayoğlu et al., 2010). However, while these concentrations of antibodies were higher, they were not statistically significant ($P > 0.05$) (Basmacioğlu Malayoğlu et al., 2010). As such, further research should be conducted to elucidate the mechanism(s) of EOs on the poultry immune system. Understanding how EOs improve the immune system of poultry may be important because *Campylobacter* colonization may elicit an immune response, and therefore there is potential to reduce *Campylobacter* concentrations through immune system modulation (Connerton et al., 2018). Essential oils may also interact with *Campylobacter* populations directly.

Direct Antimicrobial Mechanisms

Much of the mechanisms associated with antimicrobial activities of EOs have been elucidated from microorganisms other than *Campylobacter,* and thus assumptions regarding *Campylobacter* must be inferred to some extent. General antimicrobial mechanisms associated with EOs have been extensively described previously by O'Bryan et al. (2015) and will be discussed briefly in the current review with specific emphasis on *Campylobacter* where applicable. Essential oils have been shown to alter proteomes and cell morphology of pathogenic bacteria (Nazzaro et al., 2013; O'Bryan et al., 2015). Significant morphological differences in cell shape have been observed when EOs such as mint, thymol, and cinnamaldehyde have been applied to bacteria (Kwon et al., 2003; Kalchayanand et al., 2004; Hajlaoui et al., 2009). For instance, the use of cinnamaldehyde on *Bacillus cereus* inhibited cell division resulting in elongated filamentous cells that were clumped together with incomplete septa (Kwon et al., 2003). After 1 h, almost all cells were in filamentous chains with no clear septas. *Salmonella enterica* serovar Thompson grown in the presence of a sub-lethal concentration of thymol (0.01%) demonstrated an altered proteomic profile compared to the control, which included downregulation of binding and chemotaxis proteins, but resulted in upregulation of other outer membrane proteins (Di Pasqua et al., 2010). In-depth analysis using 2-D PAGE, followed by MALDI-TOF, revealed that GroEL and DnaK were upregulated in the presence of thymol. GroEL, along with GroES, as well as DnaK, along with DnaJ, prevents misfolding and proper indiscriminate assembly of polypeptides under stress conditions within the cytoplasm (Fenton and Horwich, 1997; Motojima, 2015). Changes in regulation were detected by spot detection, and the relative size of the GroEL spot was 0.109 units in the control compared to a spot size of 1.044 units. The DnaK spot was not detected in the control but exhibited a size of 0.267 in the treatment with thymol. Other downregulated proteins include CheW, which is involved in transferring sensory signals from chemoreceptors to flagellar motor proteins, and thioredoxin docking proteins (Trx1). The spot size of CheW was 0.153 units and was not detected in the thymol treated cells where the Trx1 spot was 0.223 units in the control and not detected in the thymol treatment. Trx1 is an active oxidation–reduction protein that has been found to be involved in cell division in *E. coli*, which suggests thymol may play a role in the inhibition of bacterial population growth (Kumar et al., 2004). Trx1 refolds citrate synthase, an essential enzyme in the TCA cycle, and by downregulating Trx1 with thymol, citrate synthase was not present. In addition, enzymes of the reverse TCA cycle were upregulated in the thymol treatment such as an increase of citrate lyase from 0.08 units to 0.654. Acetate kinase was also reduced from 0.567 units to 0.19 units within *Salmonella*. These results may not be replicated in *Campylobacter* due to its incomplete glycolytic pathways and studies investigating the use of EOs to alter the proteome of *Campylobacter* should be performed.

Another proposed mechanism for the effect of EOs on bacteria such as *Campylobacter* is their potential to disrupt the outer membrane and initiate cell lysis (O'Bryan et al., 2015). Attributed largely to EOs hydrophobicity, the outer membrane lipids may be disrupted, sheared, or penetrated, allowing for an increase in permeability (Fisher and Phillips, 2009; Brenes and Roura, 2010; Guinoiseau et al., 2010). Carvacrol and thymol, in 200 mg/L concentrations, have been demonstrated to inhibit *E. coli* through fluorescent flow cytometry (Xu et al., 2008). The mechanism proposed in this study, supported by Helander et al. (1998), was that EOs disrupt the lipopolysaccharides membrane structure and alter the proton gradient (Xu et al., 2008). This effect may not occur in *Campylobacter* spp. due to their reliance on fermentation pathways and would have to be investigated (Line et al., 2010). Alterations of the lipopolysaccharide membrane can still lead to disruption of the cytoplasmic membrane and cell lysis (Xu et al., 2008). Electron microscopy has demonstrated the *E. coli* treated with oregano oil resulted in cell membrane collapse and leakage of contents (Sikkema et al., 1995; De Sousa et al., 2012). Cumin derived *p*-cymene has been demonstrated to swell bacterial cell membranes and has been suggested to be used synergistically with carvacrol to lyse bacterial membranes (Ultee et al., 2002). Other phenolic compounds have been observed to demonstrate this effect on the bacterial membrane (Cosentino et al., 1999; Juliano et al., 2000; Lambert et al., 2001; Brenes and Roura, 2010).

Essential oils have also been shown to impact Gram-positive organisms (Si et al., 2006). Cinnamaldehyde and eugenol, only when used in combination, were shown to inhibit *Staphylococcus*, *Micrococcus*, and *Bacillus* (Moleyar and Narasimham, 1992). Moreover, when considering the 300 commercially viable EOs, it is quite likely not all EOs may operate under the same mechanism (Diaz-Sanchez et al., 2015). They may even operate in concert with a series of mechanisms that represent contradictory activities against bacterial cellular processes. While some EOs blends promote the growth of beneficial bacteria others have inhibited beneficial bacteria such as *Lactobacillus*, and even some *Bacillus* species (Kivanç et al., 1991; Manzanilla et al., 2001; Delaquis et al., 2002; Jamroz et al., 2003; Donsi et al., 2011). Furthermore, the antimicrobial activity may not be attributable to one specific mechanism (Skandamis et al., 2001; Carson et al., 2002). When considering all of the proposed mechanisms it seems more than likely that multiple mechanisms are responsible for the effect of EOs against pathogens including *Campylobacter* (Diaz-Sanchez et al., 2015). As a consequence, the impact of EO blends on *Campylobacter* populations may vary considerably in pre and post-harvest applications.

ANTIMICROBIAL EFFECTS OF EOs ON *Campylobacter* IN POULTRY PRE-HARVEST ENVIRONMENTS

Transmission and Colonization of *Campylobacter* in the Poultry GIT

The use of EOs in pre-harvest environments has focused on preventing pathogen colonization or reducing their concentration in the GIT (Brenes and Roura, 2010). *Campylobacter* resides in the intestinal mucosa of the avian GIT and can be rapidly transmitted throughout a flock via the drinking water and fecal-oral route (Montrose et al., 1985; Beery et al., 1988; Keener et al., 2004). *Campylobacter* virulence factors that impact colonization include, *motA*, *fliA*, *jlpA,* and *racR* and were discussed in Upadhyay et al. (2017) and reviewed in Bolton (2015). While there is still controversy over how and when *Campylobacter* colonize the ceca, the most common route is believed to be horizontal transmission throughout the flock (Cox et al., 2010; Silva et al., 2011). Vertical transmission has been reported from parent to fertile egg and studies have detected 35% inoculation of the progeny (Clark and Bueschkens, 1985; Chuma et al., 1994; Cox et al., 2010). However, in a study with 60,000 progeny-parent breeders there was no evidence of vertical transmission, and therefore more emphasis is placed on *Campylobacter* colonization occurring through horizontal or environmental transmission (Callicott et al., 2006; Silva et al., 2011). *Campylobacter* can spread through water supplies, insects, litter, rodents, fecal content, and from bird to bird contact (Aarts et al., 1995; Adkin et al., 2006; Horrocks et al., 2009). *Campylobacter* colonization is usually detected at approximately 3 weeks of age and with concentrations rapidly reaching 10^7 CFU/g (Corry and Atabay, 2001). The ceca, containing up to 10^9 CFU/g, contains the largest concentration of *Campylobacter* within the avian GIT due to the abundance of nutrients, including amino acids, and the temperature in the avian ceca being approximately 42°C, which is optimal for *Campylobacter* growth (Stern, 2008; Gerwe et al., 2010; Troxell et al., 2015). The ceca are closed pouches between the ileum and the colon (Duke, 1986). This site is an important consideration for food safety as the ceca may rupture during poultry processing leading to contamination of the finished poultry product if not properly handled (Hargis et al., 1995).

EOs and *Campylobacter* in the Ceca

When examining responses of cecal contents *in vitro,* 20 mM (approximately 0.3%) of cinnamaldehyde, thymol, eugenol, or carvacrol were all independently effective in significantly reducing *Campylobacter* concentrations after 15 s of incubation (Kollanoor-Johny et al., 2010). By 8 h incubation, 10 mM concentrations of cinnamaldehyde, thymol, eugenol, or carvacrol were sufficient in decreasing *C. jejuni* by at least 5-log colony forming units (CFUs)/mL (Kollanoor-Johny et al., 2010). Kurekci et al. (2013) spiked 3×10^8 CFU/ml of *C. jejuni* C338 into 20-day old chicken cecal contents that previously contained no detectable *Campylobacter*. One gram of cecal contents was mixed with 19 mL of an anaerobic media containing 0.05 or 0.025% lemon myrtle oil. The media comprised of $MgSO_47H_2O$, 0.5 g; $CaCl_2$, 0.02 g; K_2HPO_4, 0.75 g; $NaH_2PO_4H_2O$, 0.25 g; yeast extract 1.0 g; resazurin, 1 mg; and cysteine-HCl, 0.5 g per liter of deionized water was kept in an anaerobic chamber (Laanbroek et al., 1977). Cultures were incubated for 48 h at 39°C and plated. While the positive control retained a concentration of 6.11 log CFU/mL, broths containing EOs reduced *Campylobacter* concentrations below the limit of detection (less than 3.3 log CFU/mL).

Caprylic acid, a component of coconut oil and palm kernel oil, significantly reduced *Campylobacter* cecal concentrations when administered in feed at concentrations below 1% (Los Santos et al., 2008, 2009). This was observed in market age and 10-day old broilers and did not affect FCR or BWG. However, the *in vivo Campylobacter* reducing effects of caprylic acid have been demonstrated to be mitigated when applied as caprylate in feed and water (Hermans et al., 2010; Metcalf et al., 2011).

Arsi et al. (2014) investigated the use of thymol, carvacrol, or a combination, as a feed amendment to prevent *C. jejuni* colonization. Ten birds per treatment were inoculated on day 3 with a 5-strain mixture of *C. jejuni* that were previously isolated from chicken ceca and susceptible to ciprofloxacin or fluoroquinolone. Birds were euthanized on day 10. Cecal *Campylobacter* counts were enumerated using *Campylobacter* Line agar (Line, 2001) and confirmed using latex agglutination. Individual strains were not distinguished. Four trials were performed of this experiment with the only difference between trials was the inclusion of a 2% (Trials 1 and 2) or 0.125% treatment group and the use of EO combinations (Trials 3 and 4). While significant reductions of *Campylobacter* in the 0.25% thymol, 2% thymol, 1% carvacrol, and 0.5% thymol and carvacrol treatments existed, these reductions were not observed across all trials and were not detected in birds fed other concentrations. Thymol, at a concentration of 0.25%, reduced *Campylobacter* by 0.6 log CFU/mL cecal contents during only one trial out of four. A 2 log CFU reduction was observed with 2% thymol for only one trial out of two. Additionally, 2 log CFU/mL cecal contents were observed for one trial using 0.5% thymol and carvacrol, but this was not repeated.

Campylobacter jejuni-infected broilers in a seeder model that were given feed coated in 0.3% trans-cinnamaldehyde did not exhibit any significant reduction in *Campylobacter* cecal populations after 1 week (Hermans et al., 2011a). In this model, six pens were set up each containing nine chickens with half of the pens receiving cinnamaldehyde. At day 15, three chicks per pen were given 10^8 CFU/mL of *C. jejuni* KC 40. At day 21, the chickens were euthanized. All ceca within the treatment groups contained *C. jejuni*. When the cecal populations of *Campylobacter* were averaged per pen, significant differences between treatment and control group were not observed. Cinnamaldehyde degrades quickly in the upper GIT of piglets, and this may explain the lack of differences within the populations for the chicken-based studies as well (Michiels et al., 2008). A commercial blend of garlic and cinnamon, Alliin Plus (Orffa, Werkendam,

Netherlands), was found to cause a 1 log CFU/g reduction of *Campylobacter* cecal counts 3 days post-infection (day 11) but no significant effects were detected on day 35 or day 42 (Guyard-Nicodeme et al., 2015).

Combinations of EOs and Other Antimicrobial Compounds

Numerous alternative antimicrobials have been implemented in pre-harvest poultry environments to inhibit foodborne pathogens such as *Campylobacter* (Umaraw et al., 2017). These include bacteriophages, bacteriocins, prebiotics, probiotics, and organic acids (OAs), and EOs (van der Wielen et al., 2000; Edris, 2007; Umaraw et al., 2017). With different mechanisms of action, these remediation techniques may have synergistic potential. As such, this section will review combinations of EOs and other alternative non-EO antimicrobials, notably OAs against *Campylobacter*.

Gracia et al. (2015) evaluated the effectiveness of a 0.03% blend of thymol, eugenol, piperine, and benzoic acid or 0.08% garlic oil. Chickens were inoculated with 0.1 mL of 10^5 CFU/mL of *C. jejuni* at day 14 by Gracia et al. (2015). On days 21 to 42, birds in treatment groups were administered the EOs benzoic acid blend or garlic oil. In the control groups, *C. jejuni* populations were 7.33 and 7.38 log CFU/g. In the treatment group with the EOs blend, concentrations of *C. jejuni* were 7.66 log CFU/g, and in the garlic oil treatment, *C. jejuni* concentrations were 7.26 log CFU/g. Both these treatments were not able to statistically reduce *Campylobacter* concentrations.

An OA and EO treatment combination was administered to broilers by Thibodeau et al. (2014). This blend contained sorbate, fumarate, and thymol. Sorbate has been demonstrated to disrupt the cell wall of Gram-negative bacteria and lower pH of the GIT, while fumarate indirectly affects intestinal bacteria by lowering the pH of the stomach (Diener et al., 1993; Papatsiros et al., 2013; Dittoe et al., 2018). Because these OAs operate differently than the proposed EO mechanism, they may have synergistic potential, although fumarate has been demonstrated to be metabolized by *C. jejuni* (Hinton, 2006). Feed was amended with 500 ppm of the EOs-OA blend and provided to broilers (Thibodeau et al., 2014). On day 14, these birds were administered 1 mL of inoculum containing 10^5 CFU/mL of two strains of *Campylobacter* (designated #1 and 2). One, two, and three weeks after inoculation *Campylobacter* concentrations were enumerated in the ceca and on the whole carcass post-processing. This experiment was repeated using a different set of strains (3 and 4) with lower adhesion properties. In the trial with strains 1 and 2, counts of *Campylobacter* were not significantly different when compared to the control. In the trial with strains 3 and 4, cecal populations were significantly higher 3 weeks after inoculation by approximately 1.5 log CFU/g, but carcass rinses were significantly lower by approximately 2 log CFU/mL. This suggests the adhesion properties may impact the efficacy of the EO treatment, however other variations between the strains may be impacting the results. These adhesion properties are necessary for binding to the intestinal cell wall within poultry, which may also be impacted by EOs (Vidanarachchi et al., 2005).

Impacts on the Intestinal Mucosal Layer and Microbiota

The proposed mechanism of protecting the intestinal mucosa from colonization has been demonstrated by studies involving prebiotics, which are typically oligosaccharides utilized for the protection of the mucosal layer or improvement of the colonization of beneficial bacteria (Lee et al., 2002; Vidanarachchi et al., 2005; Johnson et al., 2015; Roto et al., 2015; Micciche et al., 2018a; Ricke, 2018). The intestinal mucosa consists of the epithelium and lymphoid tissue along with the mucus that is primarily comprised of glycoproteins referred to as mucins (Montagne et al., 2003). Mucins range from 0.5 to 20 Mda in size, and the saccharide component of the avian mucins includes fucose (15.29 ng/μg of mucin), *N*-acetyl-galactosamine (5.3 ng/μg), *N*-acetyl-glucosamine (47.72 ng/μg), galactose (24.67 ng/μg), glucose (5.15 ng/μg), and mannose (15.44 ng/μg) (Bansil and Turner, 2006; Looft et al., 2019). These carbohydrates comprise 80% of the glycoprotein weight and are oligosaccharide chains consisting of 5 to 15 monomers attached to the protein core (Bansil and Turner, 2006). Approximately 60% of the protein core consists of serine, threonine, and proline repeats and this is interspersed with approximately 10% cysteine and connect to the oligosaccharide chains via O-glycosidic bonds (Perez-Vilar and Hill, 1999; Gongqiao et al., 2003; Bansil and Turner, 2006).

Mucus protects the GIT epithelial layer from exposure to the digestive enzymes and corrosive gastric fluids and serves as a matrix for the entrapment of bacteria (Turnberg, 1987; Perez-Vilar and Hill, 1999; Lien et al., 2001). For instance, L-fucose, serine, and cysteine of mucins have been demonstrated to exhibit a positive chemotaxis response on *C. jejuni*, attracting the bacteria to the chemical compound (Hugdahl et al., 1988). *Campylobacter jejuni* have also been found to preferentially attach to avian mucins compared to cow, deer, horses, mice, sheep, pigs, and rat mucins (Naughton et al., 2013). These entrapped bacteria can, in turn, modulate gene expression of epithelial cells, impact lymphoid cells, and affect the overall health of the host by degrading complex oligosaccharides and producing short-chain fatty acids (SCFAs) (Hooper et al., 2001; Guarner and Malagelada, 2003; Sergeant et al., 2014). In chickens, the intestinal microbiota within this mucosal layer is diverse and complex, and in the ceca, the microbiome is primarily colonized by *Firmicutes, Bacteriodetes,* and *Proteobacteria* (Wei et al., 2013).

Detectable *Campylobacter* colonization usually occurs after 14 days of age and growth is often correlated with the presence of other microbiota, although *Campylobacter's* interaction with the microbiome is poorly characterized (Hermans et al., 2011b; Indikova et al., 2015; Thibodeau et al., 2015). In humans, *C. jejuni* colonization is associated with a decrease of the butyrate producer, *Faecalibacterium*, which suggests these two bacteria may share a similar ecological niche (Hansen et al., 2014; Thibodeau et al., 2015). Lower abundances of *Lactobacillus* and *Corynebacterium* have also been associated with *C. jejuni* colonization, along with higher concentrations of *Streptococcus*

and *Ruminococcaceae* (Kaakoush et al., 2014). These findings indicate that there is an interaction between the intestinal microbiota and *C. jejuni* colonization. Therefore, utilizing EOs to alter the mucosal layer, possibly through microbiome modulation, or interfering with *C. jejuni* binding properties, would be beneficial to preventing intestinal colonization and downstream product contamination.

One mechanism for bacterial adhesion to the poultry GIT is through lectin-carbohydrate receptors (Vidanarachchi et al., 2005). Mutants of *C. jejuni* without CadF protein synthesis capabilities are unable to produce a lectin protein that specifically binds to fibronectin (Ziprin et al., 1999; Rubinchik et al., 2012). CadF is a membrane-bound protein that mediates the binding of *Campylobacter* to fibronectin within the intestinal mucosa, which is necessary for *Campylobacter* colonization (Quaroni et al., 1978; Ziprin et al., 1999; Monteville et al., 2003). CadF operates by modulating the level of tyrosine phosphorylation of paxillin, which is a focal adhesion signaling molecule (Konkel et al., 2005). These mutants were unable to colonize the poultry GIT, because of the absence of the carbohydrate binding adhesion protein (Ziprin et al., 1999). Piva and Rossi (1998) postulated that oligosaccharides, such as mannan-oligosaccharides, would bind to enterocyte receptors on pathogenic cell walls which would also prevent colonization (Micciche et al., 2018a). Essential oils have not yet been found to inhibit colonization of *Campylobacter* through preventing lectin-carbohydrate binding.

Similar to prebiotics, such as pectin and oligosaccharides, EOs have been shown to improve the qualities of the mucosal layer of the intestine (Bengmark, 1998; Vidanarachchi et al., 2005; Wang et al., 2016). As with EOs impacting the immune response in layer hens against NCV, prebiotics has been shown to upregulate immune response cells in the intestinal mucosal such as CD4+ and CD8+ (Lourenço et al., 2015). Immune responses by prebiotic supplementation have been extensively reviewed in Hardy et al. (2013). Additionally, mucins, which can subvert the adherence of pathogenic *E. coli* have been upregulated by *Lactobacillus plantarum* 299V and *Lactobacillus rhamnosus* GG (Mack et al., 1999; Hardy et al., 2013). *Lactobacillus* can be promoted by prebiotics and potentially EOs, and this can potentially create a positive feedback loop as mucins have also been demonstrated to improve bacterial growth as they can be up to 90% carbohydrate by weight (Perez-Vilar and Hill, 1999; Manzanilla et al., 2004; Eeckhaut et al., 2008; Emami et al., 2012; Hardy et al., 2013; Yousaf et al., 2017).

The influence by EOs on the intestine has primarily focused on crypt depth and mucosal thickness (Vidanarachchi et al., 2005). Broiler diets supplemented with 100 mg/kg of 5% carvacrol, 3% cinnamaldehyde, and 2% of capsicum oleoresin exhibited an impact on the jejunal mucosa. A higher jejunal wall villi layer was observed along with an increase in thickness of the mucosa layer, which helps prevent bacterial colonization (Jamroz et al., 2006). This is because while the thicker mucosa may potentially entrap more bacteria, a thick mucosal layer may also prevent adhesion to the intestinal villi and subsequent GIT colonization by decreasing the proximity of bacteria to the intestinal binding sites (Turner, 2009). Broilers supplemented with thymol or garlic powder (1 g/kg) in their diet showed similar effects in the intestinal morphology (Demir et al., 2005). However, in pigs, villi length was either decreased or unaffected by supplementation of EOs (Namkung et al., 2004; Nofrarias et al., 2006; Kroismayr et al., 2008). Prevention of bacterial adhesion would be beneficial for inhibiting *Campylobacter* but could have deleterious effects on nutrient absorption due to changes within the GIT microbial community. For instance, SCFA producing bacteria, such as *Lactobacillus* and *Bifidobacterium*, have been shown to have positive impacts on nutrient absorption and overall bird health and antimicrobial strategies that prevent their growth could be detrimental. However, EOs have been reported to improve the colonization of bacteria that are non-pathogenic and may benefit the overall health of the microbiota (Wenk, 2003; Windisch and Kroismayr, 2007).

Growth rate improvement of *Lactobacillus*, *Bifidobacteria,* and other probiotic bacteria in avian hosts is viewed as a potential mechanism inhibiting avian colonization of foodborne diseases such as *Campylobacter* (Santini et al., 2010). In simulated environments, *Lactobacillus* has been shown to inhibit the colonization of undesired bacteria such as *Campylobacter* (Chang and Chen, 2000). Four strains of *Lactobacillus* (10^4/ml) and *C. jejuni* (10^6/mL) were added to a simulated chicken digestive system consisting of pH adjusted veronal buffers. Veronal buffer contains 0.15 mM $CaCl_2$, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.1% gelatin, 1.8 mM sodium barbital, and 3.1 mM barbituric acid (Sigma-Aldrich; St. Louis, MO, United States) and was subsequently adjusted to a pH of 4.5, 4.4, 2.6, 6.2, and 6.3 to represent the crop, proventriculus, gizzard, small intestine, and large intestine, respectively. A simulated chicken digestive system was also created by adjusting each veronal buffer with 0.1N HCl or with veronal buffer at pH 9.6 to simulate passage within each intestinal organ. Within individually simulated GIT compartments, and the simulated chicken digestive system as a whole, significant *Campylobacter* reductions were observed compared to the control with no addition of *Lactobacillus*. In the gizzard, while a reduction from approximately 6 log CFU/mL to approximately 4 log CFU/mL of *Campylobacter* was observed within 90 min, likely due to the low pH, a 4 log reduction was observed when 6 log CFU/mL of *Lactobacillus* was added. These results might in part be due to the ability for *Lactobacillus* to produce bacteriocins that are inhibitory to *C. jejuni* (Messaoudi et al., 2012). Stern et al. (2006) applied purified bacteriocin OR-7, produced by *Lactobacillus salivarius* NRRL B-30514, to the feed of broilers that were challenged with *C. jejuni*. Compared to controls, 6 log CFU/g cecal content reductions were observed independently of the *C. jejuni* strain utilized.

Lactobacillus has also been known to produce SCFAs that can reduce *C. jejuni* populations (Awad et al., 2018). When SCFAs were tested *in vitro*, butyrate was determined to be bactericidal toward *C. jejuni* at a concentration of 12.5 mM, and acetate and propionate were bacteriostatic at 50 mM (Van Deun et al., 2008). *Campylobacter* has been demonstrated to produce acetate as a by-product of serine catabolism and utilize it as an energy source, which could explain the variation between it and butyrate inhibition (Parker et al., 2007; Stahl et al., 2012). This does not explain the variation between

propionate and butyrate. While it has been demonstrated that *C. jejuni* can metabolize acetate and the OA lactate, no known pathways have been elucidated for the transport or metabolism of propionate or butyrate (Wright et al., 2009; Thomas et al., 2011; Anand et al., 2016).

Lactobacillus and *Enterococcus* strains, which can produce SCFAs, were tested *in vitro* and were also found to be inhibitory to *C. jejuni* (Chaveerach et al., 2004; Allameh et al., 2017). Chaveerach et al. (2004) cultured five *Enterococcus* and five *Lactobacillus* strains from healthy chickens and grew them in Mueller-Hinton broth. The broth was subsequently centrifuged, and the supernatant was separated from the bacteria. The supernatant was neutralized to a pH of 6.2 and treated with pronase and catalase to break down bacteriocins and hydrogen peroxide, respectively. Once the supernatants were confirmed to be free of bacteria, they were applied in a well-diffusion agar assay against 10 individual strains of *C. jejuni*. The supernatant with the highest bactericidal activity came from a *Lactobacillus* strain labeled P93, which produced a zone of inhibition of 9 to 15 mm and was able to impact the growth of all 10 strains of *C. jejuni*. This strain was also grown in co-culture with *C. jejuni* C2150, each at an initial inoculum of 7 log CFU/mL, and after 48 h, *Campylobacter* was not isolated from the culture. *Campylobacter jejuni* C2150 was also grown with *Lactobacillus* strain P104, which did not exhibit any antimicrobial effects in the well-diffusion agar, but by 72 h *Campylobacter* concentrations were approximately 1.5 log CFU/mL lower than the positive control at 7.5 CFU/mL. This study indicates that not only can *Lactobacillus* directly compete for nutrients and colonization niche with *Campylobacter*, but some strains can also employ antimicrobials to reduce their population numbers further.

Plant-derived compounds such as cumin, oregano, and extracts of capsaicin, cinnamaldehyde, and carvacrol have been shown to improve the growth of *Lactobacillus*, *Bifidobacteria,* and *Enterococcus*, and therefore may indirectly impact pathogen concentrations through competitive exclusion (Kivanç et al., 1991; Jamroz et al., 2003; Manzanilla et al., 2004). Manzanilla et al. (2001) demonstrated how XT, a mixture of carvacrol, cinnamaldehyde, and capsaicin, increased *Lactobacillus* cecal counts in post-weaning pigs when provided in the feed. However, these interactions are complicated as the addition of *C. jejuni* has been attributed to an increase in *Bifidobacterium* (Thibodeau et al., 2015). Further research must be performed to determine if, or which, EOs exhibit antimicrobial qualities against *Campylobacter* that may also simultaneously support GIT bacteria that are antagonistic to *Campylobacter*. While EOs do not appear to operate as substrates for *Lactobacillus* or other probiotics and are therefore not prebiotics, their indirect impacts on the intestinal mucosal layer promote an environment suitable for the growth of "beneficial" bacteria, which in turn, have the potential to reduce *Campylobacter* concentrations. Other studies found that EOs, including cumin, orange oil, and oregano, have an inhibitory effect on *Lactobacillus* spp. (Kivanç et al., 1991; Elgayyar et al., 2001; Delaquis et al., 2002; Chalova et al., 2010). As such, there are limitations for the application of EOs to poultry that must be addressed.

Potential Limitations and Future Directions for Preharvest EOs Application

There appears to be inconsistency in responses of birds infected by *Campylobacter* when administered EOs compared to *in vitro* responses as well as among individual bird trials. Understanding the underlying factors and potential limitations may give insight into how EOs can be applied in the future. One primary limitation of EOs is that they can be rapidly absorbed in the GIT (Meunier et al., 2006). This rapid absorption has been observed in pigs and humans (Kohlert et al., 2002; Meunier et al., 2006). Absorption by the stomach and small intestine before they can affect cecal concentrations may be occuring in *in vivo* studies (Arsi et al., 2014). To test this potential limitation, intestinal absorption resistant EOs derivatives can be applied. Thymol-β-d-glucopyranoside is more resistant to intestinal absorption than thymol and has been shown to have similar antimicrobial effects *in vitro* (Epps et al., 2015). However, while a 1 log reduction of *Campylobacter* was observed in the crops of market-aged broilers with this absorption-resistant compound, no significant impacts were found in the ceca with either thymol-based treatment (Epps et al., 2015).

Microencapsulation may also be utilized (Calo et al., 2015). Microencapsulation is a process in which liquid particles are surrounded in polymeric compounds (Bansode et al., 2010). Typical water-soluble coating materials can include gelatin, gum arabic, and polyacrylic acid (Jyothi et al., 2012). The intention of microencapsulation with preharvest remediation techniques is to prevent the therapeutic compound from absorption before reaching the target area within the GIT (Van Immerseel et al., 2004). For instance, Pan et al. (2014) found that encapsulated thymol inhibited pathogens more effectively in milk due to enhanced solubility. As such, microencapsulated EOs have been suggested as a methodology for improving the *in vivo* effects of EOs (Calo et al., 2015). Utilizing a feed-based microencapsulated blend of thymol and eugenol, along with propionic and sorbic acids, Grilli et al. (2013) was able to reduce *C. jejuni* in layer hens. During a 42-day trial, birds were provided this blend, labeled CTR, at varying concentrations (0.1, 0.3, 0.5, or 1.0%), and were infected with 10^7 CFU/mL *C. jejuni* on day 22. *Campylobacter* concentrations were measured by plating cecal contents onto modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA). The CTR blend was effective in reducing *C. jejuni* at all concentrations (0.1, 0.3, 0.5, or 1.0%) on days 35 and 43, with 1% CTR exhibiting a 5 log CFU/g reduction at day 42 compared to the control. At day 42 the 0.1% concentration reduced *Campylobacter* populations by 3 log CFU/g. In a second experiment utilizing either 0.1 or 0.3% of this blend, birds were given either the blend before or after the day 22 infection with *Campylobacter*. Statistically significant reductions were still observed within each treatment group, except with 0.1% CTR at day 35, but the reductions were significantly lower for the group given the treatment after infection instead of before. The CTR blend at a concentration of 0.3% given from day 0 to 21 reduced *C. jejuni* by 1.5 log CFU/g at day 35 where 3 log CFU/g reductions were observed when the 0.3% CTR blend was applied

on day 22. However, in a cecal loop model study by Hermans et al. (2011a), involving direct injection of the trans-cinnamaldehyde into ceca infected with *C. jejuni* they failed to detect significant reductions in *Campylobacter* concentrations. This indicates that the absorption of the EOs in the upper GIT is not the only reason for variation between *in vivo* and *in vitro* trials (Arsi et al., 2014). Further experiments utilizing microencapsulation of combined EO blends, along with comparisons with their unencapsulated counterparts, is necessary to determine the specific effectiveness of utilizing microencapsulation.

Additionally, the observed reductions using blends of EOs can help reduce the incidence of *Campylobacter* at time of slaughter, which can lead to reductions of human incidences of campylobacteriosis (Rosenquist et al., 2003; Chapman et al., 2016). Furthermore, an investigation into EOs impact on the GIT microbiome may also represent a pertinent future direction. For instance, increasing the concentrations of certain members of the GIT microbiome through EOs may generate a GIT less suitable or more hostile for initial *C. jejuni* colonization (Kaakoush et al., 2014). Once the interactions between *C. jejuni*, the microbiome, and EOs remediation are fully elucidated a more targeted remediation technique may be possible. For instance, specific EOs blends could be designed to modulate the microbiome to prevent *C. jejuni* colonization. While *in vivo* EOs application may not be able to eliminate *Campylobacter* concentrations at the time of slaughter, it does provide a hurdle that the bacteria must overcome before contamination of the final product (Leistner, 2000; Holley and Patel, 2005).

ANTIMICROBIAL EFFECTS OF EOs ON *Campylobacter* IN POULTRY PROCESSING

Campylobacter contamination on poultry products is one of the more common causes of campylobacteriosis in humans (Keener et al., 2004). Incidence rates in a study of 425 broiler carcasses over 12 months revealed 87.5% of the post-chill carcasses were contaminated with *Campylobacter* in a French slaughterhouse (Hue et al., 2010). In the United States, 52% of post-chill carcasses (n = 325) were contaminated with *Campylobacter* with 100% of the carcasses being contaminated pre-chill (Son et al., 2007). The most frequent method for this contamination event is for the GIT to rupture during processing and contaminants to spill onto the carcass (Berrang et al., 2001). If not properly treated during carcass rinses, cross-contamination can occur, especially within chiller tanks (Bashor et al., 2004). As such, to prevent cross-contamination, it is vital that carcass rinses and sprays be applied as appropriate sanitation techniques. Essential oils may serve as an alternative sanitizer for processing washes.

In vitro laboratory experiments may have more relevance to post-harvest interventions than pre-harvest mediations due to the complexity of the poultry GIT that cannot easily be modeled in a laboratory environment (White et al., 1997). For instance, in a series of benchtop studies orange oil was found to be inhibitory to *C. jejuni, C. coli, L. monocytogenes, Salmonella,* and *Pseudomonas* (O'Bryan et al., 2008; Nannapaneni et al., 2009; Chalova et al., 2010). Nannapaneni et al. (2009) tested seven orange oil fractions on 3 *Arcobacter* strains and 21 *Campylobacter* strains, including 14 *C. jejuni* strains, four of which were isolated from poultry. When viewed on disk diffusion agar, cold pressed (CP) terpeneless Valencia orange oil produced the largest zones of inhibition (in mm) of *C. jejuni* including those isolated in poultry, whereas other orange oil fractions produced more limited zones of inhibition. Valencia orange oil was also reported to be inhibitory toward *C. coli* and *Arcobacter*. Kurekci et al. (2013) found tea tree oil at a concentration of 0.001% to be inhibitory against two strains of *C. jejuni* on nutrient agar. Wild carrot oil, when used in agar plates as an antimicrobial against *Campylobacter* spp. including a multidrug-resistant strain, *C. jejuni* 99T403, exhibited a minimum inhibitory concentration (MIC) of 125 to 500 μg/mL depending on the species or strain (Rossi et al., 2007). Isoeugenol and E-methyl isoeugenol are extracts of carrot oil and when tested against *C. jejuni* resulted in an MIC of 125 μg/mL (Rossi et al., 2007). Thymol, in a concentration of 0.25 μmol/mL, reduced *C. jejuni* and *C. coli* by 5 logs CFU/mL in Bolton broth from an initial concentration of 7 log CFU/mL (Anderson et al., 2009; Carocho et al., 2014). An EO extract from *Origanum minutiflorum*, composed primarily of carvacrol and *p*-cymene, has been shown to be effective in inhibiting *C. jejuni* in concentrations as low as 12.5 μg/mL on Mueller-Hinton agar (Aslim and Yucel, 2008). However, these MICs were strain-specific, as of the 12 *C. jejuni* strains tested, only *C. jejuni* 118d was inhibited at the concentration of 12.5 μg/mL, while *C. jejuni* 113k, 7d, and 9a required 700 μg/mL to be inhibited (Aslim and Yucel, 2008). Cinnamon, clove, thyme, and bay leaf oils were found to be bacteriostatic at a concentration of 0.075% against *C. jejuni, S.* Enteritidis*, E. coli, Staphylococcus aureus,* and *L. monocytogenes* when tested in tryptic soy broth (TSB) (Smith-Palmer et al., 1998).

Other studies reported similar inhibitory and bacteriostatic effects of oregano, eucalyptus, marigold, ginger, jasmine, cedarwood, carrot, mugwort, bergamot, and other EOs, against *Campylobacter* and other foodborne pathogens (Friedman et al., 2002; Moreira et al., 2005; Fisher and Phillips, 2006; Thanissery et al., 2014). The MIC of EOs on other foodborne pathogens were collected and reviewed in Hyldgaard et al. (2012). Friedman et al. (2002) tested 119 EOs against *C. jejuni, E. coli* O157:H7*, L. monocytogenes,* and *S. enterica* in PBS for 60 min at 37°C (42°C for *C. jejuni*) followed by plating on appropriate media for each species, including an iron-supplemented Brucella agar for *C. jejuni.* The EO concentration that resulted in the bactericidal activity of a 50% CFU decrease relative to the control was determined for each EO bacterial species combination. Marigold taegetes (0.003%), ginger root (0.005%), jasmine (0.006%), patchouli (0.007%), and gardenia (0.007%) were the most effective oils against *C. jejuni* RM1221 followed by cedarwood (0.0075%), carrot seed (0.0078%), celery seed (0.0085%), mugwort (0.009%), spikenard (0.009%), and orange bitter oils (0.009%). In this study, 39 EOs were tested against all four foodborne pathogens. When their bactericidal activities were averaged, the five most effective EOs were cinnamaldehyde (0.03%), thymol (0.05%), Spanish oregano

(0.05%), carvacrol (0.06%), and *Oregano origanum* (0.06%). Of these five EOs, *C. jejuni* was impacted the most by the lowest percent concentration compared to the other tested foodborne pathogens.

This suggests that not only may *C. jejuni* be a prime target for EO remediation, but EO that have not been extensively tested may be more optimal candidates to utilize, such as marigold and jasmine. Disk diffusion methods indicated that lemon, sweet orange and bergamot were effective against *L. monocytogenes, S. aureus, B. cereus, E. coli* O157:H7, and *C. jejuni* (Fisher and Phillips, 2006). Each oil was added to a 2 cm disk and then placed on agar plates in the presence of the bacteria of interest, with *C. jejuni* SR 117 being plated on *Campylobacter* agar base with 5% horse blood and incubated at 42°C. No zones of inhibition were detected for *C. jejuni* when orange or citral was added. Bergamot produced a zone of inhibition of 23 mm, and lemon produced a zone of inhibition of 18 mm, which were the smallest zones compared to the other tested bacteria. The zone of inhibition for linalool was greater than 90 mm. The MIC was also determined for bergamot (greater than 4%), lemon (greater than 4%), and linalool (0.06%). While no zone of inhibition was visually detected for orange oil by Fisher and Phillips (2006), when orange oil was supplemented with thyme, the combination EOs demonstrated antimicrobial activity against *Campylobacter* (Thanissery et al., 2014). Similar to Fisher and Phillips (2006), a disk diffusion test was performed using thyme, orange, rosemary, clove, and a 1:1 ratio of thyme and orange oil. This was tested on *C. jejuni* 11601 MD, *C. jejuni* RM1221, and *C. coli* RM2228 along with a cocktail of the three strains. No visible growth on disk diffusion assay across all strains and in the cocktail was visualized when exposed to thyme or clove. For the cocktail, orange oil, rosemary, and the combination of thyme and orange oil produced zones of inhibition of 17, 11, and 20 mm, respectively. A macro-broth dilution assay was also performed using Mueller Hinton broth to determine the MIC and minimum bactericidal concentration (MBC) of these oils. However, the exact MIC and MBC was not determined as 0.0008% concentrations of the oils were sufficient for inhibiting bacterial growth.

Synergistic effects have also been detected (Nguefack et al., 2012). For instance, a combination of oregano and thyme or oregano and cinnamaldehyde required 80% less EOs to produce the same inhibitory effects in nutrient broth with a *Campylobacter* growth supplement (Navarro et al., 2015). Thymol and geraniol reduced *Clostridium difficile* in feces at 500 ppm, which was five times the concentration utilized for a significant reduction in pure cultures. When 0.16 mg/mL of rosemary oil, consisting of carnosic acid, carnosol, and rosmarinic acid was applied in the laboratory, a 2 log CFU/mL reduction in *C. jejuni* was observed in Mueller-Hinton broth when the initial concentration was at 7 log CFU/mL (Klancnik et al., 2009; Piskernik et al., 2011). However, four times the concentration was needed to achieve the same reduction in chicken meat juice, isolated from thawed previously frozen carcasses, unless supplemented with nisin, a bacteriocin (Piskernik et al., 2011). While the complete composition was not provided in Piskernik et al. (2011), it was suggested that lipids and proteins within the juice matrix might have partly inhibited the EO therapy. Burt (2004) suggested that the decreased EO effectiveness in the food matrix compared to the broth experiment may be because in an oil-in-water emulsion that allows the bacteria to grow as films and in colonies, which can shield interior cells from therapeutics. As a consequence, while *in vitro* laboratory models are important, to elucidate the potential impact of EOs, poultry product matrices as well as processing environments must be considered.

The use of EOs in the post-harvest environment has focused on their bactericidal activity within carcasses washes and finished products (Calo et al., 2015; Dima and Dima, 2015). A 0.5% 50:50 mixture of thyme oil and orange oil was used in a marinade for chicken wings dip-inoculated with 10^7 CFU/mL of a nalidixic acid resistant strain of *C. coli* (Thanissery and Smith, 2014). While cross-contamination events were observed via the marinade, *C. coli* concentrations on treated wings were reduced by 3.0 log CFU/mL, as determined through plating of rinsates. Skinless chicken breasts experimentally infected with 5×10^5 CFU/g of *C. jejuni* were subjected to stinkwort (0.2%; *Inula graveolens*), bay leaf (0.6%; *Laurus nobilis*), mastic tree (0.6%; *Pistacia lentiscus*), and winter savory (0.6%; *Atureja gontana*) (Djenane et al., 2012). After 8 days of refrigerated storage under microaerophilic conditions, greater than 5 log CFU/g reductions of *C. jejuni* were observed compared to the control inoculated with 5×10^5 CFU/g. Sensory analysis indicated these EOs improved or did not impact the odor of the refrigerated samples according to a six member trained panel. Within 60 s, 0.06% linalool oil reduced *C. jejuni* on 2 cm × 2 cm pieces of cabbage leaf and chicken skin by greater than 5 log CFU and 2 log CFU, respectively (Fisher and Phillips, 2006). Cold pressed Valencia orange oil has also been shown to reduce *C. jejuni* UAF 244 on retail chicken thighs and legs (Nannapaneni et al., 2009). Chicken thighs and legs were dipped in a 0.8% saline solution containing 10^6 CFU/mL *C. jejuni* UAF 244 for 5 min and then submerged for 2 min in 20% (v/v) Valencia orange oil or 20% (v/v) limonene. The samples were then rinsed and plated. Across both types of chicken pieces, 1.5 to 2 log CFU/mL reductions were observed, compared to the control. Treatments with limonene resulted in reductions without detectable recovery of viable bacterial cells, although the limit of detection was not provided by the authors. Moreover, while taste panels have found concentrations of orange oil up to 0.1% to be acceptable in milk, chicken patties and marinades, a 20% part dip has not been investigated for impacts on sensory characteristics (Jo et al., 2004; Fisher and Phillips, 2008; Rimini et al., 2014).

Other factors may confound EO efficacy toward *Campylobacter* in poultry products. For example, one major concern for poultry industries is the ability for pathogens to form biofilms (Srey et al., 2013). Biofilms are bacterial communities within a polysaccharide matrix that can readily form and attach on processing surfaces (Costerton, 1995; Donlan and Costerton, 2002). Biofilms can be difficult to remove by antimicrobials and processing sanitizers such as chlorine and peracetic acid, which are commonly used in the industry (Frank et al., 2003; Ryu and Beuchat, 2005; Scher et al., 2005; Deborde and Von Gunten, 2008). *Campylobacter* is known to form biofilms on

stainless steel, polystyrene, and glass (Gunther and Chen, 2009). Coriander oil and its antimicrobial component linalool were found to affect biofilm formation of *Campylobacter* planktonic cells and pre-established biofilms (Duarte et al., 2016). When coriander and linalool were used at 2 μg/mL (approximately four times the MIC) *C. jejuni* and *C. coli* biofilms were reduced in size by 70 to 80% after 48-h incubation in a crystal violet assay (Duarte et al., 2015, 2016). Even when using half the MIC, coriander, and linalool reduced the biofilm by 20 to 25% (Duarte et al., 2016). Results of biofilm inhibition varied more wildly from planktonic cells, but all concentrations (0.025 to 2 μg/mL) successfully inhibited biofilm growth compared to the control, with linalool reducing some biofilm formation between 10 and 20% of the control (Duarte et al., 2016). Thyme, oregano, and cinnamon EOs, when used at concentrations below the MIC, were also found effective against biofilms of *Acinetobacter, Sphingomonas,* and *Stenotrophomonas* spp. that were isolated from biofilms within the food industry (Szczepanski and Lipski, 2014). *Sphingomonas* biofilms were reduced by 50% by thyme oil at a concentration of 0.001% where the MIC was 0.008% (Szczepanski and Lipski, 2014). Similar results were found using thyme and balsam on *Pseudomonas* and *S. aureus* biofilms (Kavanaugh and Ribbeck, 2012; Kerekes et al., 2015).

CONCLUSIONS

The effectiveness of EOs in poultry has not been clearly defined yet. Product advantages have been noted in several studies, but studies also exist that display no impact on FCR or BWG. Other advantages of EOs include the potentially improved flavor of the carcass, antioxidant capacity, and improved feed digestibility. There is also evidence that EOs can be utilized *in vitro* to impact pathogen concentrations, including *Campylobacter*. However, this depends largely on the EO utilized as the mechanism(s) of action are not well-defined. With over 300 commercially available EOs, precisely elucidating the underlying mechanisms may prove difficult (Bajpai et al., 2012). Less information is available regarding the mechanistic role of EOs used *in vivo*. Their potential ability to improve amino acid absorption in the ileum may allow for generating a GIT environment unfavorable to *Campylobacter* in the ceca due to diminished substrate availability, which is further downstream. To fully elucidate the impact EOs have on *Campylobacter* concentrations *in vivo*, further research on the mechanism(s) and effects of EOs must be performed.

More targeted delivery of EOs to certain sites in the avian GIT may be warranted as well. Microencapsulation may help to stabilize the chemical activity of the EO until it reaches its target site in the GIT, thus ensuring less variability. Microencapsulation also holds promise in addressing the intestinal absorption of an antimicrobial. However, investigations into the duration the encapsulated EOs remains in the GIT need to be performed to see when the remediation should be administered. In post-harvest settings, further studies should be performed involving the addition of EOs to sprays, washes, or within the chiller tank.

To determine the specific antimicrobial effects of EOs, the mechanism(s) must be elucidated. This is essential because, with a wide variety of EOs, there may be multiple mechanisms at work and thus synergistic potential which cannot be determined without proper identification of the mode of inhibitory action for each individual EO. Toward this effort, molecular approaches such as transcriptomics and proteomics may be employed to determine which pathways EOs inhibit will lead to further understanding of their impact on *Campylobacter*. While there have been suggestions to utilize EOs as a hurdle technology in poultry production pre- and post-harvest, mechanisms of action against *Campylobacter* and the optimal GIT locations and processing steps must first be established before any practical recommendations can be given.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

AM was supported by a Distinguished Doctoral Fellowship and support from the Department of Food Science at the University of Arkansas.

REFERENCES

Aarts, H. J., Van Lith, L. A., and Jacobs-Reitsma, W. F. (1995). Discrepancy between Penner serotyping and polymerase chain reaction fingerprinting of *Campylobacter* isolated from poultry and other animal sources. *Lett. Appl. Microbiol.* 20, 371–374. doi: 10.1111/j.1472-765x.1995.tb01324.x

Adkin, A., Hartnett, E., Jordan, L., Newell, D., and Davidson, H. (2006). Use of systematic review to assist the development of *Campylobacter* control strategies in broilers. *J. Appl. Microbiol.* 100, 306–315. doi: 10.1111/j.1365-2672.2005.02781.x

Alcicek, A., Bozkurt, M., and Çabuk, M. (2004). The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. *S. Afr. J. Anim. Sci.* 34, 217–222.

Allameh, S. K., Ringø, E., Yusoff, F. M., Daud, H. M., and Ideris, A. (2017). Dietary supplement of *Enterococcus faecalis* on digestive enzyme activities, short chain fatty acid production, immune system response and disease resistance of Javanese carp (*Puntius gonionotus*, Bleeker 1850). *Aquac. Nutr.* 23, 331–338. doi: 10.1111/anu.12397

Allen, K. J., and Griffiths, M. W. (2001). Effect of environmental and chemotactic stimuli on the activity of the *Campylobacter jejuni flaA* sigma (28) promoter. *FEMS Microbiol. Lett.* 205, 43–48. doi: 10.1016/s0378-1097(01)00444-x

Amerah, A. M., Mathis, G., and Hofacre, C. L. (2012). Effect of xylanase and a blend of essential oils on performance and *Salmonella* colonization of broiler chickens challenged with *Salmonella* Heidelberg. *Poult. Sci.* 91, 943–947. doi: 10.3382/ps.2011-01922

Anand, S., Kaur, H., and Mande, S. S. (2016). Comparative *in silico* analysis of butyrate production pathways in gut commensals and pathogens. *Front. Microbiol.* 7:1945. doi: 10.3389/fmicb.2016.01945

Anderson, R. C., Krueger, N. A., Byrd, J. A., Harvey, R. B., Callaway, T. R., Edrington, T. S., et al. (2009). Effects of thymol and diphenyliodonium chloride

against *Campylobacter* spp. during pure and mixed culture in vitro. *J. Appl. Microbiol.* 107, 1258–1268. doi: 10.1111/j.1365-2672.2009.04308.x

Ansari-Lari, M., Hosseinzadeh, S., Shekarforoush, S. S., Abdollahi, M., and Berizi, E. (2011). Prevalence and risk factors associated with *Campylobacter* infections in broiler flocks in Shiraz, southern Iran. *Int. J. Food Microbiol.* 144, 475–479. doi: 10.1016/j.ijfoodmicro.2010.11.003

Arsi, K., Donoghue, A. M., Venkitanarayanan, K., Kollanoor-Johny, A., Fanatico, A. C., Blore, P. J., et al. (2014). The efficacy of the natural plant extracts, thymol and carvacrol against *Campylobacter* colonization in broiler chickens. *J. Food Saf.* 34, 321–325. doi: 10.1111/jfs.12129

Aslim, B., and Yucel, N. (2008). In vitro antimicrobial activity of essential oil from endemic *Origanum minutiflorum* on ciprofloxacin-resistant *Campylobacter* spp. *Food Chem.* 107, 602–606. doi: 10.1016/j.foodchem.2007.08.048

Austgulen, L. T., Solheim, E., and Schelin, R. R. (1987). Metabolism in rats of p-cymene derivates: carvacrol and thymol. *Pharmacol. Toxicol.* 61, 98–102. doi: 10.1111/j.1600-0773.1987.tb01783.x

Awad, W. A., Hess, C., and Hess, M. (2018). Re-thinking the chicken–*Campylobacter jejuni* interaction: a review. *Avian Pathol.* 47, 352–363. doi: 10.1080/03079457.2018.1475724

Bajpai, V. K., Baek, K.-H., and Kang, S. C. (2012). Control of *Salmonella* in foods by using essential oils: a review. *Food Res. Int.* 45, 722–734. doi: 10.1016/j.foodres.2011.04.052

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. (2008). Biological effects of essential oils: a review. *Food Chem. Toxicol.* 46, 446–475.

Bansil, R., and Turner, B. S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. *Curr. Opin. Colloid Interface Sci.* 11, 164–170. doi: 10.1016/j.cocis.2005.11.001

Bansode, S. S., Banarjee, S. K., Gaikwad, D. D., Jadhav, S. L., and Thorat, R. M. (2010). Microencapsulation: a review. *Int. J. Pharm. Sci. Rev. Res.* 1, 38–43.

Baratta, M. T., Dorman, H. D., Deans, S. G., Figueiredo, A. C., Barroso, J. G., and Ruberto, G. (1998). Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour Fragr. J.* 13, 235–244. doi: 10.1002/(sici)1099-1026(1998070)13%3A4%3C235%3A%3Aaid-ffj733%3E3.3.co%3B2-k

Bashor, M. P., Curtis, P. A., Keener, K. M., Sheldon, B. W., Kathariou, S., and Osborne, J. A. (2004). Effects of carcass washers on *Campylobacter* contamination in large broiler processing plants. *Poult. Sci.* 83, 1232–1239. doi: 10.1093/ps/83.7.1232

Basmacioğlu Malayoğlu, H., Baysal, Ş., Misirlioğlu, Z., Polat, M., Yilmaz, H., and Turan, N. (2010). Effects of oregano essential oil with or without feed enzymes on growth performance, digestive enzyme, nutrient digestibility, lipid metabolism and immune response of broilers fed on wheat–soybean meal diets. *Br. Poult. Sci.* 51, 67–80. doi: 10.1080/00071660903573702

Batz, M. B., Hoffmann, S., and Morris, J. G. Jr. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *J. Food Prot.* 75, 1278–1291. doi: 10.4315/0362-028X.JFP-11-418

Baurhoo, B., Ruiz-Feria, C. A., and Zhao, X. (2008). Purified lignin: nutritional and health impacts on farm animals—A review. *Anim. Feed Sci. Technol.* 144, 175–184. doi: 10.1016/j.anifeedsci.2007.10.016

Beery, J. T., Hugdahl, M. B., and Doyle, M. P. (1988). Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54, 2365–2370.

Benavides, S., Villalobos-Carvajal, R., and Reyes, J. E. (2012). Physical, mechanical and antibacterial properties of alginate film: effect of the crosslinking degree and oregano essential oil concentration. *J. Food Eng.* 110, 232–239. doi: 10.1016/j.jfoodeng.2011.05.023

Bengmark, S. (1998). Immunonutrition: role of biosurfactants, fiber, and probiotic bacteria. *Nutrition* 14, 585–594. doi: 10.1016/s0899-9007(98)00030-6

Berrang, M. E., Buhr, R. J., Cason, J. A., and Dickens, J. A. (2001). Broiler carcass contamination with *Campylobacter* from feces during defeathering. *J. Food Prot.* 64, 2063–2066. doi: 10.4315/0362-028x-64.12.2063

Bolton, D. J. (2015). *Campylobacter* virulence and survival factors. *Food Microbiol.* 48, 99–108. doi: 10.1016/j.fm.2014.11.017

Bozkurt, M., Küçükyilmaz, K., Catli, A. U., Çınar, M., Bintaş, E., and Çöven, F. (2012). Performance, egg quality, and immune response of laying hens fed diets supplemented with mannan-oligosaccharide or an essential oil mixture under moderate and hot environmental conditions. *Poult. Sci.* 91, 1379–1386. doi: 10.3382/ps.2011-02023

Brenes, A., and Roura, E. (2010). Essential oils in poultry nutrition: main effects and modes of action. *Anim. Feed Sci. Technol.* 158, 1–14. doi: 10.1016/j.anifeedsci.2010.03.007

Buchbauer, G., Jirovetz, L., and Jäger, W. (1991). Aromatherapy: evidence for sedative effects of the essential oil of lavender after inhalation. *Z. Naturforsch. C* 46, 1067–1072. doi: 10.1515/znc-1991-11-1223

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

Cabuk, M., Bozkurt, M., Alcicek, A., Akbaþ, Y., and Küçükyýlmaz, K. (2006). Effect of a herbal essential oil mixture on growth and internal organ weight of broilers from young and old breeder flocks. *S. Afr. J. Anim. Sci.* 36, 135–141.

Callaway, T. R., Carroll, J. A., Arthington, J. D., Edrington, T. S., Anderson, R. C., Ricke, S. C., et al. (2011). "Citrus products and their use against bacteria: potential health and cost benefits," in *Nutrients, Dietary Supplements, and Nutriceuticals*, eds R. R. Watson, J. K. Gerald, and V. R. Preedy (New York, NY: Humana Press), 277–286. doi: 10.1007/978-1-60761-308-4_17

Callicott, K. A., Friðriksdóttir, V., Reiersen, J., Lowman, R., Bisaillon, J. R., Gunnarsson, E., et al. (2006). Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. *Appl. Environ. Microbiol.* 72, 5794–5798. doi: 10.1128/aem.02991-05

Calo, J. R., Crandall, P. G., O'Bryan, C. A., and Ricke, S. C. (2015). Essential oils as antimicrobials in food systems–A review. *Food Control* 54, 111–119. doi: 10.1016/j.foodcont.2014.12.040

Carocho, M., Barreiro, M. F., Morales, P., and Ferreira, I. C. (2014). Adding molecules to food, pros and cons: a review on synthetic and natural food additives. *Compr. Rev. Food Sci. Food Saf.* 13, 377–399. doi: 10.1111/1541-4337.12065

Carson, C. F., Mee, B. J., and Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob. Agents Chemother.* 46, 1914–1920. doi: 10.1128/aac.46.6.1914-1920.2002

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C. A., Keseler, I. M., et al. (2015). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res.* 44, D471–D480. doi: 10.1093/nar/gkv1164

Centers for Disease Control and Prevention [CDC] (2017a). *Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data)*. Atlanta, GA: U.S. Department of Health and Human Services.

Centers for Disease Control and Prevention [CDC] (2017b). *Reports of Selected Campylobacter Outbreak Investigations*.

Centers for Disease Control and Prevention [CDC] (2018). *Campylobacter (Campylobacteriosis)*.

Cervantes, H. M. (2015). Antibiotic-free poultry production: is it sustainable? *J. Appl. Poult. Res.* 24, 91–97. doi: 10.3382/japr/pfv006

Chalova, V. I., Crandall, P. G., and Ricke, S. C. (2010). Microbial inhibitory and radical scavenging activities of cold-pressed terpeneless Valencia orange (*Citrus sinensis*) oil in different dispersing agents. *J. Sci. Food Agric.* 90, 870–876. doi: 10.1002/jsfa.3897

Chang, M. H., and Chen, T. C. (2000). Reduction of *Campylobacter jejuni* in a simulated chicken digestive tract by lactobacilli cultures. *J. Food Prot.* 63, 1594–1597. doi: 10.4315/0362-028x-63.11.1594

Chapman, B., Otten, A., Fazil, A., Ernst, N., and Smith, B. A. (2016). A review of quantitative microbial risk assessment and consumer process models for *Campylobacter* in broiler chickens. *Microb. Risk Anal.* 2, 3–15. doi: 10.1016/j.mran.2016.07.001

Chaveerach, P., Lipman, L. J. A., and Van Knapen, F. (2004). Antagonistic activities of several bacteria on in vitro growth of 10 strains of *Campylobacter jejuni/coli*. *Int. J. Food Microbiol.* 90, 43–50. doi: 10.1016/s0168-1605(03)00170-3

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

Chuma, T., Yamada, T., Yano, K., Okamoto, K., and Yugi, H. (1994). A survey of *Campylobacter jejuni* in broilers from assignment to slaughter using DNA-DNA hybridization. *J. Vet. Med. Sci.* 56, 697–700. doi: 10.1292/jvms.56.697

Clark, A. G., and Bueschkens, D. H. (1985). Laboratory infection of chicken eggs with *Campylobacter jejuni* by using temperature or pressure differentials. *Appl. Environ. Microbiol.* 49, 1467–1471.

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'Kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome* 6:88. doi: 10.1186/s40168-018-0477-5

Corcionivoschi, N., Alvarez, L. A., Sharp, T. H., Strengert, M., Alemka, A., Mantell, J., et al. (2012). Mucosal reactive oxygen species decrease virulence by disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host Microbe* 12, 47–59. doi: 10.1016/j.chom.2012.05.018

Corry, J. E. L., and Atabay, H. I. (2001). Poultry as a source of *Campylobacter* and related organisms. *J. Appl. Microbiol.* 90, 96S–114S.

Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M. L., Mascia, V., Arzedi, E., et al. (1999). *In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett. Appl. Microbiol.* 29, 130–135. doi: 10.1046/j.1472-765x.1999.00605.x

Costerton, J. W. (1995). Overview of microbial biofilms. *J. Ind. Microbiol.* 15, 137–140. doi: 10.1007/bf01569816

Cox, N. A., Richardson, L. J., Buhr, R. J., and Fedorka-Cray, P. J. (2010). *Campylobacter can Remain in Various Organs – WorldPoultry.net*.

Dang, S., Sun, L., Huang, Y., Lu, F., Liu, Y., Gong, H., et al. (2010). Structure of a fucose transporter in an outward-open conformation. *Nature* 467, 734–738. doi: 10.1038/nature09406

Davis, L., and DiRita, V. (2017). Growth and laboratory maintenance of *Campylobacter jejuni*. *Curr. Protoc. Microbiol.* 10, 8A.1.1–8A.1.7. doi: 10.1002/9780471729259.mc08a01s10

De Cesare, A., Sheldon, B. W., Smith, K. S., and Jykus, L. A. (2003). Survival and persistence of *Campylobacter* and *Salmonella* species under varying organic loads on food contact surfaces. *J. Food Prot.* 66, 1587–1594. doi: 10.4315/0362-028x-66.9.1587

De Sousa, J. P., de Araújo Torres, R., de Azerêdo, G. A., Figueiredo, R. C., da Silva Vasconcelos, M. A., and de Souza, E. L. (2012). Carvacrol and 1, 8-cineole alone or in combination at sublethal concentrations induce changes in the cell morphology and membrane permeability of *Pseudomonas fluorescens* in a vegetable-based broth. *Int. J. Food Microbiol.* 158, 9–13. doi: 10.1016/j.ijfoodmicro.2012.06.008

Deborde, M., and Von Gunten, U. R. S. (2008). Reactions of chlorine with inorganic and organic compounds during water treatment—kinetics and mechanisms: a critical review. *Water Res.* 42, 13–51. doi: 10.1016/j.watres.2007.07.025

Debruyne, L., Gevers, D., and Vandamme, P. (2008). "Taxonomy of the Family Campylobacteraceae," in *Campylobacter*, 3rd Edn, eds I. Nachamkin, C. Szymanski, and M. Blaser (Washington, DC: ASM Press), 3–25. doi: 10.1128/9781555815554.ch1

Delaquis, P. J., Stanich, K., Girard, B., and Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int. J. Food Microbiol.* 74, 101–109. doi: 10.1016/s0168-1605(01)00734-6

Demir, E., Sarica, S., Ozcan, M. A., and Suicmez, M. (2005). The use of natural feed additives as alternatives to an antibiotic growth promoter in broiler diets. *Arch. Geflugelkunde* 69, 110–116.

Denli, M., Okan, F., and Uluocak, A. N. (2004). Effect of dietary supplementation of herb essential oils on the growth performance, carcass and intestinal characteristics of quail (*Coturnix coturnix japonica*). *S. Afr. J. Anim. Sci.* 34, 174–179.

Di Pasqua, R., Mamone, G., Ferranti, P., Ercolini, D., and Mauriello, G. (2010). Changes in the proteome of *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol. *Proteomics* 10, 1040–1049. doi: 10.1002/pmic.200900568

Diaz-Sanchez, S., D'Souza, D., Biswas, D., and Hanning, I. (2015). Botanical alternatives to antibiotics for use in organic poultry production. *Poult. Sci.* 94, 1419–1430. doi: 10.3382/ps/pev014

Diener, M., Helmle-Kolb, C., Murer, H., and Scharrer, E. (1993). Effect of short-chain fatty acids on cell volume and intracellular pH in rat distal colon. *Pflügers Arch.* 424, 216–223. doi: 10.1007/bf00384345

Dima, C., and Dima, S. (2015). Essential oils in foods: extraction, stabilization, and toxicity. *Curr. Opin. Food Sci.* 5, 29–35. doi: 10.1016/j.tplants.2016.10.005

Dittoe, D. K., Ricke, S. C., and Kiess, A. S. (2018). Organic acids and potential for modifying the avian gastrointestinal tract and reducing pathogens and disease. *Front. Vet. Sci.* 5:216. doi: 10.3389/fvets.2018.00216

Díaz, E., Ferrández, A., Prieto, M. A., and García, J. L. (2001). Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* 65, 523–569. doi: 10.1128/mmbr.65.4.523-569.2001

Djenane, D., Yangueela, J., Gomez, D., and Roncales, P. (2012). perspectives on the use of essential oils as antimicrobials against *Campylobacter jejuni* CECT 7572 in retail chicken meats packaged in microaerobic atmosphere. *J. Food Saf.* 32, 37–47. doi: 10.1111/j.1745-4565.2011.00342.x

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193. doi: 10.1128/cmr.15.2.167-193.2002

Donsi, F., Annunziata, M., Sessa, M., and Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT Food Sci. Technol.* 44, 1908–1914. doi: 10.1016/j.foodchem.2018.11.078

Duarte, A., Luís, Â., Oleastro, M., and Domingues, F. C. (2016). Antioxidant properties of coriander essential oil and linalool and their potential to control *Campylobacter* spp. *Food Control* 61, 115–122. doi: 10.1016/j.foodcont.2015.09.033

Duarte, A., Martinho, A., Luís, A., Figueiras, A., Oleastro, M., Domingues, F. C., et al. (2015). Resveratrol encapsulation with methyl-b-cyclodextrin for antibacterial and antioxidant delivery applications. *LWT Food Sci. Technol.* 63, 1254–1260. doi: 10.1016/j.lwt.2015.04.004

Duke, G. E. (1986). "Alimentary canal: anatomy, regulation of feed, and motility," in *Avian Physiology*, ed. P. D. Sturkie (New York, NY: Springer-Verlag), 269–302.

Dunkley, K. D., Dunkley, C. S., Njongmeta, N. L., Callaway, T. R., Hume, M. E., Kubena, L. F., et al. (2007). Comparison of *in vitro* fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. *Poult. Sci.* 86, 801–810. doi: 10.1093/ps/86.5.801

Economou, K. D., Oreopoulou, V., and Thomopoulos, C. D. (1991). Antioxidant activity of some plant extracts of the family Labiatae. *J. Am. Oil Chem. Soc.* 68, 109–113. doi: 10.1007/bf02662329

Edris, A. E. (2007). Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother. Res.* 21, 308–323. doi: 10.1002/ptr.2072

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., et al. (2008). Arabinoxylooligosaccharides from wheat bran inhibit *Salmonella* colonization in broiler chickens. *Poult. Sci.* 87, 2329–2334. doi: 10.3382/ps.2008-00193

Elgayyar, M., Draughon, F. A., Golden, D. A., and Mount, J. R. (2001). Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *J. Food Prot.* 64, 1019–1024. doi: 10.4315/0362-028x-64.7.1019

Emami, N. K., Samie, A., Rahmani, H. R., and Ruiz-Feria, C. A. (2012). The effect of peppermint essential oil and fructooligosaccharides, as alternatives to virginiamycin, on growth performance, digestibility, gut morphology and immune response of male broilers. *Anim. Feed Sci. Technol.* 175, 57–64. doi: 10.1016/j.anifeedsci.2012.04.001

Epps, S. V., Harvey, R. B., Byrd, J. A., Petrujkiæ, B. T., Sedej, I., Beier, R. C., et al. (2015). Comparative effect of thymol or its glucose conjugate, thymol-β-D-glucopyranoside, on *Campylobacter* in avian gut contents. *J. Environ. Sci. Health Part B* 50, 55–61. doi: 10.1080/03601234.2015.965634

European Food Safety Authority [EFSA] (2010). Scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA J.* 8:1437. doi: 10.2903/j.efsa.2010.1437

Evans, W. C., and Fuchs, G. (1988). Anaerobic degradation of aromatic compounds. *Annu. Rev. Microbiol.* 42, 289–317. doi: 10.1146/annurev.micro.42.1.289

Fabricant, F. (2008). *Earl Grey Flavor, to Serve after Teatime*. New York, NY: New York Times, D2.

Facciolà, A., Riso, R., Avventuroso, E., Visalli, G., Delia, S. A., and Laganà, P. (2017). *Campylobacter*: from microbiology to prevention. *J. Prev. Med. Hyg.* 58:E79.

Fenton, W. A., and Horwich, A. L. (1997). GroEL mediated protein folding. *Prot. Sci.* 6, 743–760. doi: 10.1002/pro.5560060401

Fisher, K., and Phillips, C. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends Food Sci. Technol.* 19, 156–164. doi: 10.1016/j.tifs.2007.11.006

Fisher, K., and Phillips, C. (2009). The mechanism of action of a citrus oil blend against *Enterococcus faecium* and *Enterococcus faecalis*. *J. Appl. Microbiol.* 106, 1343–1349. doi: 10.1111/j.1365-2672.2008.04102.x

Fisher, K., and Phillips, C. A. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems. *J. Appl. Microbiol.* 101, 1232–1240. doi: 10.1111/j.1365-2672.2006.03035.x

Fitzgerald, C., and Nachamkin, I. (2011). "*Campylobacter* and *Arcobacter*," in *Manual of Clinical Microbiology*, eds J. Versalovic, K. Carroll, G. Funke, J. Jorgensen, M. L. Landry, and D. W. Warnock (Washington, DC: ASM Press), 885–899.

Frank, J. F., Ehlers, J., and Wicker, L. (2003). Removal of *Listeria monocytogenes* and poultry soil-containing biofilms using chemical cleaning and sanitizing agents under static conditions. *Food Prot. Trends* 23, 654–663.

Franz, C., Baser, K. H. C., and Windisch, W. (2010). Essential oils and aromatic plants in animal feeding–a European perspective. A review. *Flavour Fragr. J.* 25, 327–340. doi: 10.1002/ffj.1967

Frazer, A. C. (1994). "O-demethylation and other transformations of aromatic compounds by acetogenic bacteria," in *Acetogenesis*, ed. H. L. Drake (Boston, MA: Springer), 445–483. doi: 10.1007/978-1-4615-1777-1_17

Friedman, C. R., Hoekstra, R. M., Samuel, M., Marcus, R., Bender, J., Shiferaw, B., et al. (2004). Risk factors for sporadic *Campylobacter* infection in the United States: a case-control study in FoodNet sites. *Clin. Infect. Dis.* 38(Suppl._3), S285–S296.

Friedman, M., Henika, P. R., and Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65, 1545–1560. doi: 10.4315/0362-028x-65.10.1545

Gangaiah, D., Liu, Z., Arcos, J., Kassem, I. I., Sanad, Y., Torrelles, J. B., et al. (2010). Polyphosphate kinase 2: a novel determinant of stress responses and pathogenesis in *Campylobacter jejuni*. *PLoS One* 5:e12142. doi: 10.1371/journal.pone.0012142

Garénaux, A., Jugiau, F., Rama, F., De Jonge, R., Denis, M., Federighi, M., et al. (2008). Survival of *Campylobacter jejuni* strains from different origins under oxidative stress conditions: effect of temperature. *Curr. Microbiol.* 56, 293–297. doi: 10.1007/s00284-007-9082-8

Geissler, C., and Powers, H. (2011). "Chapter 3: food safety," in *Human Nutrition*, 12th Edn, eds C. Geissler and H. Powers (Philadelphia, PA: Churchill Livingstone Elsevier), 51–68.

Gerwe, T., Bouma, A., Wagenaar, J. A., Jacobs-Reitsma, W. F., and Stegeman, A. (2010). Comparison of *Campylobacter* levels in crops and ceca of broilers at slaughter. *Avian Dis.* 54, 1072–1074. doi: 10.1637/9113-101809-resnote.1

Gongqiao, X. U., Khatri, I. A., Wang, R., Rahman, S., and Forstner, J. F. (2003). N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2. *Biochem. J.* 373, 893–900. doi: 10.1042/bj20030096

Gonzalez-Molina, E., Moreno, D. A., and Garcia-Viguera, C. (2009). A new drink rich in healthy bioactives combining lemon and pomegranate juices. *Food Chem.* 115, 1364–1372. doi: 10.1016/j.foodchem.2009.01.056

Gous, R. M. (2010). Nutritional limitations on growth and development in poultry. *Livest. Sci.* 130, 25–32. doi: 10.1016/j.livsci.2010.02.007

Gracia, M. I., Millan, C., Sanchez, J., Guyard-Nicodeme, M., Mayot, J., Carre, Y., et al. (2015). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period: part B. *Poult. Sci.* 95, 886–892. doi: 10.3382/ps/pev346

Grand View Research (2018). *Essential Oils Market Size, Share & Trends Analysis Report By Product (Orange, Corn, Mint, Eucalyptus, Citronella, Pepper Mint, Lemon, Clove Leaf, Lime, Spearmint), By Application, And Segment Forecasts, 2018 - 2025*.

Grilli, E., Vitari, F., Domeneghini, C., Palmonari, A., Tosi, G., Fantinati, P., et al. (2013). Development of a feed additive to reduce caecal *Campylobacter jejuni* in broilers at slaughter age: from *in vitro* to *in vivo*, a proof of concept. *J. Appl. Microbiol.* 114, 308–317. doi: 10.1111/jam.12053

Guarner, F., and Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet* 361, 512–519. doi: 10.1016/s0140-6736(03)12489-0

Guccione, E., Del Rocio Leon-Kempis, M., Pearson, B. M., Hitchin, E., Mulholland, F., van Diemen, P. M., et al. (2008). Amino acid-dependent growth of *Campylobacter jejuni:* key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol. Microbiol.* 69, 77–93. doi: 10.1111/j.1365-2958.2008.06263.x

Guenther, E. (ed.). (1948). "The essential oils," in *Essential Oils*, (New York, NY: D. Van Nostrand), 8–52.

Guinoiseau, E., Luciani, A., Rossi, P. G., Quilichini, Y., Ternengo, S., Bradesi, P., et al. (2010). Cellular effects induced by *Inula graveolens* and *Santolina corsica* essential oils on *Staphylococcus aureus*. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 873–879. doi: 10.1007/s10096-010-0943-x

Gunther, N. W., and Chen, C. Y. (2009). The biofilm forming potential of bacterial species in the genus *Campylobacter*. *Food Microbiol.* 26, 44–51. doi: 10.1016/j.fm.2008.07.012

Guyard-Nicodeme, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., et al. (2015). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult. Sci.* 95, 298–305. doi: 10.3382/ps/pev303

Häggblom, M. M., Rivera, M. D., and Young, L. Y. (1993). Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. *Appl. Environ. Microbiol.* 59, 1162–1167.

Hajlaoui, H., Trabelsi, N., Noumi, E., Snoussi, M., Fallah, H., Ksouri, R., et al. (2009). Biological activities of the essential oils and methanol extract of two cultivated mint species (*Mentha longifolia* and *Mentha pulegium*) used in the Tunisian folkloric medicine. *World J. Microbiol. Biotechnol.* 25, 2227–2238. doi: 10.1007/s11274-009-0130-3

Hansen, A. K., Hansen, C. H. F., Krych, L., and Nielsen, D. S. (2014). Impact of the gut microbiota on rodent models of human disease. *World J. Gastroenterol.* 20:17727. doi: 10.3748/wjg.v20.i47.17727

Hardin, A., Crandall, P. G., and Stankus, T. (2010). Essential oils and antioxidants derived from citrus by-products in food protection and medicine: an introduction and review of recent literature. *J. Agric. Food Inf.* 11, 99–122. doi: 10.1080/10496501003680680

Hardy, H., Harris, J., Lyon, E., Beal, J., and Foey, A. (2013). Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. *Nutrients* 5, 1869–1912. doi: 10.3390/nu5061869

Hargis, B. M., Caldwell, D. J., Brewer, R. L., Corrier, D. E., and DeLoach, J. R. (1995). Evaluation of the chicken crop as a source of *Salmonella* contamination for broiler carcasses. *Poult. Sci.* 74, 1548–1552. doi: 10.3382/ps.0741548

Hazeleger, W., Wouters, J., Rombuts, F., and Abee, T. (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Appl. Environ. Microbiol.* 64, 3917–3922.

Helander, I. M., Alakomi, H. L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., et al. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem.* 46, 3590–3595. doi: 10.1021/jf980154m

Hermans, D., Martel, A., Van Deun, K., Verlinden, M., Van Immerseel, F., Garmyn, A., et al. (2010). Intestinal mucus protects *Campylobacter jejuni* in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. *Poult. Sci.* 89, 1144–1155. doi: 10.3382/ps.2010-00717

Hermans, D., Pasmans, F., Heyndrickx, M., Van Immerseel, F., Martel, A., Van Deun, K., et al. (2012). A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut. *Crit. Rev. Microbiol.* 38, 17–29. doi: 10.3109/1040841X.2011.615298

Hermans, D., Martel, A., Van Deun, K., Van Immerseel, F., Heyndrickx, M., Haesebrouck, F., et al. (2011a). The cinnamon-oil ingredient trans-cinnamaldehyde fails to target *Campylobacter jejuni* strain KC 40 in the broiler chicken cecum despite marked in vitro activity. *J. Food Prot.* 74, 1729–1734. doi: 10.4315/0362-028X.JFP-10-487

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., et al. (2011b). Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet. Res.* 42:82. doi: 10.1186/1297-9716-42-82

Hernandez, F., Madrid, J., Garcia, V., Orengo, J., and Megias, M. D. (2004). Influence of two plant extracts on broilers performance, digestibility, and digestive organ size. *Poult. Sci.* 83, 169–174. doi: 10.1093/ps/83.2.169

Hinton, A. (2006). Growth of *Campylobacter* in media supplemented with organic acids. *J. Food Prot.* 69, 34–38. doi: 10.4315/0362-028x-69.1.34

Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., et al. (2006). Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infect. Immun.* 74, 4694–4707. doi: 10.1128/iai.00210-06

Holley, R. A., and Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol.* 22, 273–292. doi: 10.1016/j.fm.2004.08.006

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi: 10.1126/science.291.5505.881

Horrocks, S. M., Anderson, R. C., Nielsbet, D. J., and Ricke, S. C. (2009). Incidence and ecology of *Campylobacter jejuni* and coli in animals. *Anaerobe* 15, 18–25. doi: 10.1016/j.anaerobe.2008.09.001

Hue, O., Le Bouquin, S., Laisney, M. J., Allain, V., Lalande, F., Petetin, I., et al. (2010). Prevalence of and risk factors for *Campylobacter* spp. contamination of broiler chicken carcasses at the slaughterhouse. *Food Microbiol.* 27, 992–999. doi: 10.1016/j.fm.2010.06.004

Hugdahl, M. B., Beery, J. T., and Doyle, M. P. (1988). Chemotactic behavior of *Campylobacter jejuni*. *Infect. Immun.* 56, 1560–1566.

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* 3:12. doi: 10.3389/fmicb.2012.00012

Indikova, I., Humphrey, T. J., and Hilbert, F. (2015). Survival with a helping hand: *Campylobacter* and microbiota. *Front. Microbiol.* 6:1266. doi: 10.3389/fmicb.2015.01266

Jager, W. (2010). "Metabolism of terpenoids in animal models and humans," in *Handbook of Essential Oils: Science, Technology, and Applications*, eds K. H. C. Baser and G. Buchbauer (Boca Raton, FL: CRC Press), 209–235.

Jahrmann, R. (2007). *Metabolismus von Monterpenen und Sesquiterpenen in Mensch und Säugetier: Bedeutung für die Pharmazeutische Praxis. MPharm*. Diploma thesis, University of Vienna, Vienna.

Jamroz, D., Orda, J., Kamel, C., Wiliczkiewicz, A., Wertelecki, T., and Skorupinska, J. (2003). The influence of phytogenic extracts on performance, nutrient digestibility, carcass characteristics, and gut microbial status in broiler chickens. *J. Anim. Feed Sci.* 12, 583–596. doi: 10.22358/jafs/67752/2003

Jamroz, D., Wertelecki, T., Houszka, M., and Kamel, C. (2006). Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. *J. Anim. Physiol. Anim. Nutr.* 90, 255–268. doi: 10.1111/j.1439-0396.2005.00603.x

Jang, I. S., Ko, Y. H., Kang, S. Y., and Lee, C. Y. (2007). Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Anim. Feed Sci. Technol.* 134, 304–315. doi: 10.1016/j.anifeedsci.2006.06.009

Jang, I. S., Ko, Y. H., Yang, H. Y., Ha, J. S., Kim, J. Y., Kang, S. Y., et al. (2004). Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. *Asian Australas. J. Anim. Sci.* 17, 394–400. doi: 10.5713/ajas.2004.394

Jo, C., Kang, H. J., Lee, M., Lee, N. Y., and Byun, M. W. (2004). The antioxidative potential of lyophilized citrus peel extract in different meat model systems during storage at 20 C. *J. Muscle Foods* 15, 95–107. doi: 10.1111/j.1745-4573.2004.tb00714.x

Johnson, L. P., Walton, G. E., Psichas, A., Frost, G. S., Gibson, G. R., and Barraclough, T. G. (2015). Prebiotics modulate the effects of antibiotics on gut microbial diversity and functioning in vitro. *Nutrients* 7, 4480–4497. doi: 10.3390/nu7064480

Johnson, R. (2015). *US-EU Poultry Dispute on the use of Pathogen Reduction Treatments (PRTs)*. Washington, DC: Congressional Research Service, 7–57.

Józefiak, D., Rutkowski, A., and Martin, S. A. (2004). Carbohydrate fermentation in the avian ceca: a review. *Anim. Feed Sci. Technol.* 113, 1–15. doi: 10.1016/j.anifeedsci.2003.09.007

Juliano, C., Mattana, A., and Usai, M. (2000). Composition and in vitro antimicrobial activity of the essential oil of Thymus herba-barona Loisel growing wild in Sardinia. *J. Essent. Oil Res.* 12, 516–522. doi: 10.1080/10412905.2000.9699578

Jung, H. G., and Fahey, G. C. Jr. (1983). Nutritional implications of phenolic monomers and lignin: a review 1. *J. Anim. Sci.* 57, 206–219. doi: 10.2527/jas1983.571206x

Jyothi, S. S., Seethadevi, A., Prabha, K. S., Muthuprasanna, P., and Pavitra, P. (2012). Microencapsulation: a review. *Int. J. Pharma Bio Sci.* 3, 509–531.

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

Kalchayanand, N., Dunne, P., Sikes, A., and Ray, B. (2004). Viability loss and morphology change of foodborne pathogens following exposure to hydrostatic pressures in the presence and absence of bacteriocins. *Int. J. Food Microbiol.* 91, 91–98. doi: 10.1016/s0168-1605(03)00324-6

Kassem, I. I., Chandrashekhar, K., and Rajashekara, G. (2013). Of energy and survival incognito: a relationship between viable but non-culturable cells formation and inorganic polyphosphate and formate metabolism in *Campylobacter jejuni*. *Front. Microbiol.* 4:183. doi: 10.3389/fmicb.2013.00183

Kavanaugh, N. L., and Ribbeck, K. (2012). Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol.* 78, 4057–4061. doi: 10.1128/AEM.07499-11

Keener, K. M., Bashor, M. P., Curtis, P. A., Sheldon, B. W., and Kathariou, S. (2004). Comprehensive review of *Campylobacter* and poultry processing. *Compr. Rev. Food Sci. Food Saf.* 3, 105–116. doi: 10.1111/j.1541-4337.2004.tb00060.x

Kelly, C., Gundogdu, O., Pircalabioru, G., Cean, A., Scates, P., Linton, M., et al. (2017). The *in vitro* and *in vivo* effect of carvacrol in preventing *Campylobacter* infection, colonization and in improving productivity of chicken broilers. *Foodborne Pathog. Dis.* 14, 341–349. doi: 10.1089/fpd.2016.2265

Kerekes, E. B., Vidács, A., Török Jenei, J., Gömöri, C., Takó, M., Chandrasekaran, M., et al. (2015). "Essential oils against bacterial biofilm formation and quorum sensing of food-borne pathogens and spoilage microorganisms," in *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs. Microbiology Book Series*, Vol. 5, ed. A. Méndez-Vilas (Bajadoz: Formatex Research Center), 429–437.

Keto-Timonen, R., Hietala, N., Palonen, E., Hakakorpi, A., Lindström, M., and Korkeala, H. (2016). Cold shock proteins: a minireview with special emphasis on Csp-family of enteropathogenic *Yersinia*. *Front. Microbiol.* 7:1151. doi: 10.3389/fmicb.2016.01151

Khan, I. A., and Abourashed, E. A. (2011). *Leung's Encyclopedia of Common Natural Ingredients: Used in Food, Drugs and Cosmetics*. Hoboken, NJ: John Wiley & Sons.

Kirkpinar, F., Ünlü, H. B., Serdaroğlu, M., and Turp, G. Y. (2014). Effects of dietary oregano and garlic essential oils on carcass characteristics, meat composition, colour, pH and sensory quality of broiler meat. *Br. Poult. Sci.* 55, 157–166. doi: 10.1080/00071668.2013.879980

Kivanç, M., Akgül, A., and Dogan, A. (1991). Inhibitory and stimulatory effects of cumin, oregano and their essential oils on growth and acid production of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. *Int. J. Food Microbiol.* 13, 81–85. doi: 10.1016/0168-1605(91)90140-k

Klancnik, A., Guzej, B., Kolar, H. M., Abramovic, H., and Smole Mozina, S. (2009). In vitro antimicrobial and antioxidant activity of commercial rosemary extract formulations. *J. Food Prot.* 72, 1744–1752. doi: 10.4315/0362-028x-72.8.1744

Kohlert, C., Schindler, G., Marz, R. W., Abel, G., Brinkhaus, B., Derendorf, H., et al. (2002). Systemic availability and pharmacokinetics of thymol in humans. *J. Clin. Pharmocol.* 42, 731–737. doi: 10.1177/009127002401102678

Kollanoor-Johny, A., Darre, M. J., Donoghue, A. M., Donoghue, D. J., and Venkitanarayanan, K. (2010). Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken cecal contents in vitro. *J. Appl. Poult. Res.* 19, 237–244. doi: 10.3382/japr.2010-00181

Konkel, M. E., Christensen, J. E., Keech, A. M., Monteville, M. R., Klena, J. D., and Garvis, S. G. (2005). Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein. *Mol. Microbiol.* 57, 1022–1035. doi: 10.1111/j.1365-2958.2005.04744.x

Kostaki, M., Giatrakou, V., Savvaidis, I. N., and Kontominas, M. G. (2009). Combined effect of MAP and thyme essential oil on the microbiological, chemical and sensory attributes of organically aquacultured sea bass (*Dicentrarchus labrax*) fillets. *Food Microbiol.* 26, 475–482. doi: 10.1016/j.fm.2009.02.008

Koutsos, E. A., and Arias, V. J. (2006). Intestinal ecology: interactions among the gastrointestinal tract, nutrition, and the microflora. *J. Appl. Poult. Res.* 15, 161–173. doi: 10.1093/japr/15.1.161

Kreydiyyeh, S. I., Usta, J., and Copti, R. (2000). Effect of cinnamon, clove and some of their constituents on the Na+-K+-ATPase activity and alanine absorption in the rat jejunum. *Food Chem. Toxicol.* 38, 755–762. doi: 10.1016/s0278-6915(00)00073-9

Kroismayr, A., Sehm, J., Pfaffl, M. W., Schedle, K., Plitzner, C., and Windisch, W. (2008). Effects of avilamycin and essential oils on mRNA expression of apoptotic and inflammatory markers and gut morphology of piglets. *Czech J. Anim. Sci.* 53, 377–387. doi: 10.17221/338-cjas

Kumar, J. K., Tabor, S., and Richardson, C. C. (2004). Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3759–3764. doi: 10.1073/pnas.0308701101

Kurekci, C., Padmanabha, J., Bishop-Hurley, S. L., Hassan, E., Al Jassim, R. A., and McSweeney, C. S. (2013). Antimicrobial activity of essential oils and five terpenoid compounds against *Campylobacter jejuni* in pure and mixed culture experiments. *Int. J. Food Microbiol.* 166, 450–457. doi: 10.1016/j.ijfoodmicro.2013.08.014

Kwon, J. A., Yu, C. B., and Park, H. D. (2003). Bacteriocidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. *Lett. Appl. Microbiol.* 37, 61–65. doi: 10.1046/j.1472-765x.2003.01350.x

Laanbroek, H. J., Kingma, W., and Veldkamp, H. (1977). Isolation of an aspartate fermenting, free-living *Campylobacter* species. *FEMS Microbiol. Lett.* 1, 99–102. doi: 10.1016/0378-1097(77)90010-6

Lambert, R. J. W., Skandamis, P. N., Coote, P. J., and Nychas, G. J. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91, 453–462. doi: 10.1046/j.1365-2672.2001.01428.x

Lee, H. W., Park, Y. S., Jung, J. S., and Shin, W. S. (2002). Chitosan oligosaccharides, dp 2–8, have prebiotic effect on the *Bifidobacterium bifidum* and *Lactobacillus* sp. *Anaerobe* 8, 319–324. doi: 10.1016/s1075-9964(03)00030-1

Lee, K. W., Everts, H., and Beynen, A. (2004a). Essential oils in broiler nutrition. *Int. J. Poult. Sci.* 3, 738–752. doi: 10.3923/ijps.2004.738.752

Lee, K. W., Everts, H., Kappert, H. J., van Der Kuilen, J., Lemmens, A. G., Frehner, M., et al. (2004b). Growth performance, intestinal viscosity, fat digestibility and plasma cholesterol in broiler chickens fed a rye-containing diet without or with essential oil components. *Int. J. Poult. Sci.* 3, 613–618. doi: 10.3923/ijps.2004.613.618

Lee, K. W., Everts, H., Kappert, H. J., Frehner, M., Losa, R., and Beynen, A. C. (2003). Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. *Br. Poult. Sci.* 44, 450–457. doi: 10.1080/00071660301985

Leenstra, F. R. (1986). Effect of age, sex, genotype and environment on fat deposition in broiler chickens—a review. *Worlds Poult. Sci. J.* 42, 12–25. doi: 10.1079/wps19860002

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

Levin, R. E. (2007). *Campylobacter jejuni*: a review of its characteristics, pathogenicity, ecology, distribution, subspecies characterization and molecular methods of detection. *Food Biotechnol.* 21, 271–347. doi: 10.1080/08905430701536565

Lien, K. A., Sauer, W. C., and He, J. M. (2001). Dietary influences on the secretion into and degradation of mucin in the digestive tract of monogastric animals and humans. *J. Anim. Feed Sci.* 10, 223–246.

Lin, A. E., Krastel, K., Hobb, R. I., Thompson, S. A., Cvitkovitch, D. G., and Gaynor, E. C. (2009). Atypical roles for *Campylobacter jejuni* amino acid ATP binding cassette transporter components PaqP and PaqQ in bacterial stress tolerance and pathogen-host cell dynamics. *Infect. Immunol.* 77, 4912–4924. doi: 10.1128/IAI.00571-08

Line, J. E. (2001). Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. *J. Food Prot.* 64, 1711–1715. doi: 10.4315/0362-028x-64.11.1711

Line, J. E., Hiett, K. L., Guard-Bouldin, J., and Seal, B. S. (2010). Differential carbon source utilization by *Campylobacter jejuni* 11168 in response to growth temperature variation. *J. Microbiol. Methods* 80, 198–202. doi: 10.1016/j.mimet.2009.12.011

Liu, S., Bennett, D. C., Tun, H. M., Kim, J. E., Cheng, K. M., Zhang, H., et al. (2015). The effect of diet and host genotype on ceca microbiota of Japanese quail fed a cholesterol enriched diet. *Front. Microbiol.* 6:1092. doi: 10.3389/fmicb.2015.01092

Looft, T., Cai, G., Choudhury, B., Lai, L. X., Lippolis, J. D., Reinhardt, T. A., et al. (2019). Avian intestinal mucus modulates *Campylobacter jejuni* gene expression in a host-specific manner. *Front. Microbiol.* 9:3215. doi: 10.3389/fmicb.2018.03215

Los Santos, F. S., Donoghue, A. M., Venkitanarayanan, K., Dirain, M. L., Reyes-Herrera, I., Blore, P. J., et al. (2008). Caprylic acid supplemented in feed reduces enteric *Campylobacter jejuni* colonization in ten-day-old broiler chickens. *Poult. Sci.* 87, 800–804. doi: 10.3382/ps.2007-00280

Los Santos, F. S., Donoghue, A. M., Venkitanarayanan, K., Metcalf, J. H., Reyes-Herrera, I., Dirain, M. L., et al. (2009). The natural feed additive caprylic acid decreases *Campylobacter jejuni* colonization in market-aged broiler chickens. *Poult. Sci.* 88, 61–64. doi: 10.3382/ps.2008-00228

Lourenço, M. C., Kuritza, L. N., Hayashi, R. M., Miglino, L. B., Durau, J. F., Pickler, L., et al. (2015). Effect of a mannanoligosaccharide-supplemented diet on intestinal mucosa T lymphocyte populations in chickens challenged with *Salmonella* Enteritidis. *J. Appl. Poult. Res.* 24, 15–22. doi: 10.3382/japr/pfu002

Mack, D. R., Michail, S., Wei, S., McDougall, L., and Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am. J. Physiol.* 276, G941–G950. doi: 10.1152/ajpgi.1999.276.4.G941

Manzanilla, E. G., Baucells, F., Kamel, C., Morales, J., Perez, J. F., and Gasa, J. (2001). Effects of plant extracts on the performance and lower gut microflora of early weaned piglets. *J. Anim. Sci.* 1:473. doi: 10.1111/jpn.12976

Manzanilla, E. G., Perez, J. F., Martin, M., Kamel, C., Baucells, F., and Gasa, J. (2004). Effect of plant extracts and formic acid on the intestinal equilibrium of early– weaned pigs. *J. Anim. Sci.* 82, 3210–3218. doi: 10.2527/2004.82113210x

Martucci, J. F., Gende, L. B., Neira, L. M., and Ruseckaite, R. A. (2015). Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. *Ind. Crops Prod.* 71, 205–213. doi: 10.1016/j.indcrop.2015.03.079

Messaoudi, S., Kergourlay, G., Dalgalarrondo, M., Choiset, Y., Ferchichi, M., Prévost, H., et al. (2012). Purification and characterization of a new bacteriocin active against *Campylobacter* produced by *Lactobacillus salivarius* SMXD51. *Food Microbiol.* 32, 129–134. doi: 10.1016/j.fm.2012.05.002

Metcalf, J. H., Donoghue, A. M., Venkitanarayanan, K., Reyes-Herrera, I., Aguiar, V. F., Blore, P. J., et al. (2011). Water administration of the medium-chain fatty acid caprylic acid produced variable efficacy against enteric *Campylobacter* colonization in broilers. *Poult. Sci.* 90, 494–497. doi: 10.3382/ps.2010-00891

Meunier, J. P., Cardot, J. M., Gauthier, P., Beyssac, E., and Alric, M. (2006). Use of rotary fluidized-bed technology for development of sustained-release plant extracts pellets: potential application for feed additive delivery. *J. Anim. Sci.* 84, 1850–1859. doi: 10.2527/jas.2005-361

Micciche, A. C., Foley, S. L., Pavlidis, H. O., McIntyre, D. R., and Ricke, S. C. (2018a). A review of prebiotics against *Salmonella* in poultry: current and future potential for microbiome research applications. *Front. Vet. Sci.* 5:191. doi: 10.3389/fvets.2018.00191

Micciche, A. C., Rubinelli, P. M., and Ricke, S. C. (2018b). Source of water and potential sanitizers and biological antimicrobials for alternative poultry processing food safety applications. *Front. Sustain. Food Syst.* 2:82. doi: 10.3389/fsufs.2018.00082

Michiels, J., Missotten, J., Dierick, N., Fremaut, D., Maene, P., and De Smet, S. (2008). *In vitro* degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and trans-cinnamaldehyde along the gastrointestinal tract of piglets. *J. Sci. Food Agric.* 88, 2371–2381. doi: 10.1002/jsfa.3358

Mohammadhosseini, M., Sarker, S. D., and Akbarzadeh, A. (2017). Chemical composition of the essential oils and extracts of *Achillea* species and their biological activities: a review. *J. Ethnopharmacol.* 199, 257–315. doi: 10.1016/j.jep.2017.02.010

Moleyar, V., and Narasimham, P. (1992). Antibacterial activity of essential oil components. *Int. J. Food Microbiol.* 16, 337–342. doi: 10.1016/0168-1605(92)90035-2

Montagne, L., Pluske, J. R., and Hampson, D. J. (2003). A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim. Feed Sci. Technol.* 108, 95–117. doi: 10.1016/s0377-8401(03)00163-9

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

Montrose, M. S., Shane, S. M., and Harrington, K. S. (1985). Role of litter in the transmission of *Campylobacter jejuni*. *Avian Dis.* 29, 392–399.

Moreira, M. R., Ponce, A. G., Del Valle, C. E., and Roura, S. I. (2005). Inhibitory parameters of essential oils to reduce a foodborne pathogen. *LWT Food Sci. Technol.* 38, 565–570. doi: 10.1016/j.lwt.2004.07.012

Motojima, F. (2015). How do chaperonins fold protein? *Biophysics* 11, 93–102. doi: 10.2142/biophysics.11.93

Muraoka, W. T., and Zhang, Q. (2010). Phenotypic and genotypic evidence for L-fucose utilization by *Campylobacter jejuni*. *J. Bacteriol.* 193, 1065–1075. doi: 10.1128/JB.01252-10

Murray, C. J., Barber, R. M., Foreman, K. J., Ozgoren, A. A., Abd-Allah, F., Abera, S. F., et al. (2015). Global, regional, and national disability-adjusted life years (DALYs) for 306 diseases and injuries and healthy life expectancy (HALE) for 188 countries, 1990–2013: quantifying the epidemiological transition. *Lancet* 386, 2145–2191.

Nakatsu, T., Lupo, A. T. Jr., Chinn, J. W. Jr., and Kang, R. K. (2000). "Biological activity of essential oils and their constituents," in *Studies in Natural Products Chemistry*, Vol. 21, ed. A. Rahman (Amsterdam: Elsevier), 571–631. doi: 10.1016/s1572-5995(00)80014-9

Namkung, H., Li, J., Gong, M., Yu, H., Cottrill, M., and De Lange, C. F. M. (2004). Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. *Can. J. Anim. Sci.* 84, 697–704. doi: 10.4141/a04-005

Nannapaneni, R., Chalova, V. I., Crandall, P. G., Ricke, S. C., Johnson, M. G., and O'Bryan, C. A. (2009). *Campylobacter* and *Arcobacter* species sensitivity to commercial orange oil fractions. *Int. J. Food Microbiol.* 129, 43–49. doi: 10.1016/j.ijfoodmicro.2008.11.008

National Organic Program (2018). National Organic Program, 7 C.F.R. 205.601-603.

Naughton, J. A., Mariño, K., Dolan, B., Reid, C., Gough, R., Gallagher, M. E., et al. (2013). Divergent mechanisms of interaction of *Helicobacter pylori* and *Campylobacter jejuni* with mucus and mucins. *Infect. Immun.* 81, 2838–2850. doi: 10.1128/IAI.00415-13

Nauta, M., Hill, A., Rosenquist, H., Brynestad, S., Fetsch, A., van der Logt, P., et al. (2009). A comparison of risk assessments on *Campylobacter* in broiler meat. *Int. J. Food Microbiol.* 129, 107–123. doi: 10.1016/j.ijfoodmicro.2008.12.001

Navarro, M., Stanley, R., Cusack, A., and Sultanbawa, Y. (2015). Combinations of plant-derived compounds against *Campylobacter* in vitro. *J. Appl. Poult. Res.* 24, 352–363. doi: 10.3382/japr/pfv035

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., and De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* 6, 1451–1474. doi: 10.3390/ph6121451

Netzer, F., Kuntze, K., Vogt, C., Richnow, H. H., Boll, M., and Lueders, T. (2016). Functional gene markers for fumarate-adding and dearomatizing key enzymes in anaerobic aromatic hydrocarbon degradation in terrestrial environments. *J. Mol. Microbiol. Biotechnol.* 26, 180–194. doi: 10.1159/000441946

Nguefack, J., Tamgue, O., Dongmo, J. B. L., Dakole, C. D., Leth, V., Vismer, H. F., et al. (2012). Synergistic action between fractions of essential oils from *Cymbopogon citratus*, *Ocimum gratissimum* and *Thymus vulgaris* against *Penicillium expansum*. *Food Control* 23, 377–383. doi: 10.1016/j.foodcont.2011.08.002

Nofrarias, M., Manzanilla, E. G., Pujols, J., Gibert, X., Majo, N., Segalés, J., et al. (2006). Effects of spray-dried porcine plasma and plant extracts on intestinal morphology and on leukocyte cell subsets of weaned pigs. *J. Anim. Sci.* 84, 2735–2742. doi: 10.2527/jas.2005-414

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., et al. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol. Lett.* 360, 100–112. doi: 10.1111/1574-6968.12608

O'Bryan, C. A., Crandall, P. G., Chalova, V. I., and Ricke, S. C. (2008). Orange essential oils antimicrobial activities against *Salmonella* spp. *J. Food Sci.* 73, M264–M267.

O'Bryan, C. A., Pendleton, S. J., Crandall, P. G., and Ricke, S. C. (2015). Potential of plant essential oils and their components in animal agriculture–in vitro studies on antibacterial mode of action. *Front. Vet. Sci.* 2:35. doi: 10.3389/fvets.2015.00035

Overhage, J., Steinbuchel, A., and Priefert, H. (2003). Highly efficient Í biotransformation of eugenol to ferulic acid and further conversion to vanillin in recombinant strains of *Escherichia coli*. *Appl. Environ. Microbiol.* 69, 6569–6576.

Overhage, J., Steinbüchel, A., and Priefert, H. (2006). Harnessing eugenol as a substrate for production of aromatic compounds with recombinant strains of *Amycolatopsis* sp. HR167. *J. Biotechnol.* 125, 369–376. doi: 10.1016/j.jbiotec.2006.03.024

Oyen, L. P. A., and Dung, N. X. (1999). "Essential-oil plants," in *Plant Resources of South East Asia*, eds L. P. A. Oyen and N. X. Dung (Leiden: Backhuys Publishers), 15–49.

Özek, K., Wellmann, K. T., Ertekin, B., and Tarı m, B. (2011). Effects of dietary herbal essential oil mixture and organic acid preparation on laying traits, gastrointestinal tract characteristics, blood parameters and immune response of laying hens in a hot summer season. *J. Anim. Feed Sci.* 20, 575–586. doi: 10.22358/jafs/66216/2011

Pan, K., Chen, H., Davidson, P. M., and Zhong, Q. (2014). Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties. *J. Agric. Food Chem.* 62, 1649–1657. doi: 10.1021/jf4055402

Papatsiros, V. G., Katsoulos, P. D., Koutoulis, K. C., Karatzia, M., Dedousi, A., and Christodoulopoulos, G. (2013). Alternatives to antibiotics for farm animals. *CAB Rev.* 8, 1–15.

Parker, C. T., Miller, W. G., Horn, S. T., and Lastovica, A. J. (2007). Common genomic features of *Campylobacter jejuni* subsp. *doylei* strains distinguish them from *C. jejuni* subsp. *jejuni*. *BMC Microbiol.* 7:50. doi: 10.1186/1471-2180-7-50

Parker, C. T., Quiñones, B., Miller, W. G., Horn, S. T., and Mandrell, R. E. (2006). Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. *J. Clin. Microbiol.* 44, 4125–4135. doi: 10.1128/jcm.01231-06

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Parsons, C. M. (1984). Influence of caecectomy and source of dietary fibre or starch on excretion of endogenous amino acids by laying hens. *Br. J. Nutr.* 51, 541–548.

Perez-Vilar, J., and Hill, R. L. (1999). The structure and assembly of secreted mucins. *J. Biol. Chem.* 274, 31751–31754. doi: 10.1074/jbc.274.45.31751

Phadtare, S., and Severinov, K. (2010). RNA remodeling and gene regulation by cold shock proteins. *RNA Biol.* 7, 788–795. doi: 10.4161/rna.7.6.13482

Piskernik, S., Klanènik, A., Riedel, C. T., Brøndsted, L., and Možina, S. S. (2011). Reduction of *Campylobacter jejuni* by natural antimicrobials in chicken meat-related conditions. *Food Control* 22, 718–724. doi: 10.1016/j.foodcont.2010.11.002

Piva, G., and Rossi, F. (1998). "Possible alternatives to the use of antibiotics as growth promoters: new additives," in *Proceedings of the IFIF II Conference of Mixed–Feed Manufactures in the Mediterranean*, Reus, 83–106.

Prior, R. L., and Cao, G. (2000). Analysis of botanicals and dietary supplements for antioxidant capacity: a review. *J. AOAC Int.* 83, 950–956.

Quaroni, A., Isselbacher, K. J., and Ruoslahti, E. (1978). Fibronectin synthesis by epithelial crypt cells of rat small intestine. *Proc. Natl. Acad. Sci. U.S.A.* 75, 5548–5552. doi: 10.1073/pnas.75.11.5548

Raut, J. S., and Karuppayil, S. M. (2014). A status review on the medicinal properties of essential oils. *Ind. Crops Prod.* 62, 250–264. doi: 10.1016/j.indcrop.2014.05.055

Ravindran, V., and Bryden, W. L. (1999). Amino acid availability in poultry—In vitro and in vivo measurements. *Aust. J. Agric. Res.* 50, 889–908.

Ricke, S. C. (2003). The gastrointestinal tract ecology of *Salmonella* Enteritidis colonization in molting hens. *Poult. Sci.* 82, 1003–1007. doi: 10.1093/ps/82.6.1003

Ricke, S. C. (2018). Impact of prebiotics on poultry production and food safety. *Yale J. Biol. Med.* 91, 151–159.

Ricke, S. C., Dunkley, C. S., and Durant, J. A. (2013). A review on development of novel strategies for controlling *Salmonella* Enteritidis colonization in laying hens: fiber-based molt diets. *Poult. Sci.* 92, 502–525. doi: 10.3382/ps.2012-02763

Ricke, S. C., Van der Aar, P. J., Fahey, G. C. Jr., and Berger, L. L. (1982). Influence of dietary fibers on performance and fermentation characteristics of gut contents from growing chicks. *Poult. Sci.* 61, 1335–1343. doi: 10.3382/ps.0611335

Rimini, S., Petracci, M., and Smith, D. P. (2014). The use of thyme and orange essential oils blend to improve quality traits of marinated chicken meat. *Poult. Sci.* 93, 2096–2102. doi: 10.3382/ps.2013-03601

Robbe, C., Capon, C., Coddeville, B., and Michalski, J. C. (2004). Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem. J.* 384, 307–316. doi: 10.1042/bj20040605

Rosenquist, H., Nielsen, N. L., Sommer, H. M., Norrung, B., and Christensen, B. B. (2003). Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol.* 83, 87–103. doi: 10.1016/s0168-1605(02)00317-3

Rossi, P. G., Bao, L., Luciani, A., Panighi, J., Desjobert, J. M., Costa, J., et al. (2007). (E)-Methylisoeugenol and elemicin: antibacterial components of *Daucus carota* L. essential oil against *Campylobacter jejuni*. *J. Agric. Food Chem.* 55, 7332–7336. doi: 10.1021/jf070674u

Roto, S. M., Rubinelli, P. M., and Ricke, S. C. (2015). An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. *Front. Vet. Sci.* 2:28. doi: 10.3389/fvets.2015.00028

Ruberto, G., Baratta, M. T., Deans, S. G., and Dorman, H. D. (2000). Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Med.* 66, 687–693. doi: 10.1055/s-2000-9773

Rubinchik, S., Seddon, A., and Karlyshev, A. V. (2012). Molecular mechanisms and biological role of *Campylobacter jejuni* attachment to host cells. *Eur. J. Microbiol. Immunol.* 2, 32–40. doi: 10.1556/EuJMI.2.2012.1.6

Ryu, J. H., and Beuchat, L. R. (2005). Biofilm formation by *Escherichia coli* O157: H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. *Appl. Environ. Microbiol.* 71, 247–254. doi: 10.1128/aem.71.1.247-254.2005

Sahin, O., Kassem, I. I., Shen, Z., Lin, J., Rajashekara, G., and Zhang, Q. (2015). *Campylobacter* in poultry: ecology and potential interventions. *Avian Dis.* 59, 185–200. doi: 10.1637/11072-032315-Review

Sanders, S. Q., Frank, J. F., and Arnold, J. W. (2008). Temperature and nutrient effects on *Campylobacter jejuni* attachment on multispecies biofilms on stainless steel. *J. Food Prot.* 71, 271–278. doi: 10.4315/0362-028x-71.2.271

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Scallan, E., Hoekstra, R. M., Mahon, B. E., Jones, T. F., and Griffin, P. M. (2015). An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. *Epidemiol. Infect.* 143, 2795–2804. doi: 10.1017/S0950268814003185

Scher, K., Romling, U., and Yaron, S. (2005). Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Appl. Environ. Microbiol.* 71, 1163–1168. doi: 10.1128/aem.71.3.1163-1168.2005

Schets, F. M., Jacobs-Reitsma, W. F., van der Plaats, R. Q., Heer, L. K. D., van Hoek, A. H., Hamidjaja, R. A., et al. (2017). Prevalence and types of *Campylobacter* on poultry farms and in their direct environment. *J. Water Health* 15, 849–862. doi: 10.2166/wh.2017.119

Sergeant, M. J., Constantinidou, C., Cogan, T. A., Bedford, M. R., Penn, C. W., and Pallen, M. J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. *PLoS One* 9:e91941. doi: 10.1371/journal.pone.0091941

Shin, E., Hong, H., Oh, Y., and Lee, Y. (2015). First report and molecular characterization of a *Campylobacter jejuni* isolate with extensive drug resistance from a travel-associated human case. *Antimicrob. Agents Chemother.* 59, 6670–6672. doi: 10.1128/aac.01395-15

Si, W., Gong, J., Tsao, R., Zhou, T., Yu, H., Poppe, C., et al. (2006). Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *J. Appl. Microbiol.* 100, 296–305. doi: 10.1111/j.1365-2672.2005.02789.x

Sibanda, N., McKenna, A., Richmond, A., Ricke, S. C., Callaway, T., Stratakos, A. C., et al. (2018). A review of the effect of management practices on *Campylobacter* prevalence in poultry farms. *Front. Microbiol.* 9:2002. doi: 10.3389/fmicb.2018.02002

Sikkema, J., de Bont, J. A., and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 59, 201–222.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Front. Microbiol.* 2:200. doi: 10.3389/fmicb.2011.00200

Sima, F., Stratakos, A. C., Ward, P., Linton, M., Kelly, C., Pinkerton, L., et al. (2018). A novel natural antimicrobial can reduce the *in vitro* and *in vivo* pathogenicity of T6SS positive *Campylobacter jejuni* and *Campylobacter coli* chicken isolates. *Front. Microbiol.* 9:2139. doi: 10.3389/fmicb.2018.02139

Skandamis, P., Koutsoumanis, K., Nychas, G. J. E., and Fasseas, K. (2001). Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157: H7 [food hygiene]. *Ital. J. Food Sci.* 13:65.

Smithard, R. (2002). "Chapter 14: secondary plant metabolites in poultry nutrition," in *Poultry Feedstuffs: Supply, Composition and Nutritive Value*, eds J. M. McNab and K. N. Boorman (Wallingford, CT: CABI Publishing), 237–278. doi: 10.1079/9780851994642.0237

Smith-Palmer, A., Stewart, J., and Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett. Appl. Microbiol.* 26, 118–122. doi: 10.1046/j.1472-765x.1998.00303.x

Son, I., Englen, M. D., Berrang, M. E., Fedorka-Cray, P. J., and Harrison, M. A. (2007). Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *Int. J. Food Microbiol.* 113, 16–22. doi: 10.1016/j.ijfoodmicro.2006.06.033

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

Stahl, M., Butcher, J., and Stintzi, A. (2012). Nutrient acquisition and metabolism by *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.* 2:5. doi: 10.3389/fcimb.2012.00005

Stahl, M., Friis, L. M., Nothaft, H., Liu, X., Li, J., Szymanski, C. M., et al. (2011). L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7194–7199. doi: 10.1073/pnas.1014125108

Stanley, D., Hughes, R. J., and Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl. Microbiol. Biotechnol.* 98, 4301–4310. doi: 10.1007/s00253-014-5646-2

Stern, N. J. (2008). *Salmonella* species and *Campylobacter jejuni* cecal colonization model in broilers. *Poult. Sci.* 87, 2399–2403. doi: 10.3382/ps.2008-00140

Stern, N. J., Fedorka-Cray, P., Bailey, J. S., Cox, N. A., Craven, S. E., Hiett, K. L., et al. (2001). Distribution of *Campylobacter* spp. in selected US poultry production and processing operations. *J. Food Prot.* 64, 1705–1710. doi: 10.4315/0362-028x-64.11.1705

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., et al. (2006). Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. *Antimicrob. Agents Chemother.* 50, 3111–3116. doi: 10.1128/aac.00259-06

Szczepanski, S., and Lipski, A. (2014). Essential oils show specific inhibiting effects on bacterial biofilm formation. *Food Control* 36, 224–229. doi: 10.1016/j.foodcont.2013.08.023

Thanissery, R., Kathariou, S., and Smith, D. P. (2014). Rosemary oil, clove oil, and a mix of thyme-orange essential oils inhibit *Salmonella* and *Campylobacter in vitro*. *J. Appl. Poult. Res.* 23, 221–227. doi: 10.3382/japr.2013-00888

Thanissery, R., and Smith, D. P. (2014). Marinade with thyme and orange oils reduces *Salmonella* Enteritidis and *Campylobacter coli* on inoculated broiler breast fillets and whole wings. *Poult. Sci.* 93, 1258–1262. doi: 10.3382/ps.2013-03697

Thibodeau, A., Fravalo, P., Gauthier, R., Guévremont, E., Bergeron, N., Laurent-Lewandowski, S., et al. (2014). Modification of *Campylobacter jejuni* broiler colonization by a feed additive composed of encapsulated organic acids and essential oils. *J. Agric. Sci. Technol. A* 4, 853–864.

Thibodeau, A., Fravalo, P., Yergeau, É., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS One* 10:e0131978. doi: 10.1371/journal.pone.0131978

Thomas, M. T., Shepherd, M., Poole, R. K., van Vliet, A. H., Kelly, D. J., and Pearson, B. M. (2011). Two respiratory enzyme systems in *Campylobacter jejuni* NCTC 11168 contribute to growth on L-lactate. *Environ. Microbiol.* 13, 48–61. doi: 10.1111/j.1462-2920.2010.02307.x

Tiihonen, K., Kettunen, H., Bento, M. H. L., Saarinen, M., Lahtinen, S., Ouwehand, A. C., et al. (2010). The effect of feeding essential oils on broiler performance and gut microbiota. *Br. Poult. Sci.* 51, 381–392. doi: 10.1080/00071668.2010.496446

Troxell, B., Petri, N., Daron, C., Pereira, R., Mendoza, M., Hassan, H. M., et al. (2015). Poultry body temperature contributes to invasion control through reduced expression of *Salmonella* pathogenicity island 1 genes in *Salmonella enterica* serovars Typhimurium and Enteritidis. *Appl. Environ. Microbiol.* 81, 8192–8201. doi: 10.1128/AEM.02622-15

Tschech, A., and Schink, B. (1985). Fermentative degradation of resorcinol and resorcylic acids. *Arch. Microbiol.* 143, 52–59. doi: 10.1007/bf00414768

Tsuneki, H., Ma, E. L., Kobayashi, S., Sekizaki, N., Maekawa, K., Sasaoka, T., et al. (2005). Antiangiogenic activity of β-eudesmol *in vitro* and *in vivo*. *Eur. J. Pharmocol.* 512, 105–115.

Tu, Q. V., McGuckin, M. A., and Mendz, G. L. (2008). *Campylobacter jejuni* response to human mucin MUC2: modulation of colonization and pathogenicity determinants. *J. Med. Microbiol.* 57, 795–802. doi: 10.1099/jmm.0.47752-0

Turnberg, L. A. (1987). Gastric mucus, bicarbonate and pH gradients in mucosal protection. *Clin. Invest. Med.* 10, 178–180.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* 9, 799–809. doi: 10.1038/nri2653

U.S Food and Drug Administration [FDA] (2018). *GRAS Substances (SCOGS) Database*.

Ultee, A., Bennik, M. H. J., and Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 68, 1561–1568. doi: 10.1128/aem.68.4.1561-1568.2002

Umaraw, P., Prajapati, A., Verma, A. K., Pathak, V., and Singh, V. P. (2017). Control of *Campylobacter* in poultry industry from farm to poultry processing unit: a review. *Crit. Rev. Food Sci. Nutr.* 57, 659–665. doi: 10.1080/10408398.2014.935847

Upadhyay, A., Arsi, K., Wagle, B. R., Upadhyaya, I., Shrestha, S., Donoghue, A. M., et al. (2017). Trans-cinnamaldehyde, carvacrol, and eugenol reduce *Campylobacter jejuni* colonization factors and expression of virulence genes *in vitro*. *Front. Microbiol.* 8:713. doi: 10.3389/fmicb.2017.00713

van der Aar, P. J., Fahey, G. C. Jr., Ricke, S. C., Allen, S. E., and Berger, L. L. (1983). Effects of dietary fibers on mineral status of chicks. *J. Nutr.* 113, 653–661. doi: 10.1093/jn/113.3.653

van der Wielen, P. W., Biesterveld, S., Notermans, S., Hofstra, H., Urlings, B. A., and van Knapen, F. (2000). Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. *Appl. Environ. Microbiol.* 66, 2536–2540. doi: 10.1128/aem.66.6.2536-2540.2000

Van Deun, K., Haesebrouck, F., Van Immerseel, F., Ducatelle, R., and Pasmans, F. (2008). Short-chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. *Avian Pathol.* 37, 379–383. doi: 10.1080/03079450802216603

Van Immerseel, F., Fievez, V., De Buck, J., Pasmans, F., Martel, A., Haesebrouck, F., et al. (2004). Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with *Salmonella* Enteritidis in young chickens. *Poult. Sci.* 83, 69–74. doi: 10.1093/ps/83.1.69

Vandamme, P., Dewhirst, F. E., Paster, B. J., and On, S. L. W. (2006). *Bergey's Manual of Systematic Bacteriology: The Proteobacteria (Part C)*, 2nd Edn, Vol. 2, eds G. Garrity, D. J. Brenner, J. T. Staley, N. R. Krieg, D. R. Boone, P. DeVos, et al. (Berlin: Springer Science & Business Media), 1147–1160.

Vegge, C. S., Brøndsted, L., Li, Y. P., Bang, D. D., and Ingmer, H. (2009). Energy taxis drives *Campylobacter jejuni* toward the most favorable conditions for growth. *Appl. Environ. Mmicrobiol.* 75, 5308–5314. doi: 10.1128/AEM.00287-09

Velayudhan, J., Jones, M. A., Barrow, P. A., and Kelly, D. J. (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect. Immun.* 72, 260–268. doi: 10.1128/iai.72.1.260-268.2004

Velayudhan, J., and Kelly, D. J. (2002). Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology* 148, 685–694. doi: 10.1099/00221287-148-3-685

Vidanarachchi, J. K., Mikkelsen, L. L., Sims, I. M., Iji, P. A., and Choct, H. (2005). Phytobiotics: alternatives to antibiotic growth promoters in monogastric animal feeds. *Recent Adv. Anim. Nutr.* 15, 131–144.

Villemur, R. (1995). Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. *Can. J. Microbiol.* 41, 855–861. doi: 10.1139/m95-118

Wallace, R. B. (2003). "Campylobacter," in *Foodborne Microorganisms of Public Health Significance*, 6th Edn, ed. A. D. Hocking (Sydney: Australian Institute of Food Science and Technology), 311–331.

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., et al. (2002). Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J. Clin. Microbiol.* 40, 4744–4747.

Wang, X., Farnell, Y. Z., Peebles, E. D., Kiess, A. S., Wamsley, K. G. S., and Zhai, W. (2016). Effects of prebiotics, probiotics, and their combination on growth performance, small intestine morphology, and resident *Lactobacillus* of male broilers. *Poult. Sci.* 95, 1332–1340. doi: 10.3382/ps/pew030

Wang, Y. H., Avula, B., Nanayakkara, N. D., Zhao, J., and Khan, I. A. (2013). Cassia cinnamon as a source of coumarin in cinnamon-flavored food and food supplements in the United States. *J. Agric. Food Chem.* 61, 4470–4476. doi: 10.1021/jf4005862

Wei, A., and Shibamoto, T. (2007). Antioxidant activities and volatile constituents of various essential oils. *J. Agric. Food Chem.* 55, 1737–1742. doi: 10.1021/jf062959x

Wei, S., Morrison, M., and Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poult. Sci.* 92, 671–683. doi: 10.3382/ps.2012-02822

Wenk, C. (2003). Herbs and botanicals as feed additive in monogastric animals. *Asian Australas. J. Anim. Sci.* 16, 282–289. doi: 10.5713/ajas.2003.282

White, P. L., Baker, A. R., and James, W. O. (1997). Strategies to control *Salmonella* and *Campylobacter* in raw poultry products. *Sci. Tech.* 16, 525–541. doi: 10.20506/rst.16.2.1046

Wilkinson, N., Hughes, R. J., Aspden, W. J., Chapman, J., Moore, R. J., and Stanley, D. (2016). The gastrointestinal tract microbiota of the Japanese quail, *Coturnix japonica*. *Appl. Microbiol. Biotechnol.* 100, 4201–4209. doi: 10.1007/s00253-015-7280-z

Williams, P., and Losa, R. (2001). The use of essential oils and their compounds in poultry nutrition. *Worlds Poult. Sci. J.* 17, 14–15.

Windisch, W., and Kroismayr, A. (2006). *The Effects of Phytobiotics on Performance and Gut Function in Monogastrics*. Cape Town: World Nutrition Forum, 85–90.

Windisch, W., and Kroismayr, A. (2007). Natural phytobiotics for health of young piglets and poultry: mechanisms and application. *Poult. Sci.* 86:643.

Woodward, C. L., Kwon, Y. M., Kubena, L. F., Byrd, J. A., Moore, R. W., Nisbet, D. J., et al. (2005). Reduction of *Salmonella enterica* serovar Enteritidis colonization and invasion by an alfalfa diet during molt in Leghorn hens. *Poult. Sci.* 84, 185–193. doi: 10.1093/ps/84.2.185

World Health Organization [WHO] (2013). *The Global View of Campylobacteriosis: Report of an Expert Consultation*. Utrecht: World Health Organization.

Wright, J. A., Grant, A. J., Hurd, D., Harrison, M., Guccione, E. J., Kelly, D. J., et al. (2009). Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch. *Microbiology* 155, 80–94. doi: 10.1099/mic.0.021790-0

Xu, J., Zhou, F., Ji, B. P., Pei, R. S., and Xu, N. (2008). The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett. Appl. Microbiol.* 47, 174–179. doi: 10.1111/j.1472-765X.2008.02407.x

Yousaf, M. S., Ahmad, I., Ashraf, K., Rashid, M. A., Hafeez, A., Ahmad, A., et al. (2017). Comparative effects of different dietary concentrations of β-galacto-oligosaccharides on serum biochemical metabolites, selected caecel microbiota and immune response in broilers. *J. Anim. Plant Sci.* 27, 98–105.

Zemek, J., Košíková, B., Augustin, J., and Joniak, D. (1979). Antibiotic properties of lignin components. *Folia Microbiol.* 24, 483–486. doi: 10.1007/bf02927180

Ziprin, R. L., Young, C. R., Stanker, L. H., Hume, M. E., and Konkel, M. E. (1999). The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. *Avian Dis.* 43, 586–589.

Zoetendal, E. G., Collier, C. T., Koike, S., Mackie, R. I., and Gaskins, H. R. (2004). Molecular ecological analysis of the gastrointestinal microbiota: a review. *J. Nutr.* 134, 465–472. doi: 10.1093/jn/134.2.465

Zviely, M. (2004). "Aromatic chemicals," in *Kirk-Othmer Encyclopedia of Chemical Technology*, 5th Edn, ed. A. Seidel (New York, NY: Wiley Interscience), 226–262.

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 June 2019
doi: 10.3389/fmicb.2019.01381

Microbiota Analysis for the Optimization of *Campylobacter* Isolation From Chicken Carcasses Using Selective Media

Jinshil Kim[1†], Hakdong Shin[2†], Hyeeun Park[1], Hayan Jung[2], Junhyung Kim[3], Seongbeom Cho[3], Sangryeol Ryu[1] and Byeonghwa Jeon[1*‡]*

[1]Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, South Korea, [2]Department of Food Science and Biotechnology, College of Life Science, Sejong University, Seoul, South Korea, [3]College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul, South Korea

Since contaminated poultry meat is the major source of transmitting *Campylobacter jejuni* to humans, the isolation of *Campylobacter* from poultry carcasses is frequently performed in many countries as a baseline survey to ensure food safety. However, existing isolation methods have technical limitations in isolating this fastidious bacterium, such as a growth competition with indigenous bacteria in food samples. In this study, we compared the differences in microbiota compositions between Bolton and Preston selective media, two most common selective media to isolate *Campylobacter*, and investigated how different microbiota compositions resulting from different enrichment methods may affect isolation frequencies. A next-generation sequencing (NGS) analysis of 16S *rRNA* demonstrated that Bolton and Preston-selective enrichments generated different microbiota structures that shared only 31.57% of Operating Taxonomic Unit (OTU) types. Particularly, *Escherichia* was highly prevalent in Bolton selective media, and the enrichment cultures that increase *Escherichia* negatively affected the efficacy of *Campylobacter* isolation. Furthermore, the combination of the selective media made a significant difference in the isolation frequency. The Bolton broth and Preston agar combination exhibited the highest (60.0%) frequencies of *Campylobacter* isolation, whereas the Bolton broth and Bolton agar combination showed the lowest (2.5%). These results show that each selective medium generates a unique microbiota structure and that the sequence of combining the selective media also critically affects the isolation frequency by altering microbiota compositions. In this study, we demonstrated how a microbiota analysis using NGS can be utilized to optimize a protocol for bacterial isolation from food samples.

Keywords: microbiota compositions, selective enrichment, *Campylobacter*, 16S rRNA sequencing, bacterial isolation

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Amit Morey,
Auburn University, United States
Steven Lee Foley,
National Center for Toxicological Research (FDA), United States

***Correspondence:**
Sangryeol Ryu
sangryu@snu.ac.kr
Byeonghwa Jeon
bjeon@snu.ac.kr;
bjeon@ualberta.ca;
bjeon@umn.edu

[†]These authors have contributed equally to this work

[‡]Present address:
Byeonghwa Jeon,
Division of Environmental Health Sciences, School of Public Health, University of Minnesota, St. Paul, MN, United States

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

Received: *20 November 2018*
Accepted: *03 June 2019*
Published: *21 June 2019*

Citation:
Kim J, Shin H, Park H, Jung H, Kim J, Cho S, Ryu S and Jeon B (2019) Microbiota Analysis for the Optimization of Campylobacter Isolation From Chicken Carcasses Using Selective Media. Front. Microbiol. 10:1381. doi: 10.3389/fmicb.2019.01381

INTRODUCTION

Campylobacter spp., particularly *Campylobacter jejuni*, is a leading bacterial cause of gastroenteritis worldwide (Kirk et al., 2015). As a post-infection complication, *C. jejuni* is also implicated in the development of Guillain-Barré syndrome (GBS), an acute flaccid paralysis, accounting for approximately 31% of GBS cases (Poropatich et al., 2010). As poultry is the primary host for *Campylobacter*, human campylobacteriosis is frequently caused by the consumption of contaminated poultry meat (Newell and Fearnley, 2003; Rosenquist et al., 2003). Despite the well-established commensalism between *Campylobacter* and poultry, interestingly, *Campylobacter* prevalence in poultry highly varies depending on the country; for example, 27.1% in the Netherlands (van de Giessen et al., 2006), 38.6% in Poland (Szczepanska et al., 2017), 72.9% in the UK (Kaakoush et al., 2015), and 80.9% in Cambodia (Lay et al., 2011). Although the variations may result from various factors, such as geographical differences, sampling seasons, and food product types (Sahin et al., 2015), different isolation procedures used in different laboratory settings (e.g., different selective media and enrichment methods) may also significantly affect the isolation of this fastidious bacterium (Levesque et al., 2011; Carrillo et al., 2014).

The Bolton *Campylobacter*-selective supplement, the Preston *Campylobacter*-selective supplement, and modified charcoal-cefoperazone-deoxycholate agar (mCCDA) media are frequently used to isolate *Campylobacter* from food samples (Corry et al., 1995; Bojanić et al., 2017; Narvaez-Bravo et al., 2017; Vinueza-Burgos et al., 2017). The selective enrichment of *Campylobacter* is based on the intrinsic resistance of *Campylobacter* to antimicrobials. For instance, the antibacterial component of mCCDA is cefoperazone, the Bolton *Campylobacter*-selective supplement contains three antibiotics (cefoperazone, vancomycin, and trimethoprim), and the Preston *Campylobacter*-selective supplement has three antibiotics (polymyxin B, rifampicin, and trimethoprim), and *Campylobacter* is intrinsically resistant to these antibiotics (Taylor and Courvalin, 1988; Corry et al., 1995). The International Organization for Standardization (ISO) protocol for *Campylobacter* isolation employs the enrichment with Bolton *Campylobacter*-selective broth and subsequent culture on mCCDA (ISO 10272: 2006). However, the high prevalence of antibiotic-resistant bacteria among the microbiota of poultry carcasses has significantly compromised the effectiveness of *Campylobacter* isolation using the selective media. Particularly, extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli,* which is resistant to cephalosporins, is highly prevalent in poultry (Overdevest et al., 2011). ESBL *E. coli* can grow on the Bolton selective media and mCCDA, both of which contain cefoperazone, a third-generation cephalosporin antibiotic (Hazeleger et al., 2016). Although microbiota compositions after *Campylobacter* enrichment with selective media may significantly impact the isolation frequency of *Campylobacter*, nothing is known about how different microbiota compositions may affect the efficacy of *Campylobacter* isolation.

Next-generation sequencing (NGS) technology allows for the sequencing of massive samples and is useful for the microbiota analysis (Lee et al., 2013; Bragg and Tyson, 2014). By using NGS, in this study, we investigated microbiota compositions in chicken carcasses during the selective enrichment of *Campylobacter* with Bolton and Preston selective media, two selective media most frequently used to isolate *Campylobacter*. Based on the microbiota compositions, furthermore, we optimized the procedures of *Campylobacter* isolation using the two selective media. To the best of our knowledge, this is the first study to optimize a bacterial isolation protocol based on a microbiota analysis.

MATERIALS AND METHODS

Sample Collection and Selective Enrichment

Forty whole chicken carcass samples from 23 different brands were purchased from retail stores in South Korea from March 31 to July 21, 2017. Raw whole chicken samples were selected based on the production date and the shelf-life, packaged in a polyethylene bag, and delivered to the laboratory on ice. A whole chicken carcass was divided in half and subjected to enrichment with 1 L of Bolton broth with Bolton *Campylobacter*-selective supplements (Oxoid, UK) and Preston broth with Preston *Campylobacter*-selective supplements (Thermo-Fisher Scientific, USA) in plastic bags (Ziploc®, SC Johnson Co.). Although mCCDA is frequently used to isolate *Campylobacter*, in this study, Bolton and Preston selective media were used because the antimicrobial component of mCCDA (i.e., cefoperazone) is also present in Bolton. Samples were not rinsed with peptone water not to cause microbiota changes. The bags were incubated under microaerobic conditions (4% H_2, 6% O_2, 7% CO_2, 83% N_2) at 42°C for 24 h.

DNA Extraction and 16S rRNA Amplification and Sequencing

Twenty chicken samples from 19 different brands and products were used to compare the differences in microbiota compositions after enrichment with Bolton and Preston selective media as mentioned above. Total DNA was extracted from 300 µl of each enrichment culture using a commercial DNA extraction kit (FastDNA SPIN™ kit for soil, MP Biomedical, Santa Anna, CA) according to the manufacturer's instructions. DNA was eluted in 100 µl FastDNA elution buffer, and the extracted DNA was quantified by NanoVue Plus spectrophotometry before dilution to 15 ng/µl. The V3/V4 region of the 16S rRNA gene was amplified using the universal primers 341F and 805R on the following PCR reaction conditions (95°C for 5 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C for 7 min). PCR products were purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Germany). Paired-end (2 × 301 bp) sequencing was performed commercially (Chunlab Inc., Seoul, Korea) using a MiSeq platform (Illumina, San Diego, USA).

16S rRNA Gene-Based Sequencing Analysis

The 16S rRNA gene sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software package (v1.9.1) (Caporaso et al., 2010). Quality filtered sequences (Phred ≥ Q20) were used for identifying operational taxonomic units (OTUs) with open-reference OTU picking method in accordance with

97% identity of EzTaxon database (v1.5) (Yoon et al., 2017). Chimeric sequences were removed by UCHIME (Edgar et al., 2011). All samples were rarefied to 15,030 reads per samples for bacterial diversity analysis. To evaluate alpha diversity (microbial diversity within samples) of samples, alpha rarefaction was plotted using the phylogenetic distance and the detected number of species metrics with 10 iterations. The unweighted and weighted UniFrac distances were calculated for beta diversity analysis (Lozupone et al., 2006), and PERMANOVA was used to test the dissimilarity of beta diversity between groups (Anderson, 2001). Paired *t*-test was used for the statistical analysis of paired sample sets. A non-parametric *t*-test was performed with 10,000 permutations to test significance in intra-group distances. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to identify significant differences (LDA score > 3.0) in the relative abundance of bacterial taxonomy.

Campylobacter Isolation Frequencies of the Four Media Combinations

The enrichment cultures (20 ml) were concentrated by centrifugation at 4,000×*g*, 4°C for 7 min, and pellets were resuspended with 1 ml of each supplement broth (Bolton broth or Preston broth) (Son et al., 2007). After 10-fold serial dilution with PBS, bacterial suspension (100 μl) was spread onto Bolton and Preston agars containing each *Campylobacter*-specific supplements. Four different media combinations were made in the study, including Bolton broth-Bolton agar (BB-BA), Bolton broth-Preston agar (BB-PA), Preston broth-Bolton agar (PB-BA), and Preston broth-Preston agar (PB-PA). Inoculated agar plates were incubated microaerobically at 42°C for 48 h. Based on the colony morphology, such as flat, shiny, and mucoid colonies, 10 presumptive *Campylobacter* spp. colonies were confirmed by multiplex PCR using primer sets for *Campylobacter*-specific 16S rRNA gene and four *Campylobacter* species-specific primers were used; the primer sequences were described in **Supplementary Table S3**. All *Campylobacter* isolates were grown on Mueller-Hinton (MH) agar at 42°C under microaerobic conditions and stored in MH broth with 15% glycerol at −81°C.

Taxonomical Identification of Colonies Growing on *Campylobacter*-Selective Agars

To compare the distribution of *Campylobacter* and non-*Campylobacter* strains in BB-PA and PB-BA, 15 colonies were randomly selected from a *Campylobacter*-selective agar in each media combination based on colony shape, color, size, and transparency and transferred to a fresh *Campylobacter*-selective agar plate for pure culture. After 1–2 days incubation, colonies were picked up and boiled at 95°C for 7 min, and the boiled supernatant was used for PCR amplification of 16S rRNA gene. The PCR amplicons were purified and commercially sequenced (Macrogen, Inc., South Korea) by 3730xl DNA analyzer (Thermo Fisher Scientific), and the results were analyzed using BLASTN with 16S rRNA gene database.

RESULTS

Microbiota Compositions in Chicken Carcasses After Enrichment With Bolton and Preston *Campylobacter*-Selective Media

In the 40 enrichment samples (20 samples per each selective culture), we obtained 1,741,595 sequences (paired-end, Phred ≥Q20) with an average of 43,540 reads per sample and binned into 4,508 operating taxonomic units (OTUs) (**Supplementary Table S1**). Although the bacterial alpha diversity did not show any significant differences between BB and PB (**Supplementary Figure S1**), only 31.57% (1,423) of OTU types were shared in the samples enriched with BB and PB (**Figure 1A**). The microbiota

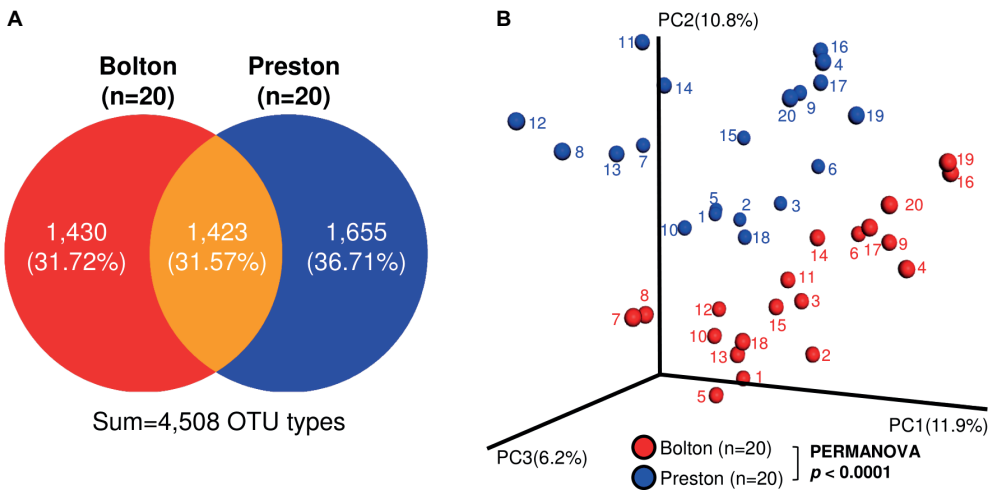

FIGURE 1 | Bacterial beta diversity in the chicken samples enriched with Bolton and Preston *Campylobacter*-selective media. **(A)** Venn diagram showing detected OTU types. **(B)** Principal Coordinate Analysis (PCoA) plot of bacterial communities in chicken samples enriched with Bolton and Preston selective media. Unweighted UniFrac distances were used to evaluate diversity between samples, and PERMANOVA was used to test the dissimilarity of bacterial population structures.

compositions after the selective enrichment with BB and PB were significantly different in bacterial structures (**Figure 1B**). Even though the same chicken carcass was divided and exposed to the two different selective enrichment conditions, there was a significant tendency to cluster depending on the type of selective media, not the sample (**Figure 1B**).

Proteobacteria and Firmicutes were the major phyla after the incubation of chicken samples in BB, whereas Proteobacteria and Fusobacteria were dominant after the PB enrichment (**Figure 2A**). At the genus level, the BB resulted in the enrichment of *Pseudomonas* (6.7%), *Acinetobacter* (1.4%), *Escherichia* (70.6%), *Phascolarctobacterium* (1.3%), *Lactobacillus* (2.5%), and *Bacteroides* (0.4%), whereas PB significantly enriched *Proteus* (5.1%), *Enterobacter* (0.6%), *Fusobacterium* (24.8%), *Erysipelothrix* (1.2%), *Coprobacillus* (2.6%), and *Clostridium_g6* (1.9%) (**Figures 2B,C, Table 1**). Notably, *Escherichia* was predominant in both BB and PB. Especially, the samples enriched by BB (70.6%) showed a higher abundance of *Escherichia* than PB (43.6%) (**Figure 3A, Table 1**). The PB enrichment exhibited a slightly higher abundance of *Campylobacter* compared to the BB enrichment, but the difference

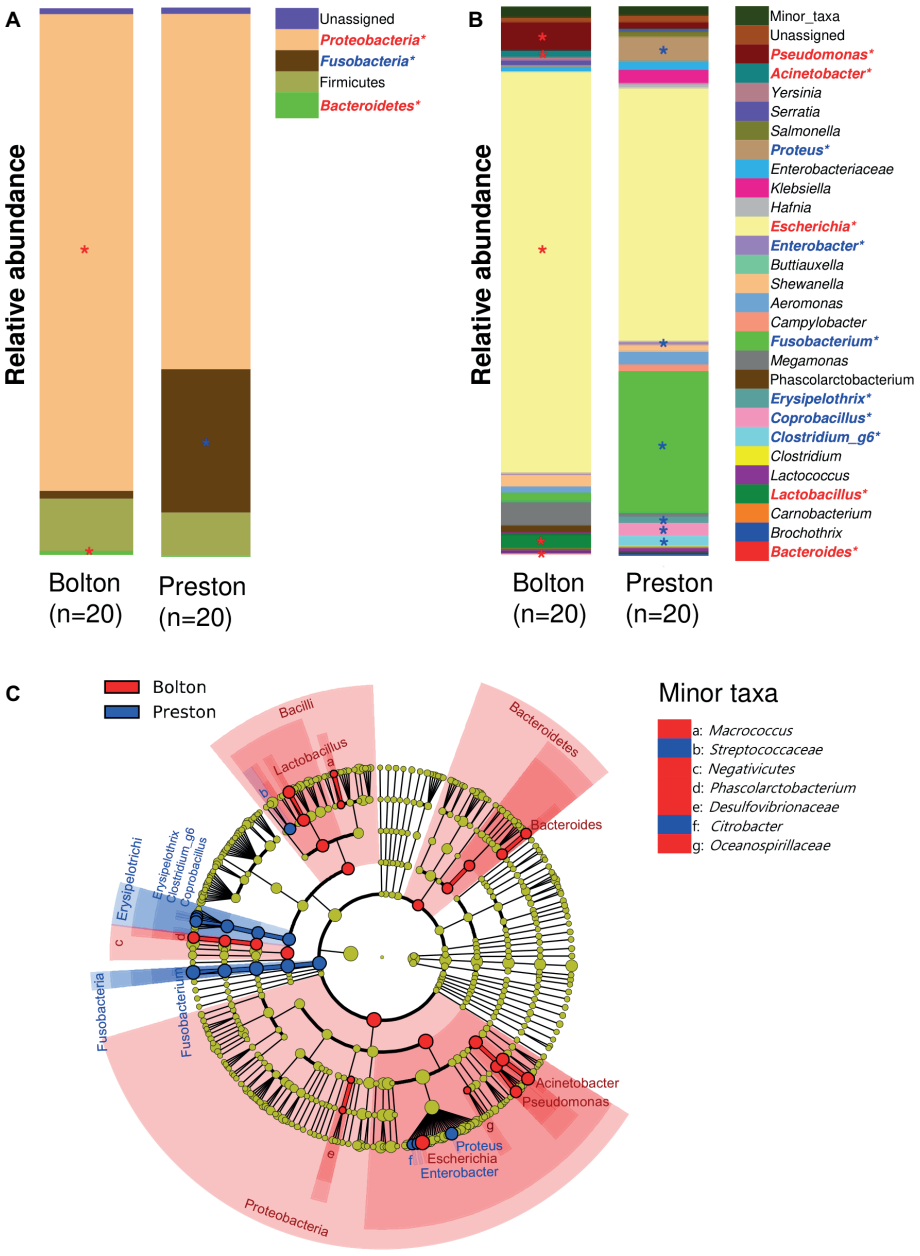

FIGURE 2 | Comparisons of bacterial taxa in chicken samples enriched with Bolton and Preston *Campylobacter*-selective media. **(A,B)** Bacterial taxonomy at the levels of phylum **(A)** and genus (top 27 bacterial taxonomies) **(B)** is indicated by a different color. *Overrepresented taxa (with LDA > 3.0) in comparison with the other selective medium (Red, Bolton media; Blue, Preston media). **(C)** Cladogram of overrepresented taxa (with LDA > 3.0) in each group. Overrepresented taxa in Bolton and Preston media were indicated in red and blue, respectively.

TABLE 1 | Relative abundance of overrepresented bacterial taxonomies (LDA > 3.0) in Bolton and Preston *Campylobacter*-selective media.

Bacterial taxonomy	Bolton broth			Preston broth		
	Average (%)	SD (%)	LDA score	Average (%)	SD (%)	LDA score
Pseudomonas	0.066993	0.107706	4.349024	0.018384	0.044282	–
Acinetobacter	0.01428	0.024536	3.796018	0.002345	0.005418	–
Escherichia	0.706185	0.205485	5.131531	0.435565	0.296455	–
Phascolarctobacterium	0.012729	0.030239	3.806587	0.000315	0.001248	–
Lactobacillus	0.025155	0.033968	4.037203	0.002922	0.011788	–
Bacteroides	0.003504	0.00653	3.260713	0.000136	0.000323	–
Proteus	0.001982	0.003262	–	0.051391	0.113173	4.363844
Enterobacter	0.001137	0.002599	–	0.006445	0.012079	3.458029
Fusobacterium	0.024574	0.101321	–	0.248409	0.322747	5.063090
Erysipelothrix	0	0	–	0.011613	0.038189	3.654410
Coprobacillus	0.000047	0.000109	–	0.026078	0.070242	4.091141
Clostridium_g6	0.00001	0.000032	–	0.018655	0.037892	3.987605

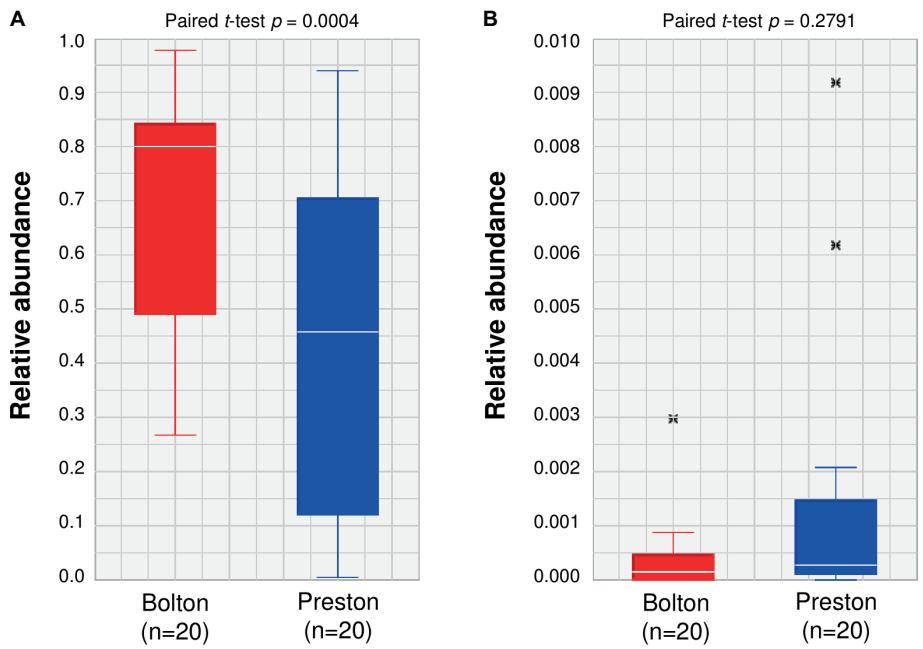

FIGURE 3 | Relative abundance of *Escherichia* **(A)** and *Campylobacter* **(B)** in chicken samples enriched by Bolton and Preston *Campylobacter*-selective media. The paired *t*-test is used to compare two population means. x: outlier samples.

was not statistically significant (**Figure 3B**). The BB-enriched group exhibited less inter-individual differences than the PB-enriched group, indicating that the microbiota of chicken carcasses after the BB enrichment were more similar to each other compared to those generated by the PB enrichment (**Supplementary Figure S2**).

Effects of the Second Selective Cultivation on the Efficacy of *Campylobacter* Isolation

For *Campylobacter* isolation, it is a common procedure to transfer the first selective enrichment culture to the second selective agar media to maximize isolation rates. To increase the selective pressure in *Campylobacter* isolation, an aliquot of each selective enrichment broth was transferred to selective agars containing the other selective supplement. For instance, an aliquot from the BB enrichment culture was transferred to Preston *Campylobacter*-selective agar (PA). Although *Escherichia* was predominantly (70.6%) enriched in BB (**Figure 3**), the prevalence of *Escherichia* was reduced to 37.8% after transfer to PA (**Table 2**). Although *Escherichia* was relatively lower (43.5%) in PB compared to BB (**Figure 3**), *E. coli* became predominant (84.3%) when transferred to Bolton *Campylobacter*-selective agar (BA) (**Table 2**). Interestingly, the proportions of *Campylobacter* spp., including *C. jejuni* and *C. coli*, increased to 37.5% (*C. jejuni* 36.7% and *C. coli* 0.8%), when BB was transferred to PA (**Table 2**). Whenever the Bolton selective supplement was used, *E. coli* became predominant, and the proportion of *Campylobacter* was reduced (**Table 2**, **Figure 3**).

The frequency of *Campylobacter* isolation was only 6.3% in Preston broth-Bolton agar (PB-BA) (**Table 2**). Based on the results, the Preston selective supplement was relatively effective in reducing *E. coli* and increasing *Campylobacter* (**Figure 3, Tables 1**, **2**). Although the same selective media (Bolton and Preston) were used, interestingly, the sequence of combining the selective media made a significant difference in the frequencies of *Campylobacter* isolation (**Table 2**). The results showed that both the type of selective media and the sequence of combining selective media may significantly affect the frequencies of *Campylobacter* isolation by changing microbiota composition.

Effects of the Sequence of Selective Medium Combinations on *Campylobacter* Isolation

To determine how the sequence of combining Bolton and Preston selective media affects the frequencies of *Campylobacter* isolation, we determined the isolation frequencies of four different combinations, including Bolton broth-Bolton agar (BB-BA), BB-PA, Preston broth-Preston agar (PB-PA), and PB-BA, using 40 retail chicken samples. Interestingly, each combination exhibited different levels of isolation frequency. Consistent with the findings above (**Table 2**), the combinations that used BA as the second selective culture medium exhibited lower isolation frequencies than those coupled with PA (**Table 3**). While BB-PA showed the highest (60.0%) frequency of *Campylobacter* isolation, BB-BA exhibited the lowest (2.5%) frequency (**Table 3**).

TABLE 2 | Proportions of bacterial species isolated with Bolton broth-Preston agar (BB-PA) and Preston broth-Bolton agar (PB-BA) combinations.

Bacterial species	BB-PA	PB-BA
Citrobacter amalonaticus	1 (0.2%)	0 (0.0%)
Escherichia coli	227 (37.8%)	506 (84.3%)
Escherichia albertii	0 (0.0%)	2 (0.3%)
Enterobacteriaceae bacterium	1 (0.2%)	0 (0.0%)
Erysipelotrichaceae bacterium	3 (0.5%)	0 (0.0%)
Erwinia sp.	0 (0.0%)	1 (0.2%)
Klebsiella pneumoniae	15 (2.5%)	17 (2.8%)
Fusobacterium mortiferum	0 (0.0%)	3 (0.5%)
Lactobacillus crispatus	1 (0.2%)	0 (0.0%)
Lactobacillus salivarius	1 (0.2%)	0 (0.0%)
Lactococcus lactis	2 (0.3%)	1 (0.2%)
Proteus mirabilis	91 (19.6%)	27 (4.5%)
Salmonella enterica	9 (1.5%)	4 (0.7%)
Staphylococcus aureus	0 (0.0%)	1 (0.3%)
Campylobacter coli	5 (0.8%)	0 (0.0%)
Campylobacter jejuni	220 (36.7%)	38 (6.3%)
Total number of isolates	**600 (100%)**	**600 (100%)**

TABLE 3 | Frequencies of *Campylobacter* isolation from retail raw chicken of four different combinations of Bolton and Preston *Campylobacter*-selective media.[†]

Species	BB-BA	BB-PA	PB-BA	PB-PA
Campylobacter coli	0/40 (0.0%)	11/40 (27.5%)	5/40 (12.5%)	1/40 (2.5%)
Campylobacter jejuni	1/40 (2.5%)	18/40 (45.0%)	7/40 (17.5%)	12/40 (27.5%)
C. coli & *C. jejuni*	0/40 (0.0%)	5/40 (12.5%)	3/40 (7.5%)	1/40 (2.5%)
Campylobacter-positive samples	1/40 (2.5%)	24/40 (60.0%)	9/40 (22.5%)	12/40 (30.0%)

[†]*Bolton broth-Bolton agar (BB-BA), Bolton broth-Preston agar (BB-PA), Preston broth-Bolton agar (PB-BA), and Preston broth-Preston agar (PB-PA).*

DISCUSSION

Bacterial isolation from food, environmental, and clinical samples is based on the selective growth and enrichment of the target bacteria using culture media supplemented with antimicrobials to which the target bacteria are intrinsically resistant. If some indigenous bacteria in the background microflora of the sample are resistant to the antimicrobials used in the selective media, the resistant bacteria may compete with target bacteria, affecting the composition of microbiota. This may consequently affect the isolation efficiency, particularly when background microflora outgrows the target bacteria. However, differences in microbiota compositions after the selective enrichment have not been taken into consideration to optimize protocols for bacterial isolation.

In this study, we found that BB markedly increased the proportion of *Escherichia* and reduced that of *Campylobacter* compared to PB (**Figure 3**). As BB contains three antibiotics (i.e., cefoperazone, vancomycin, and trimethoprim), *E. coli* growing in BB was resistant to the three antibiotics (data not shown). While vancomycin is effective against Gram-positive bacteria, Gram-negative bacteria are usually resistant to vancomycin intrinsically due to the permeability barrier of outer-membrane (Zhou et al., 2015). *E. coli* showed an increasing trend of trimethoprim resistance, and 13.8% of *E. coli* isolates from chicken are resistant to trimethoprim in the United States (Tadesse et al., 2012). In addition, cephalosporin-resistant *E. coli*, such as ESBL- and AmpC-producing *E. coli*, is highly prevalent in poultry (Overdevest et al., 2011; Reich et al., 2013; Olsen et al., 2014). Consistently, in this study, *Escherichia* was predominant in the selective enrichment media (**Figure 3A, Table 1**). The levels of *E. coli* and *C. jejuni* are originally low on chicken carcasses, which are approximately 0.8 log CFU/ml and 0.02 log CFU/ml in post-chill rinses of chicken carcasses, respectively (Altekruse et al., 2009). However, our study showed that *Escherichia* was highly enriched and became predominant after enrichment with *Campylobacter*-selective media, particularly BB (**Figure 3A, Table 1**). Furthermore, the high prevalence of *Escherichia* was accompanied by the reduction of *Campylobacter* population (**Figure 3B, Table 2**).

The different antibiotic selective pressures generated by BB and PB influenced in the formation of unique microbiota structures with the differential levels of *Campylobacter* prevalence in the enrichment cultures. Due to the fecal contamination of poultry carcasses during processing, the composition of microbiota on poultry carcasses is substantially affected by the gut microflora of poultry. Firmicutes is the predominant phylum throughout the chicken intestines from crop to large intestines (Yeoman et al., 2012) and constitutes approximately 50–90% of all taxa in the cecum (Qu et al., 2008; Danzeisen et al., 2011). According to a report from Kim et al. (Kim et al., 2017) on microbiome changes on chicken carcasses, Firmicutes are predominant in all steps of

poultry processing. In this study, consistently, Firmicutes, such as *Lactobacillus, Phascolarctobacterium, Coprobacillus,* and *Clostridium_g6*, were detected after BB and PB enrichments (**Figure 2A**). *Lactobacillus* is frequently detected in the gastrointestinal tracts of chickens and highly abundant (ca. 68%) in the duodenum and ileum (Lu et al., 2003). *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* are dominant in the ileum of chickens aged 3–21 days, and *Lactobacillus salivarius* and *Lactobacillus crispatus* are dominant *Lactobacillus* spp. in chickens aged 28–49 days (Lu et al., 2003); these *Lactobacillus* were isolated by BB-PA (**Table 2**). In the cecum of chickens, Clostridiaceae and Fusobacterium are dominant (ca. 65 and 14%, respectively) (Lu et al., 2003). *Fusobacterium* is more frequently found in the feces of chickens (Yan et al., 2017), and *Fusobacterium mortiferum* is most abundantly found in chicken feces (Oakley et al., 2013). In our study, fusobacteria were more frequently found in PB-enriched samples, compared with the BB-enriched samples (**Figures 2B,C, Table 1**), and *F. mortiferum* was isolated in PB-BA (**Table 2**). The detection of bacterial species that are originated from the gastrointestinal tracts of chickens strongly indicates fecal contamination of chicken carcasses. However, these predominant bacteria in the chicken intestines became less dominant during the enrichment step using the Bolton and Preston selective media.

Our study also highlights the need for the evaluation of bacterial competition in the microbiota of selective enrichment. While a previous report suggested that *Pseudomonas* spp. could support the survival of *C. jejuni* on chicken meat (Hilbert et al., 2010), our results showed that *Pseudomonas* was overrepresented in the BB-enriched samples with a low abundance of *Campylobacter* compared to the PB-enriched samples (**Figure 2B**). In addition, it has been reported that several *Lactobacillus* strains showed antagonistic activities against *C. jejuni* (Lehri et al., 2017), suggesting that higher abundance of *Lactobacillus* in the BB-enriched samples might affect the growth of *Campylobacter* in the BB media. Further studies are needed to elucidate the dynamics of competitive bacterial growth under selective enrichment conditions.

Interestingly, the application of the secondary selective culture generated completely different frequencies of *Campylobacter* isolation. In addition to the different capability of the selective media in enriching *Campylobacter* (**Figure 3**), the sequence of combining the selective media substantially affected the isolation frequency. Using the same selective culture media (Bolton and Preston), BB-PA exhibited significantly higher frequencies of *Campylobacter* isolation than PB-BA by 5.9-fold (**Table 3**). The ISO protocol for *Campylobacter* detection employs the enrichment with BB and subsequent culture on mCCDA that is supplemented with cefoperazone, a third-generation cephalosporin, which is one of the three antibiotics in the Bolton selective supplement. Based on the antibiotics used in the protocol, the combination of BB and mCCDA would generate a selective pressure similar to that of the BB-BA combination. To improve the isolation frequency, the revised version of ISO includes the enrichment with Preston broth and the following culture on mCCDA (ISO, 2015); this would possibly be similar to the PB-BA combination. In our study, the detection frequency of PB-BA (22.5%) was higher than that of BB-BA (2.5%), whereas the best isolation frequency was achieved in the BB-PA combination (**Table 3**).

The prevalence of *Campylobacter* in poultry is usually high in many countries; however, the prevalence level often varies depending on the country. Accurate determination of a baseline of *Campylobacter* contamination of foods is highly important, since the baseline level may affect the results of a risk assessment of *Campylobacter* contamination of poultry (Habib et al., 2008) and food safety policies pertaining to the control of *Campylobacter* in the food supply system. Although NGS is a powerful tool to investigate microbiota, it has never been used to investigate microbiota compositions during the selective culture of bacteria, although the microbiota compositions may significantly impact the frequencies of bacterial isolation. Aiming to improve the frequencies of *Campylobacter* isolation from poultry carcasses, this study provided a novel insight into how an NGS-based microbiota analysis can be employed to optimize the protocols for *Campylobacter* isolation. The same approach can be applied to the development of isolation protocols for other bacteria.

AUTHOR CONTRIBUTIONS

SC, SR, and BJ designed the study. JK, HS, HP, HJ, and JHK performed the experiments. JK, HS, SR, and BJ analyzed the data. JK, HS, and BJ wrote the manuscript. JK, HS, SC, SR, and BJ reviewed the manuscript.

FUNDING

This research was supported by a grant (16162MFDS029) from the Ministry of Food and Drug Safety in Korea. JK and HP were supported by the BK21 Plus Program of the Department of Agricultural Biotechnology, Seoul National University, Seoul, Korea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/article/10.3389/fmicb.2019.01381/full#supplementary-material

REFERENCES

Altekruse, S. F., Berrang, M. E., Marks, H., Patel, B., Shaw, W. K. Jr., Saini, P., et al. (2009). Enumeration of *Escherichia coli* cells on chicken carcasses as a potential measure of microbial process control in a random selection of slaughter establishments in the United States. *Appl. Environ. Microbiol.* 75, 3522–3527. doi: 10.1128/AEM.02685-08

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1046/j.1442-9993.2001.01070.x

Bojanić, K., Midwinter, A. C., Marshall, J. C., Rogers, L. E., Biggs, P. J., and Acke, E. (2017). Isolation of *Campylobacter* spp. from Client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand. *Zoonoses Public Health* 64, 438–449. doi: 10.1111/zph.12323

Bragg, L., and Tyson, G. W. (2014). Metagenomics using next-generation sequencing. *Methods Mol. Biol.* 1096, 183–201. doi: 10.1007/978-1-62703-712-9_15

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carrillo, C. D., Plante, D., Iugovaz, I., Kenwell, R., Belanger, G., Boucher, F., et al. (2014). Method-dependent variability in determination of prevalence of *Campylobacter jejuni* and *Campylobacter coli* in Canadian retail poultry. *J. Food Prot.* 77, 1682–1688. doi: 10.4315/0362-028X.JFP-14-133

Corry, J. E. L., Post, D. E., Colin, P., and Laisney, M. J. (1995). Culture media for the isolation of campylobacters. *Int. J. Food Microbiol.* 26, 43–76. doi: 10.1016/0168-1605(95)00044-K

Danzeisen, J. L., Kim, H. B., Isaacson, R. E., Tu, Z. J., and Johnson, T. J. (2011). Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One* 6:e27949. doi: 10.1371/journal.pone.0027949

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Habib, I., Sampers, I., Uyttendaele, M., Berkvens, D., and De Zutter, L. (2008). Baseline data from a Belgium-wide survey of *Campylobacter* species contamination in chicken meat preparations and considerations for a reliable monitoring program. *Appl. Environ. Microbiol.* 74, 5483–5489. doi: 10.1128/AEM.00161-08

Hazeleger, W. C., Jacobs-Reitsma, W. F., and Den Besten, H. M. (2016). Quantification of growth of *Campylobacter* and extended spectrum beta-lactamase producing bacteria sheds light on black box of enrichment procedures. *Front. Microbiol.* 7:1430. doi: 10.3389/fmicb.2016.01430

Hilbert, F., Scherwitzel, M., Paulsen, P., and Szostak, M. P. (2010). Survival of *Campylobacter jejuni* under conditions of atmospheric oxygen tension with the support of *Pseudomonas* spp. *Appl. Environ. Microbiol.* 76, 5911–5917. doi: 10.1128/AEM.01532-10

ISO (2015). *ISO/DIS 10272–1:2015. Microbiology of the food chain–horizontal method for detection and enumeration of campylobacter–part 1: Detection method*. (Geneva: ISO).

Kaakoush, N. O., Castano-Rodriguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi: 10.1128/CMR.00006-15

Kim, S. A., Park, S. H., Lee, S. I., Owens, C. M., and Ricke, S. C. (2017). Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Sci. Rep.* 7:43354. doi: 10.1038/srep43354

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Lay, K. S., Vuthy, Y., Song, P., Phol, K., and Sarthou, J. L. (2011). Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. *J. Vet. Med. Sci.* 73, 325–329. doi: 10.1292/jvms.10-0373

Lee, C.-Y., Chiu, Y.-C., Wang, L.-B., Kuo, Y.-L., Chuang, E. Y., Lai, L.-C., et al. (2013). Common applications of next-generation sequencing technologies in genomic research. *Transl. Cancer Res.* 2, 33–45. doi: 10.3978/j.issn.2218-676X.2013.02.09

Lehri, B., Seddon, A. M., and Karlyshev, A. V. (2017). *Lactobacillus fermentum* 3872 as a potential tool for combatting *Campylobacter jejuni* infections. *Virulence* 8, 1753–1760. doi: 10.1080/21505594.2017.1362533

Levesque, S., St-Pierre, K., Frost, E., Arbeit, R. D., and Michaud, S. (2011). Determination of the optimal culture conditions for detecting thermophilic campylobacters in environmental water. *J. Microbiol. Methods* 86, 82–88. doi: 10.1016/j.mimet.2011.04.001

Lozupone, C., Hamady, M., and Knight, R. (2006). UniFrac--an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinform.* 7:371. doi: 10.1186/1471-2105-7-371

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., and Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl. Environ. Microbiol.* 69, 6816–6824. doi: 10.1128/AEM.69.11.6816-6824.2003

Narvaez-Bravo, C., Taboada, E. N., Mutschall, S. K., and Aslam, M. (2017). Epidemiology of antimicrobial resistant *Campylobacter* spp. isolated from retail meats in Canada. *Int. J. Food Microbiol.* 253, 43–47. doi: 10.1016/j.ijfoodmicro.2017.04.019

Newell, D. G., and Fearnley, C. (2003). Sources of *Campylobacter* colonization in broiler chickens. *Appl. Environ. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Oakley, B. B., Morales, C. A., Line, J., Berrang, M. E., Meinersmann, R. J., Tillman, G. E., et al. (2013). The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PLoS One* 8:e57190. doi: 10.1371/journal.pone.0057190

Olsen, R. H., Bisgaard, M., Lohren, U., Robineau, B., and Christensen, H. (2014). Extended-spectrum beta-lactamase-producing *Escherichia coli* isolated from poultry: a review of current problems, illustrated with some laboratory findings. *Avian Pathol.* 43, 199–208. doi: 10.1080/03079457.2014.907866

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., et al. (2011). Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerg. Infect. Dis.* 17, 1216–1222. doi: 10.3201/eid1707.110209

Poropatich, K. O., Walker, C. L., and Black, R. E. (2010). Quantifying the association between *Campylobacter* infection and Guillain-Barre syndrome: a systematic review. *J. Health Popul. Nutr.* 28, 545–552. doi: 10.3329/jhpn.v28i6.6602

Qu, A., Brulc, J. M., Wilson, M. K., Law, B. F., Theoret, J. R., Joens, L. A., et al. (2008). Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One* 3:e2945. doi: 10.1371/journal.pone.0002945

Reich, F., Atanassova, V., and Klein, G. (2013). Extended-spectrum beta-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, Germany. *Emerg. Infect. Dis.* 19, 1253–1259. doi: 10.3201/eid1908.120879

Rosenquist, H., Nielsen, N. L., Sommer, H. M., Nørrung, B., and Christensen, B. B. (2003). Quantitative risk assessment of human campylobacteriosis associated with thermophilic Campylobacter species in chickens. *Int. J. Food Microbiol.* 83, 87–103. doi: 10.1016/S0168-1605(02)00317-3

Sahin, O., Kassem, I., Shen, Z., Lin, J., Rajashekara, G., and Zhang, Q. (2015). *Campylobacter* in poultry: ecology and potential interventions. *Avian Dis.* 59, 185–200. doi: 10.1637/11072-032315-Review

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Son, I., Englen, M. D., Berrang, M. E., Fedorka-Cray, P. J., and Harrison, M. A. (2007). Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *Int. J. Food Microbiol.* 113, 16–22. doi: 10.1016/j.ijfoodmicro.2006.06.033

Szczepanska, B., Andrzejewska, M., Spica, D., and Klawe, J. J. (2017). Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from children and environmental sources in urban and suburban areas. *BMC Microbiol.* 17:80. doi: 10.1186/s12866-017-0991-9

Tadesse, D. A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M. J., et al. (2012). Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. *Emerg. Infect. Dis.* 18, 741–749. doi: 10.3201/eid1805.111153

Taylor, D. E., and Courvalin, P. (1988). Mechanisms of antibiotic-resistance in *Campylobacter* species. *Antimicrob. Agents Chemother.* 32, 1107–1112. doi: 10.1128/AAC.32.8.1107

van de Giessen, A. W., Bouwknegt, M., Dam-Deisz, W. D., Van Pelt, W., Wannet, W. J., and Visser, G. (2006). Surveillance of *Salmonella* spp. and *Campylobacter* spp. in poultry production flocks in The Netherlands. *Epidemiol. Infect.* 134, 1266–1275. doi: 10.1017/S0950268806005905

Vinueza-Burgos, C., Wautier, M., Martiny, D., Cisneros, M., Van Damme, I., and De Zutter, L. (2017). Prevalence, antimicrobial resistance and genetic diversity of *Campylobacter coli* and *Campylobacter jejuni* in Ecuadorian broilers at slaughter age. *Poult. Sci.* 96, 2366–2374. doi: 10.3382/ps/pew487

Yan, W., Sun, C., Yuan, J., and Yang, N. (2017). Gut metagenomic analysis reveals prominent roles of *Lactobacillus* and cecal microbiota in chicken feed efficiency. *Sci. Rep.* 7:45308. doi: 10.1038/s41598-017-18049-8

Yeoman, C. J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N. D., and White, B. A. (2012). The microbiome of the chicken gastrointestinal tract. *Anim. Health Res. Rev.* 13, 89–99. doi: 10.1017/S1466252312000138

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. doi: 10.1099/ijsem.0.001755

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., et al. (2015). Synergistic interactions of vancomycin with different antibiotics against *Escherichia coli*: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type *E. coli*. *Antimicrob. Agents Chemother.* 59, 276–281. doi: 10.1128/AAC.03502-14

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: 26 July 2019
doi: 10.3389/fmicb.2019.01509

A Historical Review on Antibiotic Resistance of Foodborne *Campylobacter*

Yichao Yang[1†‡], Kristina M. Feye[2†‡], Zhaohao Shi[3], Hilary O. Pavlidis[4], Michael Kogut[2], Amanda J. Ashworth[5] and Steven C. Ricke[2]*

[1]Department of Poultry Science, University of Arkansas Fayetteville, Fayetteville, AR, United States, [2]Southern Plains Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, College Station, TX, United States, [3]Center of Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [4]Diamond V, Cedar Rapids, IA, United States, [5]Poultry Production and Product Safety Research Unit (USDA-ARS), Fayetteville, AR, United States

Campylobacter is one of the most commonly reported foodborne human bacterial gastrointestinal pathogens. *Campylobacter* is the etiological agent of campylobacteriosis, which is generally a self-limited illness and therefore does not require treatment. However, when patients are immunocompromised or have other co-morbidities, antimicrobial treatment may be necessary for clinical treatment of campylobacteriosis, macrolides and fluoroquinolones are the drugs of choices. However, the increase in antimicrobial resistance of *Campylobacter* to clinically important antibiotics may become insurmountable. Because of the transmission between poultry and humans, the poultry industry must now allocate resources to address the problem by reducing *Campylobacter* as well as antimicrobial use, which may reduce resistance. This review will focus on the incidence of antibiotic-resistant *Campylobacter* in poultry, the clinical consequences of this resistance, and the mechanisms of antibiotic resistance associated with *Campylobacter*.

Keywords: *Campylobacter*, antibiotic resistance, macrolide, fluoroquinolone resistance, historical review

OPEN ACCESS

Edited by:
Adolfo J. Martinez-Rodriguez, Instituto de Investigación en Ciencias de la Alimentación (CIAL), Spain

Reviewed by:
Heriberto Fernandez, Austral University of Chile, Chile
Ozan Gundogdu, London School of Hygiene and Tropical Medicine, United Kingdom

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

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

[‡]*Co-first authors*

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

Received: *28 May 2019*
Accepted: *17 June 2019*
Published: *26 July 2019*

Citation:
Yang Y, Feye KM, Shi Z, Pavlidis HO, Kogut M, Ashworth JA and Ricke SC (2019) A Historical Review on Antibiotic Resistance of Foodborne Campylobacter. Front. Microbiol. 10:1509. doi: 10.3389/fmicb.2019.01509

THE IMPORTANCE OF ANTIBIOTIC-RESISTANT *CAMPYLOBACTER*

Campylobacteriosis is usually a self-limited illness, and patients with prolonged symptomology are usually placed on a macrolide or fluoroquinolone antibiotic regimen (Salazar-Lindo et al., 1986; Allos and Blaser, 2010). However, antibiotic resistance of *Campylobacter* to these important classes of antibiotics, especially fluoroquinolones, is on the rise (US Food and Drug Administration, 2014; Bolinger et al., 2018). Resistance in two important Anti-*Campylobacter* antibiotics, azithromycin (macrolide) and ciprofloxacin (fluoroquinolone), has increased, resulting in approximately 310,000 cases of potentially untreatable infections, leading to 28 deaths in the United States annually (CDC, 2019).

The rise in antibiotic resistance has been geographically heterologous, with trends in both good and bad antibiotic stewardship mirrored in the agricultural and private sector. Information learned through epidemiological studies in higher income countries is targeted toward stopping resistance. Specifically, the antibiotic stewardship programs like the National Healthcare Safety Network, Gonococcal Isolate Surveillance Program, National Tuberculosis Surveillance System,

and the Emerging Infections Program have all increased awareness of the prescription behavior, patient compliance, and resistance pattern of important clinical pathogens (Ventola, 2015a,b). Many of these programs have targeted practitioners to stop over-prescribing antibiotics. Instead, data have directly resulted in a more tuned regimen program for multiple classes of clinically important antibiotics and led to improve treatment success (Fridkin et al., 2014; Ventola, 2015a,b). Specific to food, the National Antimicrobial Resistance Monitoring System was established in 1996. This program distributes information and conducts research on foodborne pathogens from retail meat and the risk may or may not pose to the community as a whole (Zhao et al., 2006; Ventola, 2015a,b). The World Health Organization has had additional resolutions to improve antibiotic stewardship as the European Union and other groups worldwide (Ventola, 2015a,b). In more developed countries, organizations track the spread of antibiotic-resistant foodborne pathogens, which in turn enable regulatory agencies to change management strategies. This awareness has brought about changes that may ultimately reduce the risk of antibiotic resistance.

While antibiotic stewardship is still a challenge in high income countries, it is not the same obstacle as what is faced in lower to middle income countries. In these countries, agriculture is exploding, and there are very few, if any, regulations on the market to control the use of antibiotics. As these countries also typically have high incidence of diarrheal disease that impacts growth and development as well as reduces vaccine efficiency, the rise in any antibiotic resistance is alarming (McCormick and Lang, 2016). In particular, countries such as Brazil, Russia, India, China, and South Africa (BRICS) require antibiotics to meet the agricultural paradigm in place (Van Boeckel et al., 2014).

In the BRICS countries, poor hygiene, lack of access to potable water, and the absolutely uncontrolled use of antibiotics all contribute to this issue of spreading disease and rise in resistance (Van Boeckel et al., 2014; Frost et al., 2019). For instance, in India, *Campylobacter* isolates are often multiple-drug resistant and have phenotypic resistance to ciprofloxacin, tetracycline, furazolidone, ampicillin, gentamicin, and erythromycin (Jain et al., 2005). In Central America, trends hold true as well, with antibiotic-resistant *Campylobacter* on the rise (Toledo et al., 2018).

Besides reducing the treatment failures for diarrheal diseases in low income countries, preventing the rise in antibiotic-resistant *Campylobacter* is essential as certain populations that are more at risk for severe complications. These populations, such as the immunocompromised or the elderly, will likely be placed on antibiotic regimens in order to prevent bacteremia and sequelae. Specifically, HIV patients are particularly vulnerable as infections can be intractable with bacteremia, ultimately requiring macrolide or fluoroquinolone therapy (Hussein et al., 2016). Another potential outcome of campylobacteriosis includes when *Campylobacter* disseminates to the periphery and causes profound disease. *Campylobacter fetus,* a rare and animal associated *Campylobacter* species epithet, causes spondylodiscitis in HIV and aged patients that commonly have other comorbidities (diabetes mellitus), where common therapeutics are not effective (Olaiya et al., 2018). Therefore, antibiotic-resistant strains of *Campylobacter* are absolutely problematic clinically as treatment resistance could have significant consequences for immunocompromised patients. As a result, understanding how resistance occurs historically is important in order to potentially find novel patterns and lessons in controlling *Campylobacter*.

A controversial avenue for the introduction of antibiotic-resistant *Campylobacter* strains to humans is through the consumption of meat, including poultry (Thakur et al., 2010; Barton, 2014; US Food and Drug Administration, 2014; Mäesaar et al., 2016). *Campylobacter* is present as a commensal organism in the gastrointestinal tracts (GITs) of poultry and can be antibiotic resistant (Zhao et al., 2010; Kojima et al., 2015; Ohishi et al., 2017; Raeisi et al., 2017). A Polish study indicated that as many as 94.4% of *Campylobacter jejuni* isolated from chicken are resistant to at least one class of antibiotic (Wieczorek et al., 2017). However, the link between agriculture and human clinical isolates of *Campylobacter* remains controversial. This review will focus on the mechanisms associated with antibiotic resistance, the controversial link between the use of antibiotics in agriculture and clinical resistance, and the current antibiotics available as well as the challenges faced with the ever-present rise in antibiotic-resistant isolates.

ANTIBIOTIC RESISTANCE MECHANISMS

Antibiotics inhibit the growth and proliferation of microorganisms by binding to a specific target central to the microbial molecular biology and inhibiting the targets normal homeostatic activity or otherwise preventing the activity of an antibiotic. These effects can result in either bacteriostatic (cessation of replication) or bactericidal (killing) effects on the microorganism. There are four common mechanisms associated with antibiotic resistance: alteration of the antibiotic target, inactivation of the drug, decreasing membrane permeability, and the expression of antimicrobial efflux pumps (Iovine, 2013). In *Campylobacter*, mechanisms of antibiotic resistance are no different and are on occasion multimodal.

One of the most common mechanisms associated with antibiotic resistance is the ability of microorganisms to alter their membrane permeability, which ultimately prevents diffusion of the antibiotic into the intracellular environment. Porins are transmembrane proteins that create molecular pores allowing for the diffusion of chemical compounds that otherwise cannot cross the cell membrane, including antibiotics, into the periplasmic and intracellular environment (Galdiero et al., 2012). *Campylobacter* reduces membrane permeability *via* changing the expression of porins (Page et al., 1989; Pumbwe et al., 2004). In many instances, the natural and unique porins expressed by *Campylobacter* naturally prevent the entry of most antibiotics with molecular weights greater than 360 KDas (Page et al., 1989). By altering porin expression pattern, *Campylobacter* can reduce antibiotic diffusion to target within the intracellular and periplasmic space.

Another common mechanism used by *Campylobacter* for resistance is the expression of efflux pumps, which in many

cases result in multidrug-resistant phenotypes. Efflux pumps can be expressed by both Gram-positive and Gram-negative prokaryotes and actively transport structurally variable molecules like antibiotics from the periplasmic or cytoplasmic space to the external environment of the cell (Pagès and Amaral, 2009; Nikaido and Pagès, 2012; Handzlik et al., 2013; Blair et al., 2014; Yao et al., 2016). An example of this includes the efflux pumps used by *Campylobacter* that removes the aminoglycosides from the intracellular space, preventing the antibiotic from reaching the ribosome and exerting its effects. Potential novel approaches to combating this kind of resistance include potentiators that act as efflux pump antagonist, which ultimately shuts down resistance (Mamelli et al., 2003; Payot et al., 2004). Efflux pumps are especially concerning as they are not specific and may successfully efflux multiple classes of antibiotics. Yao et al. (2016) found that the emergence of a super efflux pump variant, named *RE-CmeABC*, directly confers multidrug resistance in *Campylobacter* as it can non-specifically efflux multiple classes of antibiotics. *RE-CmeABC* is a particularly dangerous genetic element as it is encoded in a plasmid and subject to horizontal transfer.

Mutations in the target site reduce affinity and avidity for the antibiotic, which ultimately makes that class of antibiotics ineffective (Vetting et al., 2011). For example, fluoroquinolone resistance is due to a point mutation in the topoisomerase site where the antibiotic typically binds and renders it ineffective. Point mutations occur naturally as a consequence of normal biological replication and can be favored or disfavored by environmental conditions. By changing that target site, the point mutation reduces the binding affinity of the fluoroquinolone (Ling et al., 2003). In environments where antibiotics are non-existent, resistant point mutations are not likely a favored or even result in a neutral mutation. However, if antibiotics are present, the surviving bacterial progeny must confer that mutation for resistance and selective pressure results in the mutation becoming dominant (Ling et al., 2003). Additionally, the biotransformation of an antibiotic by the bacteria intracellularly can also render it ineffective. Bacterial enzymes in the bacteria modify side chains of chemical groups on the antibiotic, which consequentially reduce antibiotic-binding affinity to the target site. A classic example of this important mechanism occurs in aminoglycoside antibiotics, which have numerous hydroxyl and amide groups that are vulnerable to modification once in bacterial systems (Norris and Serpersu, 2013). Once modified, the aminoglycosides are ineffective.

The rise in multiple antibiotic-resistant *Campylobacter* is mechanistically multimodal. Numerous studies point to multimodal resistance becoming an important component of *Campylobacter* drug resistance. Hao et al. (2016) found that *C. jejuni* 1655 had several mutations at different target sties for antibiotics, including Thr-86-Ile mutation in *gyrA* and the A2075G mutation in *23S rRNA*, *tetO*, *aphA*, and *aadE* genes. Additionally, *C. jejuni* 1655 carried a *pTet* plasmid. All together, these mutations and plasmids result in a multidrug resistant phenotype to fluoroquinolone, macrolide, tetracycline, and aminoglycoside classes of antibiotics. Therefore, identifying one mechanism of resistance as the resistance "smoking gun" is not likely. Clinically, data indicates that *Campylobacter* should be subjected to antibiograms prior to the initiation of clinical therapeutic regimens to select antibiotics; the pathogen is susceptible in order to ensure that positive therapeutic outcomes are possible.

AGRICULTURE, ANTIBIOTICS, AND *CAMPYLOBACTER*

Significant press and public attention have, likely unfairly, pointed to the agricultural industry as the root cause of antibiotic resistance. However, this is still a controversial notion, with data resulting from multiple studies ultimately being non-conclusive. Multiple studies have been conducted to determine whether or not this link is real. Ultimately, this may be a case of correlation not indicating causation. While the rise in resistance in the clinical sector is mirrored in the agricultural sector, these may be very independent events or perhaps existing in a gray area. The judicious use of antibiotics as a whole may ultimately combat antibiotic resistance.

In order to determine if there was a link between antibiotic-resistant *Campylobacter* in poultry with human campylobacteriosis, a study was conducted that isolated *Campylobacter* strains from poultry and human clinical campylobacteriosis samples (Wieczorek et al., 2018). While some correlations existed, the link was neither direct nor concrete and remains controversial. Additionally, Silva et al. (2016) found that there is no relationship between *Campylobacter* isolated from human and poultry sources based on pulse field gel electrophoresis analysis. More research is needed to fill the gap between animal use antibiotics and human infection of antibiotic-resistant pathogens.

The paralleled rise in resistance is illustrated by the rise of quinolone-resistant strains in the veterinary and clinical setting. It is true that in the early 1990s, Endtz et al. (1991) found a rise in resistant strains isolated from poultry products from 0 to 14% between 1982 and 1989. Correspondingly, quinolone-resistant *Campylobacter* isolates increased from 0 to 11% isolated from human sources. In order to determine if agricultural isolates impacted human disease outcomes, Zhao et al. (2015) used pulsed-field gel electrophoresis (PFGE) to subtype *Campylobacter* isolates from humans and retail poultry and observed that isolates between the two sets displayed the same resistance phenotypes and PFGE patterns. Data indicated that the human isolates were likely linked to the contaminated poultry products. However, this is not entirely correlative and the exposure of humans to resistant *Campylobacter* does not come without its own nuances. Zhao et al. (2015) also observed that human isolates of *Campylobacter* tended to be more genetically diverse and resistant than the retail chicken isolates. This amplification of drug resistance in clinical patients may be due to the horizontal transfer of mobile genetic elements encoding antibiotic resistance in the human gastrointestinal tract (Barnes et al., 1972; Eckburg et al., 2005).

In order for causation to be correlative, a direct link must be established and consistent between agriculture and clinical isolates, which remains to be the case with *Campylobacter*. The missing link likely speaks to the complexities associated with mobile genetic elements making that direct link unlikely or that the correlation ultimately does not mean causation. Some models have shown that antibiotics fed to food animals present a low risk of clinical treatment failure associated with antibiotic resistance. For example, Hurd et al. (2004) conducted a risk assessment modeling the effects of tylosin and tilmicosin, two macrolide antibiotics, on *Campylobacter* spp. and *E. faecium*. The scientists administered both types of veterinary antibiotics to swine, cattle, or poultry for therapeutic, prophylactic, and growth promotion. The antimicrobial resistance determinant was considered the most likely hazard factor for causing human illness, which is supported by U.S. Food and Drug Administration's Center of Veterinary Medicine. This farm-to-patient risk assessment model indicated that the use of macrolides resulted in less than 1 in 10 million rate of failure in treating *Campylobacter* with these two antibiotics. While resistance is a problem in both sectors, the common use of macrolides used in food animals does not impact antibiotic resistance in human campylobacteriosis.

ANTIBIOTICS USED TO TREAT CAMPYLOBACTERIOSIS IN THE CLINICAL SETTING

The CDC has recommended treatment regimens be initiated for *Campylobacter* if the patients have weakened immune systems or are experiencing profound effects. Commonly, macrolides and fluoroquinolones are prescribed with macrolides being preferred due to their low resistance rate (CDC, 2019). It has been documented that there are a greater frequency in identifying fluoroquinolone-resistant isolates compared to macrolide-resistant *Campylobacter* isolates. That may be due to the mutation frequency of the 50S ribosomal subunit that confers resistance occurring in approximately 10 mutations per *Campylobacter* cell per generation. The mutation frequency for the 50S ribosomal subunit is 10,000-fold lower than the mutation frequency of the gyrase and topoisomerase genes that confer fluoroquinolone resistance (Yan et al., 2006; Lin et al., 2007). The increased rate in mutation frequency likely corresponds to the rise in ciprofloxacin-resistant isolates *Campylobacter,* which was from 13% in 1997 to 19% in 2001 (Gupta et al., 2004). This is compared to the steady prevalence of 2% erythromycin, a macrolide, resistant positive isolates of *Campylobacter* during the same time period (Gupta et al., 2004). This trend has continued through time as currently the prevalence of fluoroquinolone resistance is 35.4% in *C. jejuni* and 74.4% in *Campylobacter coli* in the United States (Tang et al., 2017).

MACROLIDES

A course of the macrolide azithromycin is the gold standard chemotherapeutic regimen to treat *Campylobacter* infections, which effectively reduces bacterial shedding and the length of the illness (Kuschner et al., 1995). The breakpoint concentration associated with azithromycin indicates that 30 to 500/d for 3 days of azithromycin was effective for the eradication of *Campylobacter* and acceleration of a patient's recovery time (Kuschner et al., 1995; Vukelic et al., 2010). This, as well as the low incidence of natural resistance, has led to azithromycin being the drug of choice (DuPont, 2007).

Another option for therapeutic regimens in clinical medicine includes the use of erythromycin (Guerrant et al., 2001). Efficacy is lost when the antibiotic regimen is not started at the onset of symptoms and may require prolonged treatment (Guerrant et al., 2001). Advantages of using erythromycin include the low frequency of natural resistance of *Campylobacter* to erythromycin (Smith et al., 1999; Bardon et al., 2009), with antibiogram analysis of 1,808 isolates from Finnish patients between 2003 and 2005 reporting a resistance prevalence to erythromycin at 1.1% (Lehtopolku et al., 2010). Clindamycin is a lincomycin antibiotic, and Wagner et al. (2003) first proposed this as a potential therapeutic for campylobacteriosis. This alternative was evaluated by determining the minimum inhibitory concentration for five common antibiotics. The researchers observed that only 2% of the *Campylobacter* isolates were resistant to clindamycin, while greater than 45% of isolates exhibited resistance against four fluoroquinolone antibiotics tested (Wagner et al., 2003).

FLUOROQUINOLONES

Fluoroquinolones may be used to treat *Campylobacter* but are problematic due to the resistance profile of clinical *Campylobacter* isolates (Nord and Edlund, 1991). The clinical isolates associated with active campylobacteriosis exhibit greater frequencies of quinolone resistance, increasing as much as 20-fold in the 1990s in Sweden (Wretlind et al., 1992; Gibreel et al., 1998). As with macrolide resistance, fluoroquinolone resistance is naturally occurring as a point mutation, ultimately leading to treatment failure and symptomatic relapse (Segreti et al., 1992; Sanders et al., 2007). A classic example of this issue is norfloxacin, fluoroquinolone widely used to treat enteric infections (Sjögren et al., 1997). Epidemiological evidence indicates that resistance can occur within 1 day of therapy (Sjögren et al., 1997). In fact, resistance to multiple fluoroquinolones exhibits a sharp increase in *Campylobacter* isolates, such as the rise of nalidixic acid resistance from 8.2% in 1990 to 26.3% in 2004 (Gallay et al., 2007), and a similar trend of quinolone resistance occurred in Germany (Luber et al., 2003).

There are promising fluoroquinolones for use in the clinical setting, yet mounting resistance continues to be a significant theme in the historical perspective associated with *Campylobacter* fluoroquinolone resistance. For instance, moxifloxacin is a fourth-generation synthetic fluoroquinolone that effectively kills *Campylobacter* and is difficult to mount resistance toward, as compared to ciprofloxacin (Wagner et al., 2003). Additionally, the use of levofloxacin in clinical cases of campylobacteriosis

is promising (de la Cabada Bauche and DuPont, 2011). The effectiveness of a single dose treatment of 500 mg/d of levofloxacin is similar to 1,000 mg/d of azithromycin but with fewer side effects (Sanders et al., 2007). However, as with most fluoroquinolones, resistance has been observed when compared with azithromycin (Sanders et al., 2007).

Resistance against other fluoroquinolones has been widespread. A common fluoroquinolone of choice for medical practitioners, ciprofloxacin, has been reported in developing countries with levels ranging from 30 to greater than 84% (Hoge et al., 1998; Pandey et al., 2010; Meng et al., 2011). As with other fluoroquinolones, the frequency of isolating ciprofloxacin-resistant strains of *Campylobacter* is rising. In Peru, Pollett et al. (2012) observed that between 2001 and 2010, the prevalence of ciprofloxacin-resistant *Campylobacter* isolates rose from 73.1 to 89.9% in the area of Lima and from 24.1 to 48.9% in the Iquitos region. Similar to the clinical setting, an increased prevalence of fluoroquinolone-resistant *Campylobacter* strains has been recovered from food animals in developed countries (Taylor et al., 2008). Nannapaneni et al. (2005) isolated ciprofloxacin-resistant *Campylobacter* isolates from retail raw chicken carcasses in the United States with numbers ranging from 57% in 2001 to 96% in 2003. This is likely due to the relationship developing countries have with antibiotics, which ultimately results in poor antibiotic stewardship and an ultimately significant increase in antibiotic resistance. Therefore, increase in both clinical and veterinary isolates of *Campylobacter* in the developing world is anecdotal of a larger problem in countries where the use of antibiotics in both independent settings, the clinical and the veterinary, is poorly controlled and executed.

MODELS FOR CONTROLLING *CAMPYLOBACTER*

The intensive use of antibiotics has been suggested to have an increased number of resistant strains of mutants and a decreased effectiveness of antibiotics (Wegener, 2003; Ventola, 2015a,b). The World Health Organization (WHO) has concluded that increases in antibiotic resistance represent a considerable, worldwide threat to public health (WHO, 2014). In order to attempt to preserve the efficacy of clinical antibiotics, the European Union banned the use of clinical antibiotics for growth promotion in 1999 (Casewell et al., 2003). In the United States, the Institute of Medicine recommend to reduce or eliminate the use of antibiotics in feed in 1980 and 1989, and also it was supported by a Council for Agricultural Science and Technology (1981) and a Committee on Drug Use in Food Animals Panel on Animal Health, Food Safety, and Public Health (1998). The Veterinary Feed Directive was recently implemented in the United States to limit the use of human clinical antibiotics as well as provide stricter guidelines for the use of antibiotics in food animals and is viewed as the first step to solving a significant veterinary and human clinical problem (Veterinary Feed Directive (VFD), 2017).

TABLE 1 | Veterinary feed directive antibiotics.

	Medically important drugs	**Non-medically important drugs**
Therapeutic uses	Allow under veterinary supervision	Allow under veterinary supervision
Production use	No longer allowed	Allowed
Drugs	1. Penicillin 2. Cephalosporins 3. Quinolones 4. Fluoroquinolones 5. Tetracyclines 6. Macrolides 7. Sulfas 8. Glycopeptides	1. Bambermycin 2. Carbadox 3. Ionophores 4. Pleuromutilin 5. Polypeptides

However, despite the regulations, it is still under debate about the relationship between resistance microorganisms selected when an antibiotic was used as the growth promoter in food animals and the antibiotic-resistant infections in humans (Phillips et al., 2004; Vaughn and Copeland, 2004). At the time of increasing concern about the spread of antibiotic resistance pathogens in humans, U.S. Food and Drug Administration initiated the Veterinary Feed Directive (VFD), which requires a prescription from a veterinarian to use medically important antibiotics in infected animals. These medically important antibiotics are not allowed to be used as growth promoters. The regulation information has been listed in **Table 1**.

As with other bacteria, the use of the correct antibiotic with the correct species epithet is important and is a tenant of good antimicrobial stewardship. When analyzing antibiotic-resistant isolates of *Campylobacter* isolated from live broilers, Li et al. (2017) reported that *C. coli* exhibited significantly greater prevalence of antibiotic resistance than *C. jejuni* to clindamycin, gentamicin, and kanamycin, but less resistance to florfenicol. This finding demonstrated that while a particular antimicrobial may be effective for reducing *C. jejuni*, it may not be as effective for treating *C. coli* and vice versa. Therefore, while campylobacteriosis tends to be binned within a single category, the identification of the specific species epithet is important for treatment success. By becoming more aware of the potential differences in resistance across species epithets, properly prescribing the correct treatment regimen may further enhance antimicrobial stewardship.

CONCLUSION

Ultimately, it is poorly understood as to how fluid the *Campylobacter* genome is and the full risk associated with antibiotic resistance in the agricultural setting. Additionally, the link between agricultural antimicrobial abuse and treatment failures in the clinical setting still fails to be established. With increased antibiotic vigilance, research into the mechanisms that drive antibiotic resistance in *Campylobacter*, and the potential development of novel antimicrobial strategies, it may be possible to mitigate the effect of antibiotic resistance

independently across the veterinary and clinical sectors. While resistance is not conferred between poultry and the clinical patient, the exposure to the clinical patient does occur through poultry. The rearrangement and transference of mobile genetic elements within the human host, the environment, and the poultry itself likely make the "smoking gun" improbable.

However, the establishment and prescription of a single-hurdle approach to control this foodborne pathogen and ultimately reduce the risk to the human population are unlikely. Therefore, not only does research need to focus on the development of new antibiotics to help patients in the clinical setting, it needs to focus on preventing the reservoir for campylobacteriosis, poultry, from continuing to serve as such. Whether or not reducing *Campylobacter* resistance in poultry affects the war on antibiotic resistance is unknown and will remain a mystery.

AUTHOR CONTRIBUTIONS

YY, KF, SR, AA, MK, and HP provided the framework and concept for the publication. YY produced the first version of the manuscript. KF completed, edited, and restructured the manuscript for publication. KF submitted the manuscript and handled all communications with the reviewers. All authors reviewed the manuscript prior to submission.

FUNDING

YY, KF, MK, and SR were not provided financial assistance to complete this review. HP is an employee of Diamond V Mills (Cedar Rapids, IA, USA).

REFERENCES

Allos, B. M., and Blaser, M. J. (2010). "*Campylobacter jejuni* and related species" in *Principles and practice of infectious diseases,* 7th Edn. eds. G. L. Mandell, J. E. Bennett and R. Dolin (Philadelphia, PA: Churchill Livingstone), 2793–2802.

Bardon, J., Kolar, M., Cekanova, L., Hejnar, P., and Koukalova, D. (2009). Prevalence of *Campylobacter jejuni* and its resistance to antibiotics in poultry in the Czech Republic. *Zoonoses Public Health* 56, 111–116. doi: 10.1111/j.1863-2378.2008.01176.x

Barnes, E. M., Mead, G., Barnuml, D., and Harry, E. (1972). The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *Br. Poult. Sci.* 13, 311–326.

Barton, M. D. (2014). Impact of antibiotic use in the swine industry. *Curr. Opin. Microbiol.* 19, 9–15. doi: 10.1016/j.mib.2014.05.017

Blair, J. M., Richmond, G. E., and Piddock, L. J. (2014). Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future Microbiol.* 9, 1165–1177. doi: 10.2217/fmb.14.66

Bolinger, H. K., Zhang, Q., Miler, W. G., and Kathariou, S. (2018). Lack of evidence for *erm*(B) infiltration into erythromycin-resistant *Campylobacter coli* and *Campylobacter jejuni* from commercial Turkey production in Eastern North Carolina: a major Turkey-growing region in the United States. *Foorborne Pathog. Dis.* 15, 1–3. doi: 10.1089/fpd.2018.2477

Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* 52, 159–161. doi: 10.1093/jac/dkg313

CDC (2019). Fast Facts 2019. Available at: https://www.cdc.gov/campylobacter/faq.html (Accessed July 2, 2019).

Committee on Drug Use in Food Animals Panel on Animal Health, Food Safety, and Public Health (1998). *Use of drugs in food animals: Benefits and risks*. Washington, DC: National Academy Press.

Council for Agricultural Science and Technology (1981). *Antibiotics in animal feeds. Report 88*. CAST: Ames, IA.

de la Cabada Bauche, J., and DuPont, H. L. (2011). New developments in traveler's diarrhea. *Gastroenterol. Hepatol.* 7, 88–95.

DuPont, H. L. (2007). Azithromycin for the self-treatment of traveler's diarrhea. *Clin. Infect. Dis.* 44, 347–349. doi: 10.1086/510594

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591

Endtz, H. P., Ruijs, G. J., van Klingeren, B., Jansen, W. H., van der Reyden, T., and Mouton, R. P. (1991). Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. *J. Antimicrob. Chemother.* 27, 199–208. doi: 10.1093/jac/27.2.199

Fridkin, S., Baggs, J., Fagan, R., Magill, S., Pollack, L. A., Malpiedi, P., et al. (2014). Vital signs: improving antibiotic use among hospitalized patients. *MMWR Morb. Mortal. Wkly. Rep.* 63, 194–200. https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6309a4.html.

Frost, I., Van Boeckel, T. P., Pires, J., Craig, J., and Laxminarayan, R. (2019). Global geographic trends in antimicrobial resistance: the role in internaitonal travel. *J. Travel Med.* doi: 10.1093/jtm/taz036

Galdiero, S., Falanga, A., Cantisani, M., Tarallo, R., Elena Della Pepa, M., D'Oriano, V., et al. (2012). Microbe-host interactions: structure and role of Gram-negative bacterial porins. *Curr. Protein Pept. Sci.* 13, 843–854. doi: 10.2174/138920312804871120

Gallay, A., Prouzet-Mauléon, V., Kempf, I., Lehours, P., Labadi, L., Camou, C., et al. (2007). *Campylobacter* antimicrobial drug resistance among humans, broiler chickens, and pigs, France. *Emerg. Infect. Dis.* 13, 259–266. doi: 10.3201/eid1302.060587

Gibreel, A., Sjögren, E., Kaijser, B., Wretlind, B., and Sköld, O. (1998). Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. *Antimicrob. Agents Chemother.* 42, 3276–3278. doi: 10.1128/AAC.42.12.3276

Guerrant, R. L., Van Gilder, T., Steiner, T. S., Thielman, N. M., Slutsker, L., Tauxe, R. V., et al. (2001). Practice guidelines for the management of infectious diarrhea. *Clin. Infect. Dis.* 32, 331–351. doi: 10.1086/318514

Gupta, A., Nelson, J. M., Barrett, T. J., Tauxe, R. V., Rossiter, S. P., Friedman, C. R., et al. (2004). Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. *Emerg. Infect. Dis.* 10, 1102–1109. doi: 10.3201/eid1006.030635

Handzlik, J., Matys, A., and Kieć-Kononowicz, K. (2013). Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus*. *Antibiotics* 2, 28–45. doi: 10.3390/antibiotics2010028

Hao, H., Ren, N., Han, J., Foley, S. L., Iqbal, Z., Cheng, G., et al. (2016). Virulence and genomic feature of multidrug resistant *Campylobacter jejuni* isolated from broiler chicken. *Front. Microbiol.* 7:1605. doi: 10.3389/fmicb.2016.01605

Hoge, C. W., Gambel, J. M., Srijan, A., Pitarangsi, C., and Echeverria, P. (1998). Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. *Clin. Infect. Dis.* 26, 341–345. doi: 10.1086/516303

Hurd, H. S., Doores, S., Hayes, D., Mathew, A., Maurer, J., Silley, P., et al. (2004). Public health consequences of macrolide use in food animals: a deterministic risk assessment. *J. Food Prot.* 67, 980–992. doi: 10.4315/0362-028X-67.5.980

Hussein, K., Raz-Pasteur, A., Schachor-Meyouhas, Y., Geffen, Y., Oren, I., and Kassis, I. (2016). *Campylobacter* bacteraemia: 16 years of experience in a single centre. *Infect. Dis.* 46, 796–799. doi: 10.1080/23744235.2016.1195916

Iovine, N. M. (2013). Resistance mechanisms in *Campylobacter jejuni*. *Virulence* 4, 230–240. doi: 10.4161/viru.23753

Jain, D., Sinha, S., Prasad, K. N., and Pandey, C. M. (2005). *Campylobacter* species and drug resistance in north Indian rural community. *Trans. R. Soc. Trop. Med. Hyg.* 99, 207–214. doi: 10.1016/j.trstmh.2004.09.006

Kojima, C., Kishimoto, M., and Ezaki, T. (2015). Distribution of antimicrobial resistance in *Campylobacter* strains isolated from poultry at a slaughterhouse and supermarkets in Japan. *Biocontrol Sci.* 20, 179–184. doi: 10.4265/bio.20.179

Kuschner, R. A., Trofa, A. F., Thomas, R. J., Hoge, C. W., Pitarangsi, C., Amato, S., et al. (1995). Use of azithromycin for the treatment of *Campylobacter* enteritis in travelers to Thailand, an area where ciprofloxacin resistance is prevalent. *Clin. Infect. Dis.* 21, 536–541. doi: 10.1093/clinids/21.3.536

Lehtopolku, M., Nakari, U.-M., Kotilainen, P., Huovinen, P., Siitonen, A., and Hakanen, A. J. (2010). Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: in vitro activities of 20 antimicrobial agents. *Antimicrob. Agents Chemother.* 54, 1232–1236. doi: 10.1128/AAC.00898-09

Li, B., Ma, L., Li, Y., Jia, H., Wei, J., Shao, D., et al. (2017). Antimicrobial resistance of *Campylobacter* species isolated from broilers in live bird markets in Shanghai, China. *Foodborne Pathog. Dis.* 14, 96–102. doi: 10.1089/fpd.2016.2186

Lin, J., Yan, M., Sahin, O., Pereira, S., Chang, Y.-J., and Zhang, Q. (2007). Effect of macrolide usage on emergence of erythromycin-resistant *Campylobacter* isolates in chickens. *Antimicrob. Agents Chemother.* 51, 1678–1686. doi: 10.1128/AAC.01411-06

Ling, J., Chan, E., Lam, A., and Cheng, A. (2003). Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. *Antimicrob. Agents Chemother.* 47, 3567–3573. doi: 10.1128/AAC.47.11.3567-3573.2003

Luber, P., Wagner, J., Hahn, H., and Bartelt, E. (2003). Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. *Antimicrob. Agents Chemother.* 47, 3825–3830. doi: 10.1128/AAC.47.12.3825-3830.2003

Mäesaar, M., Kramarenko, T., Meremäe, K., Sõgel, J., Lillenberg, M., Häkkinen, L., et al. (2016). Antimicrobial resistance profiles of *Campylobacter* spp. isolated from broiler chicken meat of Estonian, Latvian and Lithuanian origin at Estonian retail level and from patients with severe enteric infections in Estonia. *Zoonoses Public Health* 63, 89–96. doi: 10.1111/zph.12208

Mamelli, L., Amoros, J.-P., Pagès, J.-M., and Bolla, J.-M. (2003). A phenylalanine–arginine β-naphthylamide sensitive multidrug efflux pump involved in intrinsic and acquired resistance of *Campylobacter* to macrolides. *Int. J. Antimicrob. Agents* 22, 237–241. doi: 10.1016/S0924-8579(03)00199-7

McCormick, B. J. J., and Lang, D. R. (2016). Diarrhea disease and enteric infections in LMIC communities: how big is the problem? *Trop. Dis. Travel Med. Vaccines* 2. doi: 10.1186/s40794-016-0028-7

Meng, C. Y., Smith, B. L., Bodhidatta, L., Richard, S. A., Vansith, K., Thy, B., et al. (2011). Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. *Pediatr. Infect. Dis. J.* 30, 331–335. doi: 10.1097/INF.0b013e3181fb6f82

Nannapaneni, R., Story, R., Wiggins, K. C., and Johnson, M. G. (2005). Concurrent quantitation of total *Campylobacter* and total ciprofloxacin-resistant *Campylobacter* loads in rinses from retail raw chicken carcasses from 2001 to 2003 by direct plating at 42 C. *Appl. Environ. Microbiol.* 71, 4510–4515. doi: 10.1128/AEM.71.8.4510-4515.2005

Nikaido, H., and Pagès, J.-M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol. Rev.* 36, 340–363. doi: 10.1111/j.1574-6976.2011.00290.x

Nord, C., and Edlund, C. (1991). Ecological effects of antimicrobial agents on the human intestinal microflora. *Microb. Ecol. Health Dis.* 4, 193–207.

Norris, A. L., and Serpersu, E. H. (2013). Ligand promiscuity through the eyes of the aminoglycoside N3 acetyltransferase IIa. *Protein Sci.* 22, 916–928. doi: 10.1002/pro.2273

Ohishi, T., Aoki, K., Ishii, Y., Usui, M., Tamura, Y., Kawanishi, M., et al. (2017). Molecular epidemiological analysis of human-and chicken-derived isolates of *Campylobacter jejuni* in Japan using next-generation sequencing. *J. Infect. Chemother.* 23, 165–172. doi: 10.1016/j.jiac.2016.11.011

Olaiya, D., Fok, R., Chakrabarti, P., Sharma, H., and Greig, J. (2018). *Campylobacter* fetus spondylodiscitis: A case report and review of the literature. *IDCases* e00468. doi: 10.1016/j.idcr.2018.e00468

Page, W. J., Huyer, G., Huyer, M., and Worobec, E. (1989). Characterization of the porins of *Campylobacter jejuni* and *Campylobacter coli* and implications for antibiotic susceptibility. *Antimicrob. Agents Chemother.* 33, 297–303. doi: 10.1128/AAC.33.3.297

Pagès, J.-M., and Amaral, L. (2009). Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. *Biochim. Biophys. Acta* 1794, 826–833. doi: 10.1016/j.bbapap.2008.12.011

Pandey, P., Bodhidatta, L., Lewis, M., Murphy, H., Shlim, D. R., Cave, W., et al. (2010). Travelers' diarrhea in Nepal: an update on the pathogens and antibiotic resistance. *J. Travel Med.* 18, 102–108. doi: 10.1111/j.1708-8305.2010.00475.x

Payot, S., Avrain, L., Magras, C., Praud, K., Cloeckaert, A., and Chaslus-Dancla, E. (2004). Relative contribution of target gene mutation and efflux to fluoroquinolone and erythromycin resistance, in French poultry and pig isolates of *Campylobacter coli*. *Int. J. Antimicrob. Agents* 23, 468–472. doi: 10.1016/j.ijantimicag.2003.12.008

Phillips, I., Casewell, M., Cox, T., DeGroot, B., Friis, C., Jones, R., et al. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* 53, 28–52. doi: 10.1093/jac/dkg483

Pollett, S., Rocha, C., Zerpa, R., Patiño, L., Valencia, A., Camiña, M., et al. (2012). *Campylobacter* antimicrobial resistance in Peru: a ten-year observational study. *BMC Infect. Dis.* 12:193. doi: 10.1186/1471-2334-12-193

Pumbwe, L., Randall, L. P., Woodward, M. J., and Piddock, L. J. (2004). Expression of the efflux pump genes *cmeB*, *cmeF* and the porin gene *porA* in multiple-antibiotic-resistant *Campylobacter jejuni*. *J. Antimicrob. Chemother.* 54, 341–347. doi: 10.1093/jac/dkh331

Raeisi, M., Khoshbakht, R., Ghaemi, E. A., Bayani, M., Hashemi, M., Seyedghasemi, N. S., et al. (2017). Antimicrobial resistance and virulence-associated genes of *Campylobacter* spp. isolated from raw milk, fish, poultry, and red meat. *Microb. Drug Resist.* 23, 925–933. doi: 10.1089/mdr.2016.0183

Salazar-Lindo, E., Sack, R. B., Chea-Woo, E., Kay, B. A., Piscoya, Z. A., Leon-Barua, R., et al. (1986). Early treatment with erythromycin of *Campylobacter jejuni*-associated dysentery in children. *J. Pediatr.* 109, 355–360. doi: 10.1016/S0022-3476(86)80404-8

Sanders, J. W., Frenck, R. W., Putnam, S. D., Riddle, M. S., Johnston, J. R., Ulukan, S., et al. (2007). Azithromycin and loperamide are comparable to levofloxacin and loperamide for the treatment of traveler's diarrhea in United States military personnel in Turkey. *Clin. Infect. Dis.* 45, 294–301. doi: 10.1086/519264

Segreti, J., Gootz, T. D., Goodman, L. J., Parkhurst, G. W., Quinn, J. P., Martin, B. A., et al. (1992). High-level quinolone resistance in clinical isolates of *Campylobacter jejuni*. *J. Infect. Dis.* 165, 667–670. doi: 10.1093/infdis/165.4.667

Silva, D. T., Tejada, T. S., Blum-Menezes, D., Dias, P. A., and Timm, C. D. (2016). *Campylobacter* species isolated from poultry and humans, and their analysis using PFGE in southern Brazil. *Int. J. Food Microbiol.* 217, 189–194. doi: 10.1016/j.ijfoodmicro.2015.10.025

Sjögren, E., Lindblom, G., and Kaijser, B. (1997). Norfloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli* isolates from Swedish patients. *J. Antimicrob. Chemother.* 40, 257–261. doi: 10.1093/jac/40.2.257

Smith, K. E., Besser, J. M., Hedberg, C. W., Leano, F. T., Bender, J. B., Wicklund, J. H., et al. (1999). Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992–1998. *N. Engl. J. Med.* 340, 1525–1532. doi: 10.1056/NEJM199905203402001

Tang, Y., Dai, L., Sahin, O., Wu, Z., Liu, M., and Zhang, Q. (2017). Emergence of a plasmid-borne multidrug resistance gene *cfr*(C) in foodborne pathogen *Campylobacter*. *J. Antimicrob. Chemother.* 72, 1581–1588. doi: 10.1093/jac/dkx023

Taylor, N., Davies, R., Ridley, A., Clouting, C., Wales, A., and Clifton-Hadley, F. (2008). A survey of fluoroquinolone resistance in *Escherichia coli* and thermophilic *Campylobacter* spp. on poultry and pig farms in Great Britain. *J. Appl. Microbiol.* 105, 1421–1431. doi: 10.1111/j.1365-2672.2008.03877.x

Thakur, S., Zhao, S., McDermott, P. F., Harbottle, H., Abbott, J., English, L., et al. (2010). Antimicrobial resistance, virulence, and genotypic profile comparison of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and retail meats. *Foodborne Pathog. Dis.* 7, 835–844. doi: 10.1089/fpd.2009.0487

Toledo, Z., Simaluiza, R. J., and Fernandez, H. (2018). Occurance and antibiotic resistance of *Campylobacter jejuni* and *Campylobacter coli* from domestic animals from Southern Ecuador. *Microbiology* 48:e20180003. doi: 10.1590/0103-8478cr20180003

US Food and Drug Administration (2014). *NARMS integrated report: 2014. The National Antimicrobial Resistance Monitoring System (NARMS): Enteric bacteria*. Silver Spring, MD: US Food and Drug Administration. https://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM528861.pdf

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2014). Global trends in antimicrobial use in food animlas. *PNAS* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Vaughn, M. B., and Copeland, D. (2004). "Is there human health harm following fluoroquinolone use in poultry?" in *Proceedings of the 53rd Western Poultry Disease Conference, Sacramento, CA*. 27–29.

Ventola, C. L. (2015a). The antibiotic resistance crisis: part 1: causes and threats. *Phar. Ther.* 40, 277–283.

Ventola, C. L. (2015b). The antibiotic resistant crisis, part 2: management strategies and new agents. *Phar. Ther.* 40, 344–352.

Veterinary Feed Directive (VFD) (2017). FDA. Available at: https://www.fda.gov/animal-veterinary/development-approval-process/veterinary-feed-directive-vfd (Accessed July 2, 2019).

Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., and Blanchard, J. S. (2011). Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. *J. Biol. Chem.* doi: 10.1074/jbc.M111.226936

Vukelic, D., Trkulja, V., and Salkovic-Petrisic, M. (2010). Single oral dose of azithromycin versus 5 days of oral erythromycin or no antibiotic in treatment of *Campylobacter* enterocolitis in children: a prospective randomized assessor-blind study. *J. Pediatr. Gastroenterol. Nutr.* 50, 404–410. doi: 10.1097/MPG.0b013e3181a87104

Wagner, J., Jabbusch, M., Eisenblätter, M., Hahn, H., Wendt, C., and Ignatius, R. (2003). Susceptibilities of *Campylobacter jejuni* isolates from Germany to ciprofloxacin, moxifloxacin, erythromycin, clindamycin, and tetracycline. *Antimicrob. Agents Chemother.* 47, 2358–2361. doi: 10.1128/AAC.47.7.2358-2361.2003

Wegener, H. C. (2003). Antibiotics in animal feed and their role in resistance development. *Curr. Opin. Microbiol.* 6, 439–445. doi: 10.1016/j.mib.2003.09.009

WHO (2014). *Antimicrobial resistance: Global report on surveillance*. Geneva, Switzerland: World Health Organization.

Wieczorek, K., Denis, E., Lachtara, B., and Osek, J. (2017). Distribution of *Campylobacter jejuni* multilocus sequence types isolated from chickens in Poland. *Poult. Sci.* 96, 703–709. doi: 10.3382/ps/pew343

Wieczorek, K., Wołkowicz, T., and Osek, J. (2018). Antimicrobial resistance and virulence-associated traits of *Campylobacter jejuni* isolated from poultry food chain and humans with diarrhea. *Front. Microbiol.* 9:1508. doi: 10.3389/fmicb.2018.01508

Wretlind, B., Strömberg, A., Östlund, L., Sjögren, E., and Kaijser, B. (1992). Rapid emergence of quinolone resistance in *Campylobacter jejuni* in patients treated with norfloxacin. *Scand. J. Infect. Dis.* 24, 685–686.

Yan, M., Sahin, O., Lin, J., and Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J. Antimicrob. Chemother.* 58, 1154–1159. doi: 10.1093/jac/dkl412

Yao, H., Shen, Z., Wang, Y., Deng, F., Liu, D., Naren, G., et al. (2016). Emergence of a potent multidrug efflux pump variant that enhances *Campylobacter* resistance to multiple antibiotics. *MBio* 7, e01543–e01516. doi: 10.1128/mBio.01543-16

Zhao, S., McDermott, P. F., Friedman, J., Abbot, S., Ayers Glenn, A., Hall-Robinson, E., et al. (2006). Antimicrobial resistance and genetic relatedness among Salmonella from retail foods of animal origin: NARMs retail meat surveillance. *Foodborne Pathog. Dis.* 3, 106–117. doi: 10.1089/fpd.2006.3.106

Zhao, S., Mukherjee, S., Chen, Y., Li, C., Young, S., Warren, M., et al. (2015). Novel gentamicin resistance genes in *Campylobacter* isolated from humans and retail meats in the USA. *J. Antimicrob. Chemother.* 70, 1314–1321. doi: 10.1093/jac/dkv001

Zhao, S., Young, S., Tong, E., Abbott, J., Womack, N., Friedman, S., et al. (2010). Antimicrobial resistance of *Campylobacter* isolates from retail meat in the United States between 2002 and 2007. *Appl. Environ. Microbiol.* 76, 7949–7956. doi: 10.1128/AEM.01297-10

Conflict of Interest Statement: HP is an employee of Diamond V.

The remaining 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 August 2019
doi: 10.3389/fmicb.2019.01940

A Mathematical Model of *Campylobacter* Dynamics Within a Broiler Flock

Thomas Rawson[1]*, *Marian Stamp Dawkins*[2] and *Michael B. Bonsall*[1]

[1] Mathematical Ecology Research Group, Department of Zoology, University of Oxford, Oxford, United Kingdom,
[2] Department of Zoology, University of Oxford, John Krebs Field Station, Oxford, United Kingdom

Globally, the bacterial genus *Campylobacter* is one of the leading causes of human gastroenteritis, with its primary route of infection being through poultry meat. The application of biosecurity measures is currently limited by a lack of understanding of the transmission dynamics within a flock. Our work is the first to undertake a mathematical modeling approach to *Campylobacter* population dynamics within a flock of broilers (chickens bred specifically for meat). A system of stochastic differential equations is used to model the routes of infection between co-housed birds. The presented model displays the strong correlation between housing density and *Campylobacter* prevalence, and shows how stochastic variation is the driving factor determining which strains of *Campylobacter* will emerge first within a flock. The model also shows how the system will rapidly select for phenotypic advantages, to quickly eliminate demographically-weaker strains. A global sensitivity analysis is performed, highlighting that the growth and death rate of other native bacterial species likely contributes the greatest to preventing flock outbreaks, presenting a promising approach to hypothesizing new methods of combatting disease transmission.

Keywords: *Campylobacter*, mathematical modeling, stochastic differential equations, bacterial population dynamics, microbiome

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Antonio Valero,
Universidad de Córdoba, Spain
Xiaonan Lu,
University of British Columbia, Canada

***Correspondence:**
Thomas Rawson
thomas.rawson@zoo.ox.ac.uk

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

Received: *11 April 2019*
Accepted: *07 August 2019*
Published: *21 August 2019*

Citation:
Rawson T, Dawkins MS and Bonsall MB (2019) A Mathematical Model of Campylobacter Dynamics Within a Broiler Flock. Front. Microbiol. 10:1940. doi: 10.3389/fmicb.2019.01940

1. INTRODUCTION

Campylobacter is recognized as the leading cause of human gastroenteritis in the developed world (Ghareeb et al., 2013). While several transmission routes have been noted over the years (Nauta et al., 2005), poultry meat has been overwhelmingly attributed as the leading route of ingestion for humans [EFSA Panel on Biological Hazards (BIOHAZ), 2011]. An ongoing study by Public Health England has highlighted the extent to which *Campylobacter* spp. have dominated our commercial poultry. Seventy-three percent of supermarket chicken carcasses were found to contain *Campylobacter* and 7% of the outer packaging was similarly contaminated (Jorgensen et al., 2015). An estimated 450,000 people across the United Kingdom are infected every year, with 10% of these infections resulting in hospitalization (Strachan and Forbes, 2010). The immediate impact of infection is rarely fatal in the developed world, characterized by stomach cramps and diarrhea, however the resulting sequelae, while rare, are far more serious. *Campylobacteriosis* leaves the host ∼100 times more likely to develop the auto-immune disorder Guillain-Barré syndrome (McCarthy and Giesecke, 2001).

While the bacteria provoke an aggressive response in human hosts, the most common species, *Campylobacter jejuni*, is considered commensal within its most common host, broiler chickens. The term "broiler" refers to any chicken bred and raised specifically for meat production. Once *Campylobacter* is present in a flock, full colonization of all birds occurs very rapidly (Evans and Sayers, 2000). From the introduction of one infected bird, it can take only a single week for an entire flock to become infected (Stern N.J. et al., 2001). The bacteria are spread via the fecal-oral route. After becoming infected, the newly-infected host broiler spends a brief period in a non-infectious incubation period, before excreting the bacteria in its fecal and cecal matter. Surrounding susceptible broilers are then exposed to this by ingesting the surrounding feed and water (Shanker et al., 1990). While the direct cause of introduction to the flock is uncertain, an exhaustive review by Adkin et al. (2006) considered that horizontal transmission is by far the most likely route, primarily being brought into a susceptible flock from some other source on the farm, such as the enclosures of other farm animals. This is as opposed to vertical transmission from breeder flocks, which are themselves often fully colonized by *Campylobacter* spp. Nevertheless, there may be a combination of both routes of entry into a flock, which deserves greater consideration.

Campylobacter is very rarely observed to colonize the gut of very young chickens (0–2 weeks of age) (Newell and Wagenaar, 2000). This is theorized to be the result of a supply of innate maternal antibodies acquired during a pre-laying period. This immunity has been shown to have significant bactericidal properties (Sahin et al., 2001).

Despite numerous intervention measures being trialed and employed on farms, little impact has been seen in reducing outbreak incidence (Hermans et al., 2011). This is due in part to the aggressive rate of proliferation once *Campylobacter* has entered a flock, coupled with persisting uncertainty in the exact route of primary infection. Specifically designed prevention methods are also marred by genetic variation and plasticity of *Campylobacter* spp. (Tresse et al., 2017).

Of increasing concern is the growing trend of antimicrobial resistance in *campylobacteriosis* outbreaks. Roughly 90% of the antibiotics applied in agriculture are used only to promote growth or as prophylactic agents, as opposed to being used to treat infection (Khachatourians, 1998). This overzealous use has been a major contributing factor to the continuing spread of antibiotic resistance. Ge et al. (2003) conducted a study showing that 94% of tested raw chicken samples were resistant to at least one of seven antibiotics being tested, 54% of which showed resistance to erythromycin, the antibiotic most commonly used to treat *campylobacteriosis*. These antimicrobial-resistant strains cause more prolonged and severe illness in humans (Travers and Michael, 2002) and create a scenario where *in-vitro* susceptibility testing may be necessary before any drugs may be prescribed.

Despite a wealth of empirical investigations, there is a lack of knowledge synthesizing these empirical findings through theoretical modeling frameworks. Only two studies have considered a theoretical approach to understanding *Campylobacter* spp. outbreaks; Hartnett et al. (2001) and Van Gerwe et al. (2005), who built a basic susceptible-infected (SI) model and a probabilistic model, respectively. Both frameworks only consider a model on the scale of a flock through basic susceptible-infected interactions. These approaches are not sophisticated enough to develop any meaningful theories on *Campylobacter* dynamics, as they do not represent or convey any specific interbacterial actions by *Campylobacter* populations. The lack of modeling approaches is likely due in part to the inherent challenges of mathematically simulating a gut microbiome. Over 100 different bacterial genera have been isolated from the intestines of chickens (Pan and Yu, 2014), all with a range of individual ecological interactions with one-another. Questions must then be asked regarding how to simulate the temporal and spatial impact of gut motility on the development of a microbial community. Despite these challenges, simplified models of stochastic differential equations have proved effective in capturing the often frenetic bacterial population dynamics within the gut (Wiles et al., 2016).

Here, we introduce a framework of stochastic differential equations that captures the basic interactions that are known to be observed within the broiler gut. Using this framework we simulate the propagation of multiple strains of *Campylobacter* through multiple birds in a flock. In the analysis presented below we observe key dynamical behavior commonly observed through experimentation, which can now be mechanistically explained using this theoretical framework. The theoretical insights derived from this model can be used to refine current hypotheses regarding *Campylobacter* transmission and inform future experimental and control efforts. The model will likely be of use to experimentalists and risk assessors in theorizing the impact of potential new disease prevention methods on the bacterial transmission dynamics.

2. MODELING FRAMEWORKS

2.1. Deterministic Model

Before presenting the stochastic differential equation framework, we begin by introducing the underlying deterministic core of the framework and the particular interactions modeled. Consider four variables to describe the bacterial populations within a broiler's digestive tract. C, the proportion of a single bird's gut flora made up of *Campylobacter*. B, the proportion of the gut flora made up of other bacterial species competing for space and resources. P, the proportion of the gut containing host defense peptides (HDPs) (this may also be interpreted as other plausible forms of host autoimmune response). Lastly, M, the proportion of the gut containing innate maternal antibodies. These all take values ranging such that $0 \leq C, B, P, M \leq 1$. The set of ODEs describing the dynamics follows:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = r_1 C\left(1 - (C + \alpha_1 B)\right) - \gamma CP - d_1 C - \beta CB - \sigma CM, \tag{1}$$

$$\frac{\mathrm{d}B}{\mathrm{d}t} = r_2 B\left(1 - (B + \alpha_2 C)\right) - d_2 B, \tag{2}$$

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \xi CP(1 - P) - d_3 P, \tag{3}$$

$$\frac{\mathrm{d}M}{\mathrm{d}t} = -d_4 M. \quad (4)$$

All rate constants are defined below in **Table 1**. Model boundedness is shown in Appendix S1 (**Supplementary Material**). The first term $\left(r_1 C\left(1-(C+\alpha_1 B)\right)\right)$ in Equation (1) describes the logistic growth of *Campylobacter* to a relative carrying capacity of 1, while in competition with other bacteria *B*. Competition for resources is the key to success within the gut. *Campylobacter* is known to be an effective colonizer (Stahl et al., 2012), as it is very effective at drawing zinc and iron from its environment (Gielda and DiRita, 2012; Raines et al., 2016). The second term (γCP) in Equation (1) models the inhibitory effect of host defense peptides, *P*. These peptides are created in response to challenge by *Campylobacter*, as shown by Cawthraw et al. (1994). The third term ($d_1 C$) of Equation (1) simply describes the natural death rate of *Campylobacter*. The fourth term (βCB) simulates an important interbacterial interaction; that some of the most abundant competing bacteria in the microbiome have an inhibitory effect on *Campylobacter* (Schoeni and Doyle, 1992). The final term (σCM) of Equation (1) represents the strong bactericidal abilities of the bird's maternal antibodies. All chickens hatch with an initial supply of antibodies that depletes over time, gone by about 3 weeks of age (Sahin et al., 2001) (most broilers are slaughtered at 5 or 6 weeks of age, however some organic and free-range flocks are slaughtered at approximately 8 weeks). These antibodies have a strong inhibitory effect on *Campylobacter*, and many studies are unable to detect *Campylobacter* (by culture methods) in birds under 2 weeks of age under commercial conditions (Sahin et al., 2015). However, forced inoculation of high-quantities of *Campylobacter* soon after hatching can still result in expression of the bacteria (Welkos, 1984).

Equations (2), (3), and (4) follow a similar logic to Equation (1). Other bacteria, *B*, grow in competition with *Campylobacter* to a carrying capacity. Defense peptides, *P*, grow in response to *Campylobacter* expression (not in competition for resources), and the population of maternal antibodies, *M*, does not grow. All variables decay at a rate proportional to their respective populations.

Note that the above model could be reduced by amalgamating terms in Equations (1) and (2), however we choose to keep these separate to (i) keep biological processes clearly defined, and (ii) make further model development and sensitivity analyses clearer.

Ignoring the trivial cases of complete domination by either *C* or *B*, the basic dynamical behavior observed for this simplified model is illustrated in **Figure 1**. Notably, *Campylobacter* is absent from the microbiome until the maternal antibody population has been exhausted. At this point a sudden, temporary, surge in the population of *Campylobacter* is observed. This phenomena is due to the very low population of HDPs, caused by the strong effect of the initial maternal antibodies. The HDP population then quickly

TABLE 1 | Model parameters and baseline values.

Expression	Description	Value
r_{C_j}	Growth rate for *Campylobacter* strain *j*.	0.3009
r_2	Growth rate for other bacteria (*B*).	0.1407
α_1	*Campylobacter* competition coefficient.	0.9744
α_2	Other bacteria competition coefficient.	1
γ_{C_j}	Rate of inhibition by host defense peptides (*P*) on *Campylobacter* strain *j*.	0.6358
ξ_j	Rate of host defense peptide growth in response to *Campylobacter* strain *j*.	0.7411
b	Rate of broiler shedding *Campylobacter* into the environment, E_j.	10
Ω	Total environmental carrying capacity of *Campylobacter*.	200,000*
d_{C_j}	Death rate of *Campylobacter* strain *j*.	0.0185
d_2	Death rate of other bacteria.	0.0212
d_3	Decay rate of host defense peptides.	0.0463
d_4	Decay rate of maternal antibodies.	0.0046
d_5	Death rate of *Campylobacter* in the environment.	0.05
β_{C_j}	Rate of inhibition by other bacteria on *Campylobacter* strain *j*.	0.0276
σ_{C_j}	Rate of inhibition by maternal antibodies on *Campylobacter* strain *j*.	0.0661
η_{C_j}	Scaling factor applied to stochastic *Campylobacter* growth in the gut.	0.01
η_{BC_j}	Scaling factor applied to stochastic *Campylobacter* inhibition by other competing bacteria.	0.0847
η_2	Scaling factor applied to stochastic competing bacteria (*B*) growth.	0.01
η_3	Scaling factor applied to stochastic host defense peptide (*P*) growth.	0.01
η_4	Scaling factor applied to stochastic maternal antibody (*M*) decay.	0.01
η_5	Scaling factor applied to stochastic *Campylobacter* growth in the environment.	0.01

*Descriptions for all parameter values appearing in the final stochastic model, Equations (14–18). Baseline values are given, used for model validation and simulation case studies. Values were calculated by using simulated annealing to identify a parameter set that best fits the experimental data of Achen et al. (1998) (**Figure 2**). See Appendix S2 in **Supplementary Material** for an explanation of simulated annealing. * Ω value is dependent on the experiment specifics for model validation, but flock case studies consider a flock of 400 chickens, and an Ω value of 200,000.*

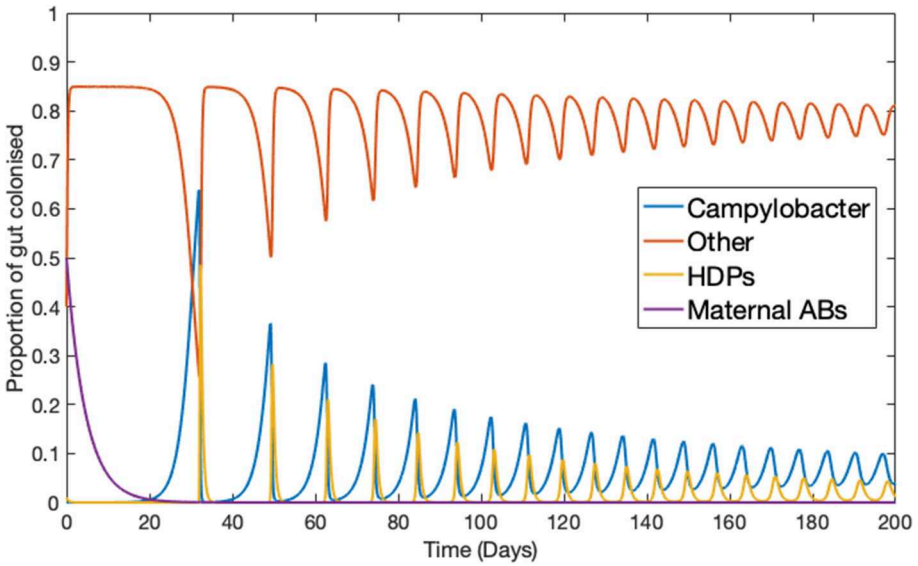

FIGURE 1 | Deterministic model for one chicken. An example of the typical dynamical behavior observed for simulations of Equations (1–4). Parameters defined in **Table 1**.

rises to meet this sudden challenge, bringing the *Campylobacter* population back to a lower level in an oscillating manner, where it eventually reaches a steady-state equilibrium. This behavior is commonly observed in experimental studies (Achen et al., 1998; Newell and Fearnley, 2003).

From this simple core of four equations we adapt the model to allow for N unique strains of *Campylobacter*, by describing each strain as a separate variable. Equation (1) is repeated for each individual strain, while altering the growth rate terms to reflect the fact that all strains will also be in competition with one another. This alteration is represented by the following set of ODEs:

$$\frac{\mathrm{d}C_j}{\mathrm{d}t} = r_{C_j} C_j \left(1 - \left(\sum_{j=1}^{N} C_j + \alpha_1 B\right)\right) - \gamma_{C_j} C_j P - d_{C_j} C_j - \beta_{C_j} C_j B - \sigma_{C_j} C_j M, \tag{5}$$

$$\frac{\mathrm{d}B}{\mathrm{d}t} = r_2 B \left(1 - \left(B + \alpha_2 \sum_{j=1}^{N} C_j\right)\right) - d_2 B, \tag{6}$$

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \sum_{j=1}^{N} \xi_j C_j P(1-P) - d_3 P, \tag{7}$$

$$\frac{\mathrm{d}M}{\mathrm{d}t} = -d_4 M. \tag{8}$$

Here C_j represents the jth strain of *Campylobacter*, where $j \in \{1, 2, ..., N\}$, and N is the total number of strains. As such this adjusted model is composed of $N + 3$ variables. The next alteration is to allow for multiple birds and the ability for *Campylobacter* to move from one bird to another. This is done by repeating the $N + 3$ equations presented in Equations (5)–(8) for each bird, and introducing new variables to display the saturation of *Campylobacter* strains in the shared living space.

As such, the newly adjusted model to describe the population dynamics of N strains of *Campylobacter* within L broilers, is written as,

$$\frac{\mathrm{d}C_{ij}}{\mathrm{d}t} = r_{C_j} C_{ij} \left(1 - \left(\sum_{j=1}^{N} C_{ij} + \alpha_1 B_i\right)\right) - \gamma_{C_j} C_{ij} P_i - d_{C_j} C_{ij} - \beta_{C_j} C_{ij} B_i - \sigma_{C_j} C_{ij} M_i + a\frac{E_j}{\Omega}, \tag{9}$$

$$\frac{\mathrm{d}B_i}{\mathrm{d}t} = r_2 B_i \left(1 - \left(B_i + \alpha_2 \sum_{j=1}^{N} C_{ij}\right)\right) - d_2 B_i, \tag{10}$$

$$\frac{\mathrm{d}P_i}{\mathrm{d}t} = \sum_{j=1}^{N} \xi_j C_{ij} P_i (1 - P_i) - d_3 P_i, \tag{11}$$

$$\frac{\mathrm{d}M_i}{\mathrm{d}t} = -d_4 M_i, \tag{12}$$

$$\frac{\mathrm{d}E_j}{\mathrm{d}t} = \sum_{i=1}^{L} b C_{ij} \left(1 - \frac{E_j}{\Omega}\right) - d_5 E_j. \tag{13}$$

Here then, C_{ij} represents the proportion of the ith broiler's gut bacteria which is composed of *Campylobacter* strain j. B_i is the proportion of the ith broiler's gut bacteria made up of other bacterial species competing for space and resources. P_i, the proportion of the ith broiler's gut containing host defense peptides. M_i is the proportion of the ith broiler's gut containing innate maternal antibodies. Here $i \in \{1, 2, ..., L\}$, where L is the total number of broilers. E_j represents the amount of *Campylobacter* strain j that is currently in the flock's enclosed living space. We assume a living space of fixed size shared by

all broilers. As such, Ω represents this total size, or carrying capacity for strains. The first term in Equation (13) shows that the amount of strain j in the environment is increased by being shed from birds that are already infected with strain j at a rate b. Note from the final term $\left(a\frac{E_j}{\Omega}\right)$ in Equation (9) that birds may then ingest strain j from the environment at a rate $\frac{a}{\Omega}$. This route of infection simulates the fecal-oral route of infection, but may be interpreted as some other intermittent transmission stage between birds. As such we do not remove *Campylobacter* from the environment (E_j) upon an ingestion event, as the possibility of further environmental contamination is not yet understood and may indeed depend on the specific route of infection. The model is now composed of $L(N + 3) + N$ equations, for N strains of *Campylobacter*, and L individual broilers.

2.2. Stochastic Model

While several important biological phenomena can be discovered and better understood with the model in its current, deterministic, form, there are key reasons to pursue a stochastic framework. First, having one variable alone to represent the multitudes of bacterial species that make up the constantly-evolving gut microbiome is, of course, a significant simplification. In practice, these other bacterial species competing with *Campylobacter* will be constantly changing, both in resurgences of population and in how they interact with *Campylobacter*. Adding stochastic elements to these populations and interactions is a small step toward capturing some of this more unpredictable behavior. Indeed the biomass of *Campylobacter* measurable in fecal and cecal matter has been observed to fluctuate widely (Morishita et al., 1997; Achen et al., 1998). Secondly, the density dependent assumptions made when formulating the initial deterministic model, that is that interaction rates are directly proportional to the variable populations, are assumptions that break down for smaller populations. The simulations undertaken often display bacterial populations at very small quantities, especially in the initial period dominated by maternal antibodies. A stochastic system behaves very differently under these circumstances and means that the model is more likely to display cases of strain extinction, a phenomena that the deterministic model cannot capture. Indeed, the very nature of *Campylobacter* infections is one that is often described in the language of probability. The all-or-nothing nature of flock infections means that we often must ask what measures can reduce the likelihoods of infections, rather than the magnitude. Through a stochastic framework we explore multiple realizations of potential outcomes, and investigate reducing the likelihood of outbreaks in a flock of broilers. The case studies presented below highlight the need for a stochastic modeling approach to accurately capture the multi-strain dynamics of *Campylobacter* within the gut. This approach allows for the simulation of turn-over and resurgence of dominant strains, an experimentally observed behavior (Colles and Maiden, 2014) that cannot be captured by a deterministic system.

For the stochastic framework, Equations (9–13) are adjusted to the following set of stochastic differential equations,

$$\begin{aligned} \mathrm{d}C_{ij} = &\left[r_{C_j} C_{ij} \left(1 - \left(\sum_{j=1}^{N} C_{ij} + \alpha_1 B_i \right) \right) - \gamma_{C_j} C_{ij} P_i - d_{C_j} C_{ij} \right. \\ &\left. - \beta_{C_j} C_{ij} B_i - \sigma_{C_j} C_{ij} M_i + a(E_j) \right] \mathrm{d}t \\ &+ \left[\eta_{C_j} C_{ij} + \lambda_j(t) - \eta_{BC_j} C_{ij} B_i \right] \mathrm{d}W_t, \end{aligned} \tag{14}$$

$$\begin{aligned} \mathrm{d}B_i = &\left[r_2 B_i \left(1 - \left(B_i + \alpha_2 \sum_{j=1}^{N} C_{ij} \right) \right) - d_2 B_i \right] \mathrm{d}t \\ &+ \left[\eta_2 B_i \right] \mathrm{d}W_t, \end{aligned} \tag{15}$$

$$\mathrm{d}P_i = \left[\sum_{j=1}^{N} \xi_j C_{ij} P_i (1 - P_i) - d_3 P_i \right] \mathrm{d}t + \left[\eta_3 P_i \right] \mathrm{d}W_t, \tag{16}$$

$$\mathrm{d}M_i = \left[-d_4 M_i \right] \mathrm{d}t + \left[\eta_4 M_i \right] \mathrm{d}W_t, \tag{17}$$

$$\mathrm{d}E_j = \left[\sum_{i=1}^{L} b C_{ij} \left(1 - \frac{E_j}{\Omega} \right) - d_5 E_j \right] \mathrm{d}t + \left[\eta_5 E_j \right] \mathrm{d}W_t, \tag{18}$$

where $\lambda_j(t)$ is defined by;

$$\lambda_j(t) = \begin{cases} 0, & \text{if } C_{ij}(t) = 0. \\ 2.873 \times 10^{-4}, & \text{otherwise.} \end{cases}$$

and where $a(E_j)$ is defined by;

$$a(E_j) = \begin{cases} 0.015, & \text{if } X < \frac{E_j}{\Omega} \text{ for random variable } X \sim \mathcal{U}(0,1). \\ 0, & \text{otherwise.} \end{cases}$$

Here W_t denotes a Wiener process (standard Brownian motion process). These stochastic increments are scaled by the respective population size and constants η_2 through to η_5. These constants dictate the variance of their respective Wiener processes, defining the range of stochasticity attributed to the growth rate of their respective variables. The changes and additions shown in Equation (14) warrant further explanation. The sixth term ($a(E_j)$) in Equation (14) (the last of the deterministic terms), has been changed from a constant rate of ingestion from the environment, as seen in Equation (9), to instead have ingestion modeled by a chance to ingest *Campylobacter* depending on the amount of that strain in the environment, E_j. The greater E_j is, the more likely it is for ingestion to occur.

The eighth term $[\lambda_j(t)]$ in Equation (14) is a stochastic term independent of the population of C_{ij}. This is introduced to allow for the possibility of extinction events, should the population of C_{ij} reach a particularly low threshold. This threshold is decided by the value taken by $\lambda_j(t)$, in this case 2.873×10^{-4}. As with all other parameter values displayed in **Table 1**, the values used in the expressions $\lambda_j(t)$ and $a(E_j)$ are calculated through model

validation against the studies presented below in section 2.3. Finally, the ninth term of Equation (14) considers stochasticity surrounding the interactions between C_{ij} and the competing bacteria B_i. This term allows for instances when the particular biodiversity and spatial structure of the gut microbiome may be more inhibitory toward *Campylobacter*, or perhaps actually assisting its growth instead.

Several interesting dynamical behaviors can be observed using this model, which are highlighted through some specific question-led case studies. **Table 1** defines all parameters presented in the final stochastic model [(14–18)] as well as a baseline of parameter values that were used in model validation against real world data sets (presented below). The model is constructed to an arbitrary timescale, however the parameter values given in **Table 1** ensure that multiple oscillations in the *Campylobacter* population can be observed in the below case studies, a phenomena observed in the lifespan of broilers (Morishita et al., 1997).

Note that throughout we have chosen to use a *Campylobacter* competition coefficient of $\alpha_1 = 0.9744 < 1$. This choice is justified in that bacterial populations can inhabit multiple intestinal niches that cannot be colonized by other competing bacteria. Indeed competitive exclusion therapies have been far less effective in tackling *Campylobacter* compared to other foodborne illnesses such as *Salmonella* (Stern N. et al., 2001). The deterministic model is solved using the ode45 solver, a fifth-order Runge-Kutta method in Matlab. The stochastic model is solved numerically using the Euler-Maruyama method (Higham, 2001) with $N = 2^{14}$ timesteps, also programmed in Matlab. The code used to produce all figures presented is available at: https://osf.io/b3duc/.

We also note that while the model is general enough that it is not specific to any one species of *Campylobacter*, two of the three datasets that were used to tune our model parameter values were from studies unique to the most common species, *Campylobacter jejuni* (Achen et al., 1998; Stern N.J. et al., 2001). As such we may expect slightly different parameter values to be applicable for other species of *Campylobacter*. However, from testing the model, the results we present below are ones that are generally observed for a range of parameter values, and as such are relevant findings to the whole genus.

2.3. Model Validation

We test our model by comparing its predictions against three experimental studies on *Campylobacter* expression and spread. Firstly, we consider the work of Achen et al. (1998). Achen et al. performed an experiment with 24 broilers, who were kept in individual, isolated wire-bottomed cages. Birds were confirmed as free of *Campylobacter* before being inoculated with a *C. jejuni* suspension. A cloacal swab was then obtained from each bird every day for 42 days, to monitor whether or not each bird was shedding *Campylobacter*. **Figure 2** shows their experimental results alongside the predictions made by our model.

Specifically, the blue line represents the modal value of the percentage of the 24 birds shedding across a thousand simulations, with error bars depicting the standard deviation across these simulations. Achen et al. (1998) also reports how most birds would shift from phases of positive shedding to negative shedding, a phenomena also captured by the oscillating behavior displayed by the model. Sampling via culture methods like those performed in this experiment is prone to false-negative results for samples with very low quantities of *Campylobacter* (Acke et al., 2009). Therefore, for this model validation, we considered a broiler as being clear of *Campylobacter* if its proportion of *Campylobacter* (variable C) was below 0.005. This was considered a more accurate measure to correspond with the experimental data. While our model was constructed to an arbitrary timescale, comparing to this real-world data set it was found that our time axis is best rescaled by a factor of 0.021 to align with the measure of days used in these studies. This corrected timescale is used for all subsequent case studies within this paper.

Secondly, we consider the experiment conducted by Stern N.J. et al. (2001). Multiple separate pens were prepared, each containing 70 broilers, all free of *Campylobacter*. A *Campylobacter*-positive seeder bird was then added to the flock. Different pens had seeder birds introduced at different points in time. Three, five, and seven days after a seeder bird was introduced, a sample of chickens were tested for *Campylobacter* to estimate the percentage of the flock that was currently *Campylobacter*-positive. We plot our model predictions against Stern et al.'s experimental data below in **Figure 3**. To match the housing density of the experiment, a value of $\Omega = 45,369$ was used for the model. An error band is plotted around our model prediction displaying the standard deviation of values across 100 simulations.

Lastly we simulated the experiment performed by Van Gerwe et al. (2005). Four flocks of 400 birds were set up in individual enclosures from day of hatch. Four birds in each flock were then inoculated with a *Campylobacter* suspension and returned to the flock. Birds were then sampled from each flock throughout the next few weeks to record the percentage of flock infection. **Figure 4** plots their experimental data against our model prediction. For the experiments shown in **Figures 4A,B**, the four seeder birds were inoculated at day of hatch, and chickens were sampled by cloacal swabbing. For the experiments shown in **Figures 4C,D**, the seeder birds were inoculated one day after hatch, and the flock was analyzed by collecting fresh fecal samples.

3. SIMULATIONS

We now use a series of (simulated) case studies to investigate key dynamical behaviors and predictions from the model.

3.1. Staggered Strain Infection

In this first example, the deterministic model for multiple strains in one broiler (Equations 5–8) is considered. Five strains of *Campylobacter* within one chicken are simulated, all with the exact same respective rate constants as shown in **Table 1**. **Figure 5A** shows the results when all five strains are introduced at $t = 0$ days, with the same initial inoculation amount of $C_i(0) = 0.0001$. **Figure 5B** shows instead when each strain is introduced in intervals of $t = 5$ days. Therefore, only strain 1 is introduced at $t = 0$ days, strain 2 is introduced at $t = 5$ days and so on until finally strain

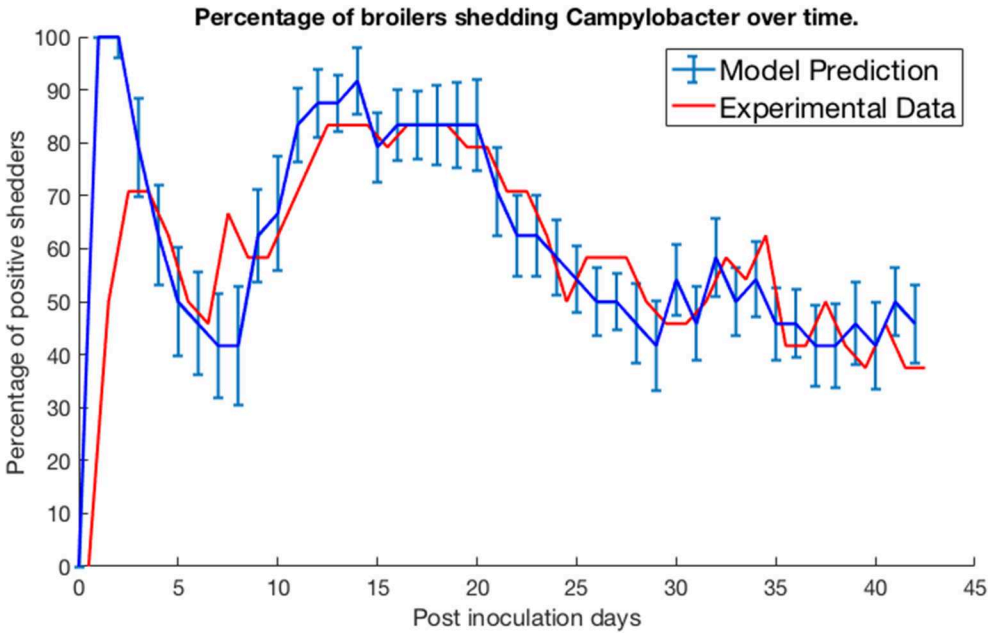

FIGURE 2 | Model validation against data of Achen et al. (1998). The percentage of a group of isolated broilers shedding *Campylobacter* across several weeks following inoculation.

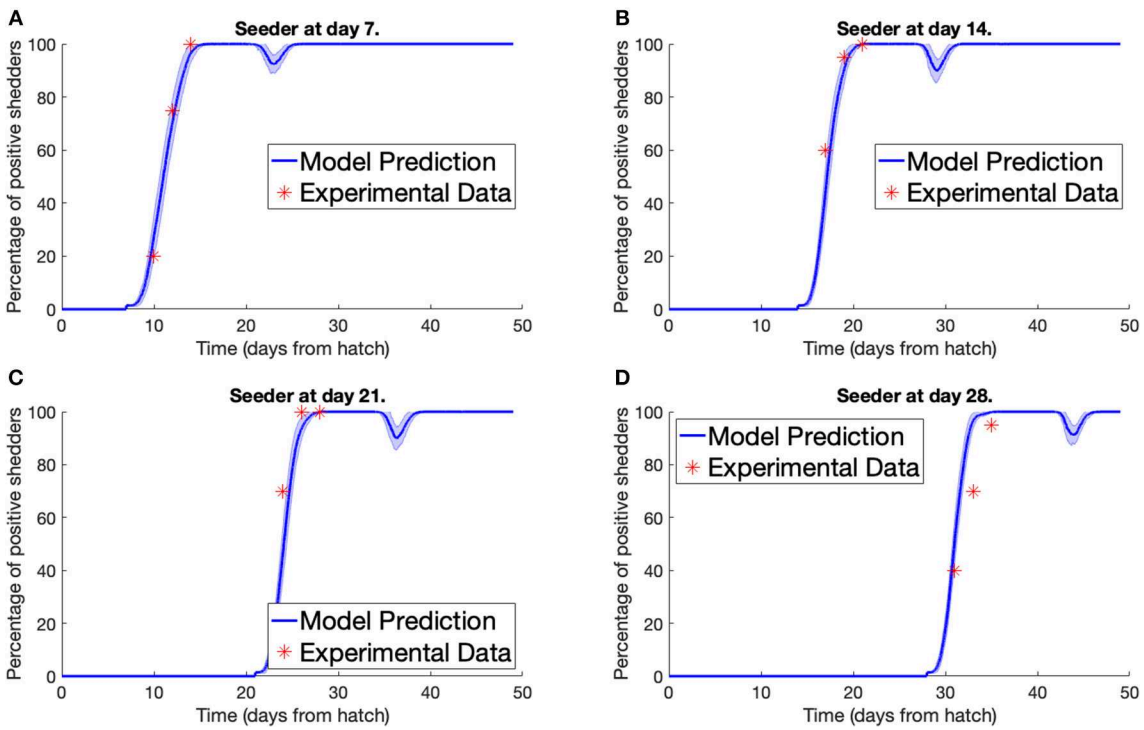

FIGURE 3 | Model validation against data of Stern N.J. et al. (2001). The percentage of a flock of broilers shedding *Campylobacter* across several weeks after introduction of a *Campylobacter*-positive seeder bird at **(A)** 7 days, **(B)** 14 days, **(C)** 21 days, **(D)** 28 days.

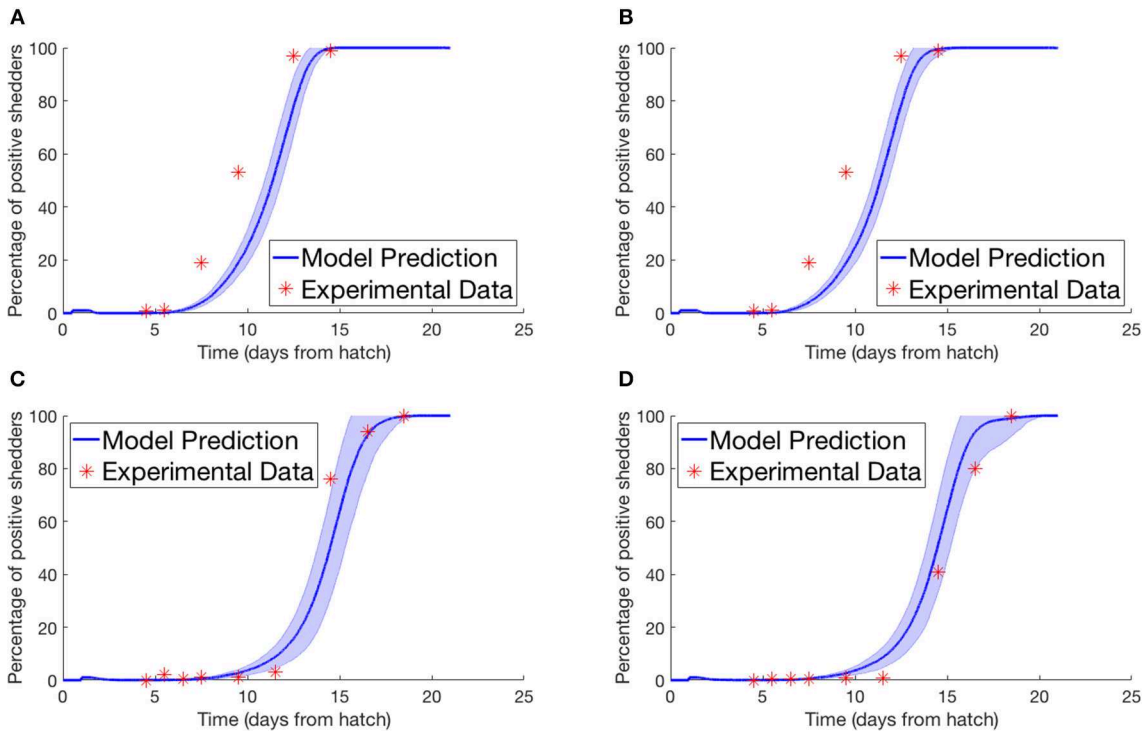

FIGURE 4 | Model validation against data of Van Gerwe et al. (2005). The percentage of a flock of broilers shedding *Campylobacter* across several weeks after introduction of a *Campylobacter*-positive seeder bird. **(A,B)** Seeder bird introduced at day of hatch, samples collected via cloacal swab, **(C,D)** seeder bird introduced one day after hatch, samples collected via fresh fecal droppings.

5 is introduced at $t = 20$ days. In both cases the other three variables are initialized at $B(0) = 0.4$, $P(0) = 0.01$, and $M(0) = 0.5$.

While the maternal antibodies (M) are not plotted on these figures, they approach 0 at approximately $t = 20$ days, as can be seen by the following surge in *Campylobacter* populations following this point in **Figure 5**. While, unsurprisingly, all strains perform identically in **Figure 5A** (where strains are initialized at the same point in time), a more curious dynamic is observed in **Figure 5B**. The strain that performs best and exists at the highest proportion in the staggered release example is strain 2, the second strain to be introduced. The reason for this is that strain 1, present at $t = 0$ days, is initially suppressed by the maternal antibodies (parameter M), reducing the proportion of strain 1. As a result, when strain 2 is introduced, it is able to capitalize on the severely reduced amount of strain 1, and the reduced amount of maternal antibodies, to quickly grow and dominate the competitive space. Strain 2's increased presence then puts future strains at a disadvantage as it has already had the opportunity to establish itself within the gut. These results suggest that dominant *Campylobacter* strains can prevent new strains from taking hold. Moreover, there is an optimal point in time for inoculation to occur for a strain to become dominant, as shown in **Figure 5B** where strain 2 is consistently occupying a higher proportion of the gut than other strains.

3.2. Stochastic Model—One Strain in One Broiler

The stochastic model (Equations 14–17) is run to simulate one strain of *Campylobacter* within one broiler. In this scenario, we ignore the environmental variable E (Equation 18), as its input is negligible for only one broiler. The rate constants are kept at the same values as used previously, defined in **Table 1**, with the additions of the stochastic variance scaling rate constants, parameters that limit the variance of the stochastic additions. These are set as $\eta_{C_j} = \eta_2 = \eta_3 = \eta_4 = 0.01$, and $\eta_{BC_j} = 0.0847$. η_{BC_j} is set higher than the other stochastic rate constants to capture the greater unpredictability surrounding these bacterial interactions. Four different realizations of this model are presented in **Figure 6**, all initialized at $C(0) = 0.02$, $B(0) = 0.4$, $P(0) = 0.01$, $M(0) = 0.5$.

Empirical studies measuring the amount of *Campylobacter* in the fecal matter of isolated broilers have shown a spectrum of results. Some broilers display sustained high populations, others express initial peaks followed by great reduction and potentially later resurgence, and sometimes extinction cases are observed (Achen et al., 1998). All these dynamical behaviors can be observed in different realizations of this model (**Figure 6**). **Figure 6A** shows an instance where a broiler is consistently infected and shedding into the environment, unable to effectively clear the *Campylobacter* from its system. **Figure 6B** instead shows an instance where a broiler has multiple periods of high infection

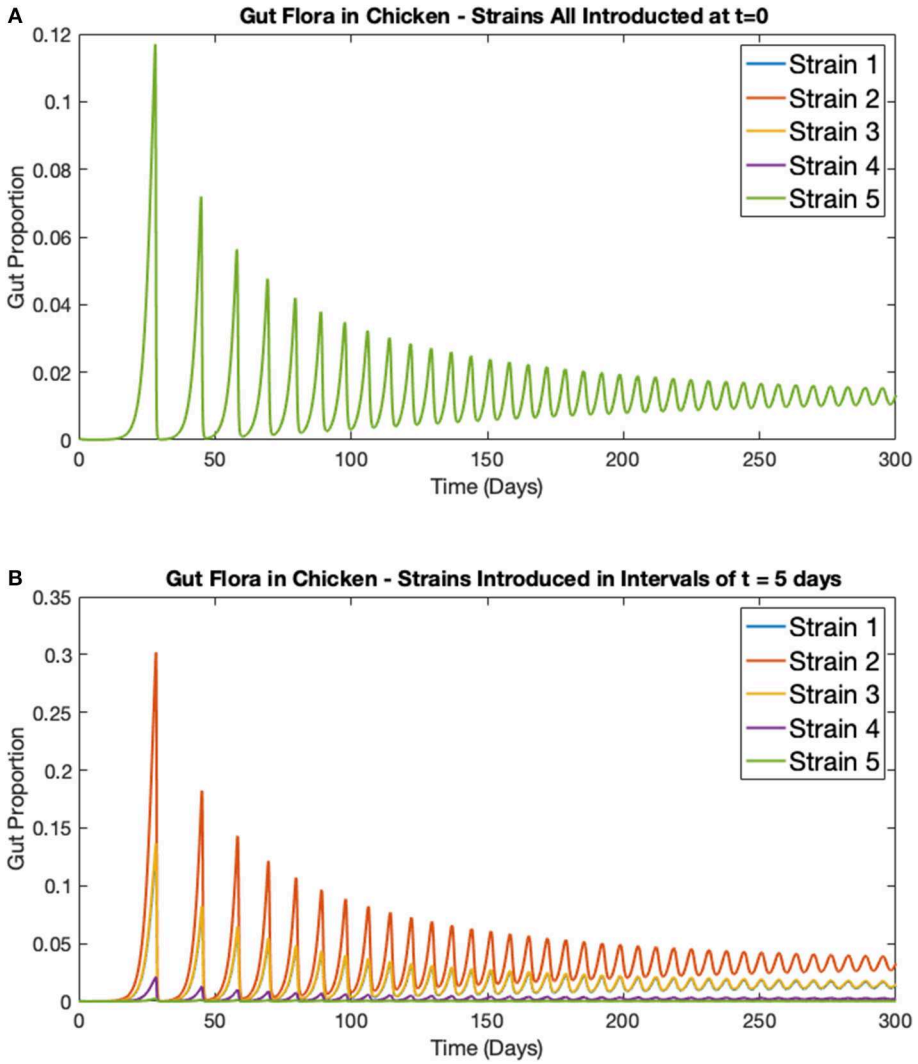

FIGURE 5 | Simulations of multiple *Campylobacter* strains within one broiler. Population growth of five strains of *Campylobacter* within one broiler that are **(A)** all introduced at $t = 0$ days **(B)** introduced in intervals of $t = 5$ days. Strains are initialized at $C_j(t) = 0.0001$ at their respective time of introduction. Other variables are initialized at $B(0) = 0.4$, $P(0) = 0.01$, and $M(0) = 0.5$. Note that the single green line in **Figure 5A** is due to overlap, all five strains exhibit the exact same dynamical behavior, as would be expected.

and shedding, before being able to clear the infection. **Figure 6C** shows an instance where after one initial peak in *Campylobacter* expression, a broiler is able to quickly clear infection. Finally, **Figure 6D** shows an instance where the broiler successfully clears *Campylobacter* at the initial point of inoculation. All these realizations are run with the same parameters given in **Table 1**, demonstrating the benefit of a stochastic framework being able to better capture the more diverse range of possible events. This broad array of dynamical profiles is not observed in commercial broiler flocks, a phenomena that is demonstrated in the following case study.

3.3. Stochastic Model—One Strain in Multiple Broilers

The previous scenario is now extended to consider multiple broilers. **Figure 7** presents the results for one *Campylobacter* strain in a flock of 400 broilers. We use the parameter values stated in **Table 1**. The total size of the enclosure, or the carrying capacity of E, is set at $\Omega = 200,000$. This value is considered in cm^2, and so with 400 broilers, translates to 500 cm^2 per broiler. EU directive 2007/43/CE states that broilers may never be stocked at more than 42 kg/m^2 (Council of European Union, 2007). Assuming a targeted bird weight of 1.5 kg, this translates to 357 cm^2 per bird. This simulation models slightly more space allowed to each bird than the limit. The death rate of *Campylobacter* in the environment is set at $d_5 = 0.05$, higher than the death rate within a broiler as, despite their many survival mechanisms (Murphy et al., 2006) *Campylobacter* is susceptible to many exterior environmental stresses (Park, 2002) and is exceptionally fragile outside of its host. The simulation began with no *Campylobacter* in the surrounding environment [$E(0) = 0$] and the other initial conditions are set the same as for the

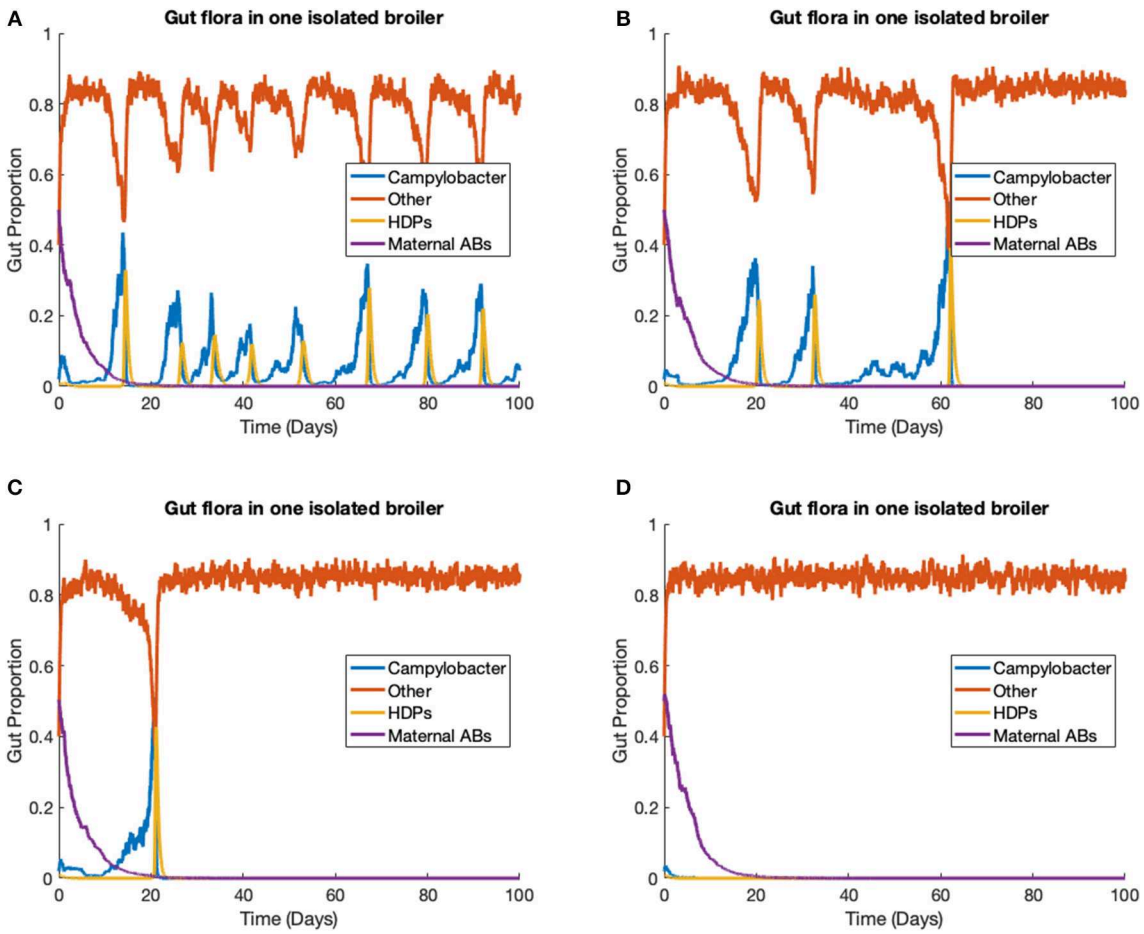

FIGURE 6 | Stochastic simulations of one *Campylobacter* strain within one broiler. **(A–D)** Four different realizations of a stochastic model simulating one strain of *Campylobacter* within one isolated broiler.

previous example, with the exception that two of the 400 broilers start with an initial condition of $C_1(0) = C_2(0) = 0.02$, while the others are initialized without any *Campylobacter*. These results are shown in **Figure 7**.

While birds who are not initialized with *Campylobacter* become infected at a slightly later time, the dynamical behavior is very similar across all birds in the flock. Multiple realizations do not display the broader spectrum of behavior observed in the one broiler case (**Figure 6**). The implication is that housing a greater number of birds causes more homogeneous dynamical behavior, and indeed the wide variety of *Campylobacter* expression seen in the isolated bird experiments of Achen et al. (1998) is not so commonly observed in experiments with group-housed birds (Van Gerwe et al., 2005).

3.4. Stochastic Model—Five Strains in Multiple Broilers

We extend the previous scenario to investigate dynamics of multiple strains. Five strains of competing *Campylobacter* are modeled within the same flock of 400 birds. The same constants are used as in the previous scenario, with each strain having identical rate constants. One key difference is that all broilers are initialized without any *Campylobacter*, instead an initial amount is present in the environment. Each strain of *Campylobacter* in the environment is initialized at $E_1(0) = E_2(0) = E_3(0) = E_4(0) = E_5(0) = 100$. The results of this simulation are shown in **Figure 8**.

On average, all strains perform equally well across the flock, as shown in **Figure 8E**. All strains slowly converge to roughly equal amounts in the environment, reflecting an equal presence on average across all birds in the flock. However, when observing the *Campylobacter* proportions within individual broilers, one or two strains will tend to dominate early on in colonizing a broiler's gut, which can in turn prevent other strains from taking hold (seen most clearly in **Figure 8D**). This dynamical behavior was first observed in our deterministic simulations (see **Figure 5B**), however unlike in the deterministic case, stochastic events can cause dominant *Campylobacter* strains to reduce in population, presenting an opportunity for a different strain to establish itself.

This phenomena is more clearly seen if the timescale of the simulation is extended, as illustrated in **Figure 9**.

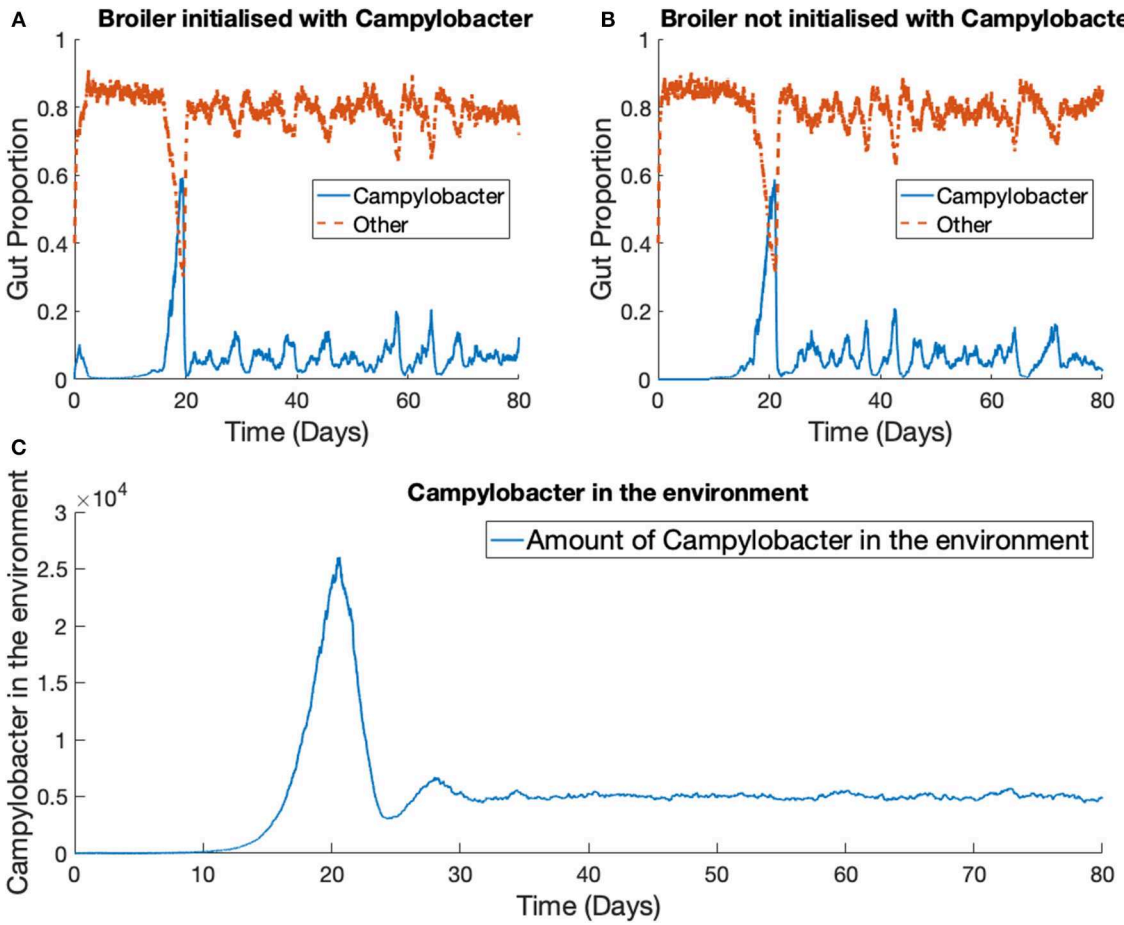

FIGURE 7 | Stochastic simulations of one *Campylobacter* strain within multiple broilers. The proportion of a broiler's gut containing *Campylobacter* for **(A)** a broiler in the flock initialized with a small proportion of *Campylobacter* **(B)** a broiler in the flock initialized with no *Campylobacter*. **(C)** Shows how much of the environment (total size of 200,000) contains *Campylobacter*. This is variable E in the model.

Although the average population of strains across the flock is equal, the stochastic model shows that a single strain of *Campylobacter* tends to dominate the gut of individual broilers at any one time. Although there are brief periods where strains exist in equal amounts, eventually the balance shifts again to longer periods of dominance by one or perhaps two strains.

Disadvantaged strains of *Campylobacter* are quickly eliminated. **Figure 10** shows the results for a simulation where strain 4's growth rate, r_{C_4}, is reduced from 0.3009 to 0.295, and strain 5's growth rate, r_{C_5}, is reduced to 0.29. Strains 1, 2, and 3 are kept with a growth rate of 0.3009. As **Figure 10** shows, the weaker strains are unable to outcompete the other three and are quickly eliminated. Changing other demographic parameters of a strain achieve a similar result of driving a strain to extinction, the phenomenon is not unique to only altering the growth rate. Making only very small reductions to the growth rate can result in a strain surviving at a lower average population size, although this may only be due to the time needed for extinction to occur being too long to observe in these simulations.

4. SENSITIVITY ANALYSIS

A powerful use of this model is to conduct a robust sensitivity analysis to identify the parameters of greatest impact in driving outbreaks of *Campylobacter*. We adopt a variance-based analysis of the model, and investigate the likelihood of flocks remaining free of *Campylobacter* based on a random assignment of parameter values.

We consider the case of a flock of broilers infected with a single strain of *Campylobacter*, the scenario shown in section 2.3. Model parameters are sampled randomly from a uniform range, and the model is run multiple times for these values. We then record how many of these stochastic runs resulted in the flock successfully eliminating *Campylobacter* infections, before drawing a new random sample of parameters values and repeating as necessary. Eventually we finish with a final data set which we display an example of below in **Figure 11**.

As such, the most "important" parameters will be the ones which exhibit a strong trend in their scatter plot. A seemingly randomly distributed scatter plot would indicate a parameter

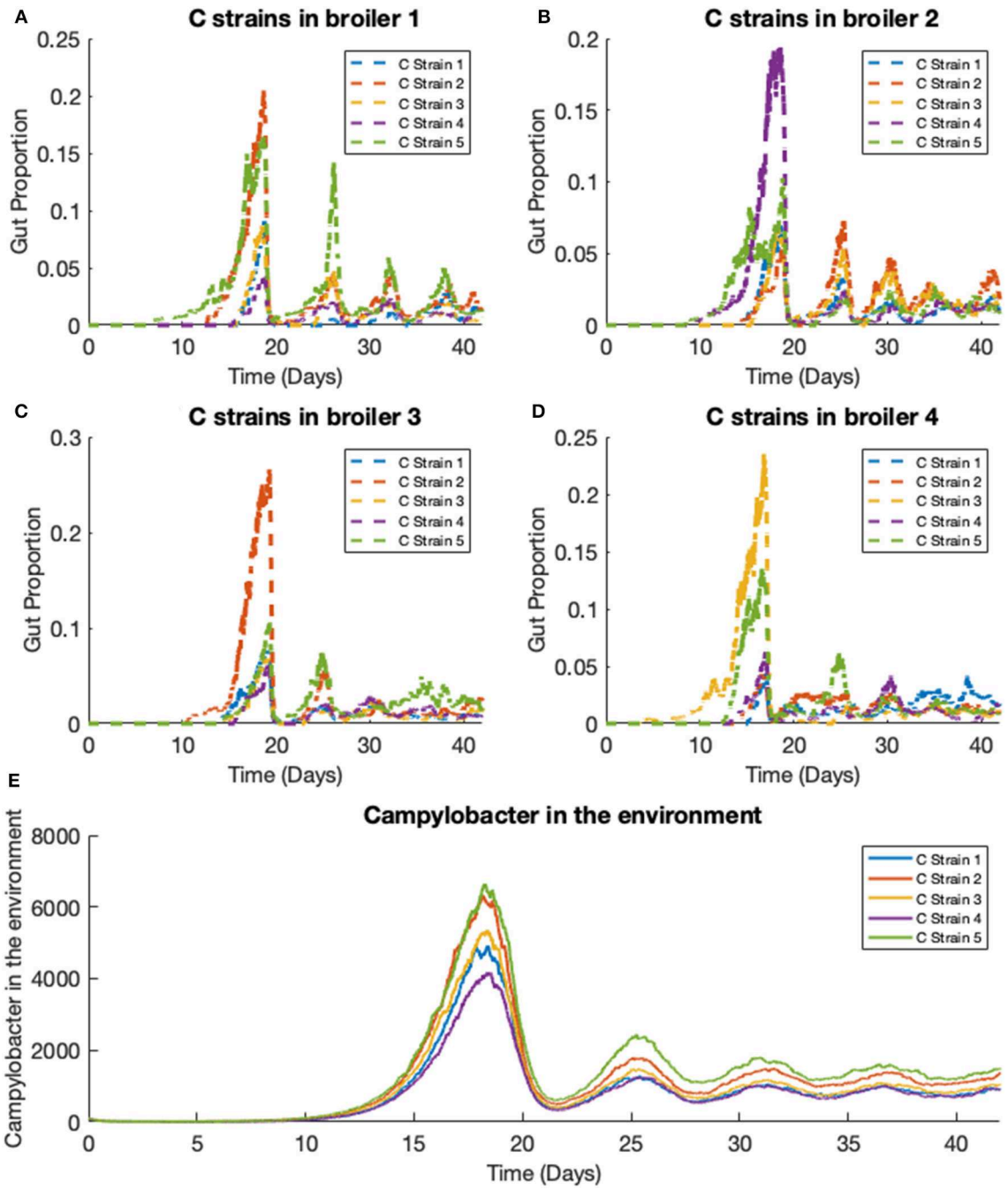

FIGURE 8 | Stochastic simulations of multiple *Campylobacter* strains within multiple broilers. **(A–D)** The proportion of four different broilers' microbiomes that contain five strains of *Campylobacter*. All birds are within the same flock. **(E)** Shows how much of the environment (total size of 200,000) contains the five strains of *Campylobacter*. These are variables E_j in the model.

value which has little impact on our output. To report more accurately this measure we use the first-order sensitivity index, S_i, and the total effect index, S_{T_i}, defined as:

$$S_i = \frac{V_{X_i}(E_{X_{\sim i}}(Y|X_i))}{V(Y)}, \qquad S_{T_i} = \frac{E(V(Y|X_{\sim i}))}{V(Y)},$$

where X_i denotes parameter i, and Y denotes the model output. $X_{\sim i}$ denotes the vector of all factors but X_i. $V(\cdot)$ denotes the variance, and $E(\cdot)$ the expectation. Specifically $E(A|B)$ denotes the expectation of variable A when B is held fixed. In short S_i will measure the changes observed in the output when parameter X_i is kept fixed, while S_{T_i} measures the changes to the output when all other parameters are kept fixed. A full derivation and explanation

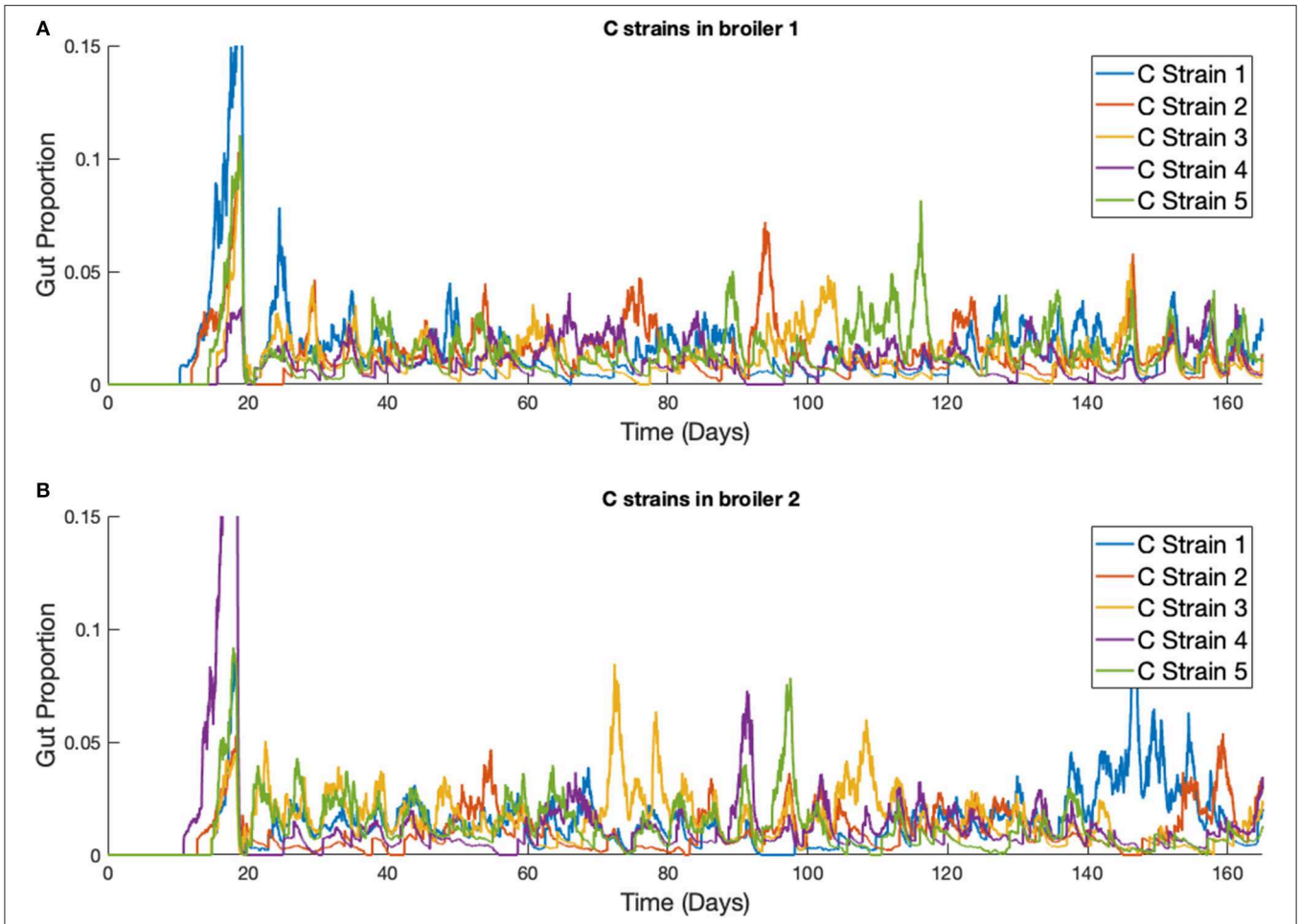

FIGURE 9 | Stochastic simulations of multiple *Campylobacter* strains within multiple broilers across a greater timescale. **(A,B)** The proportion of two different broilers' microbiomes that contain five identical strains of *Campylobacter*.

can be found in Saltelli et al. (2008). In short, both are values that range from zero to one, that explain the impact of a parameter on the model output. The higher the value, the more "important" the parameter is. S_{T_i} is considered a stronger metric, as it also considers the higher-order impact of a parameter, whereas S_i only considers the immediate first-order impact. As such S_i would be a sufficient measure for a linear model, but for a more complex model such as the one presented in this paper, S_{T_i} can better reveal the impact that each parameter plays. An initial sensitivity analysis was run for 20 parameters with 2,000 parameter set samples, drawn from a quasi-random Sobol set (Saltelli et al., 2008). The results of this analysis are displayed in **Table 2**, and the code used to produce them is available to access at: https://osf.io/b3duc/.

Specifically, our objective function will run the stochastic model for a flock of chickens with the random parameter set drawn. If this model run results in no *Campylobacter* being present in the flock, it is considered to have successfully eliminated infection. The model is run 20 times with this parameter set, and the proportion of these 20 runs that results in an elimination of *Campylobacter* is the final output value, the "probability of flock clearing infection."

Note that some of the values in **Table 2** are negative, despite S_i and S_{T_i} being limited to being between zero and one. This is due to the computational error in estimating the value, however the ordering of parameters for these particular runs will not be affected by this error. **Table 2** shows that the S_{T_i} values associated with most parameters ranges between 0 and 0.1. The "most important" parameters however have a wider spread of associated S_{T_i} values.

The main result from this analysis is that the growth, death and inhibition rates of the other bacteria present in a broiler's gut (parameters r_2, d_2, and β_C) collectively carry the largest impact in eliminating *Campylobacter* from a flock. As such, we can begin to consider which preventative methods could best take advantage of this heightened sensitivity.

5. DISCUSSION

Here, we have investigated the dynamics of *Campylobacter* across a range of model applications. Our framework reveals

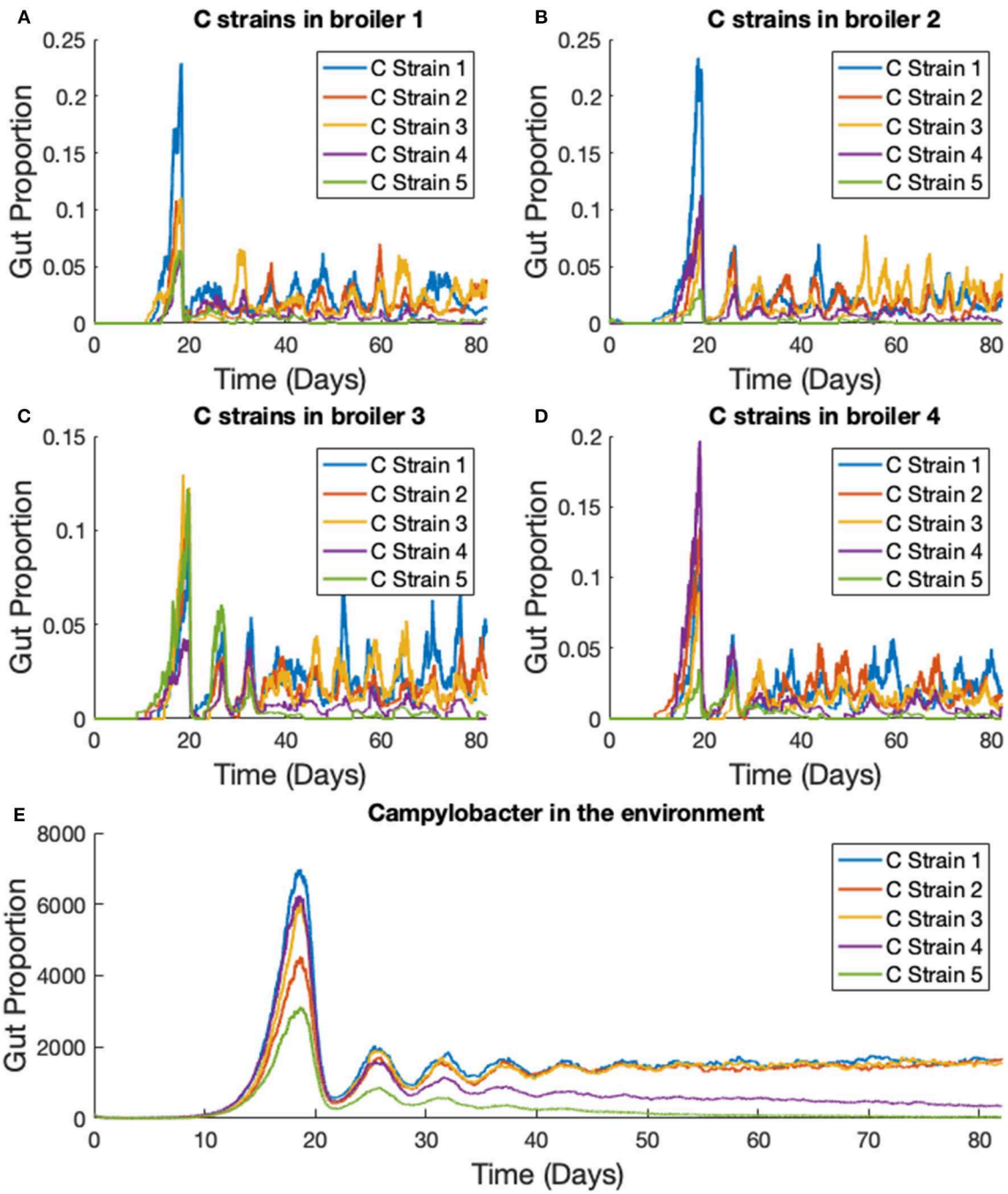

FIGURE 10 | Stochastic simulations of multiple *Campylobacter* strains, differing in growth rates, within multiple broilers. **(A–D)** The proportion of four different broilers' microbiomes that contain five strains of *Campylobacter*. Strain 4 has had it's growth rate reduced from 0.3009 to 0.295 and strain 5 has had its growth rate reduced to 0.29. Strains 1, 2, and 3 have a growth rate of 0.3009. **(E)** Shows how much of the environment (total size of 200,000) contains the five strains of *Campylobacter*. These are variables E_j in the model.

several key dynamics of microbial interaction that explain many experimentally observed phenomena. This presents promising new approaches to understanding and tackling this bacteria.

First, the most apparent prediction is that the *Campylobacter* population is successfully suppressed by the innate maternal antibodies (an experimentally observed phenomenon; Connerton et al., 2018), until these antibodies are eventually removed from the system. At this point an initial surge in the population of *Campylobacter* is observed, before it comes to rest at a lower level, reaching an equilibrium with the broiler's immune-response. This can be seen in all of the above figures, but most clearly in **Figure 1**. This initial surge creates an interesting

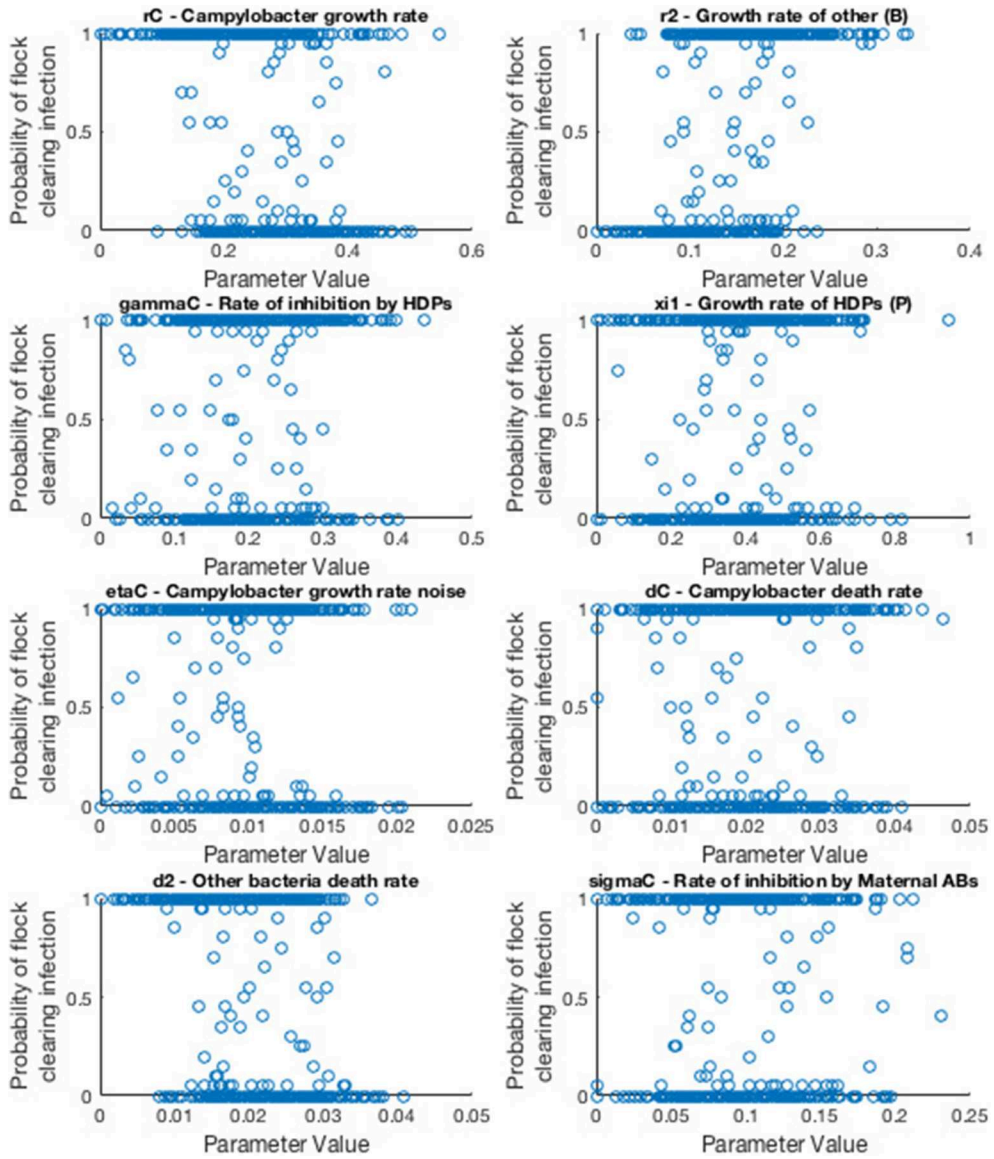

FIGURE 11 | Scatter plots displaying probability of a flock clearing *Campylobacter* infection against randomly sampled parameter values. Each scatter plot depicts the results for a specific parameter value. Probability is calculated by running the model for a sampled parameter set 20 times, and recording how many of those runs resulted in the flock not becoming infected with *Campylobacter*.

opportunity for certain strains of *Campylobacter* to emerge as an early dominating strain. **Figure 5B** shows that, due to the antibacterial properties of a broiler's maternal antibodies, any strains that infect a broiler early on in its lifespan will be heavily inhibited. This creates a brief window at the point in which maternal antibodies have depleted, whereby any new strain introduced is observed to quickly colonize and dominate the gut flora, suppressing other strains (see **Figure 10C**). This hypothesis has been verified experimentally (Connerton et al., 2018).

The proposition of damped oscillations between *Campylobacter* population size and the host's immune-response is better reinforced by observations that host antibody populations will also oscillate in birds infected with *Campylobacter* (Cawthraw et al., 1994). This basic interaction has been experimentally observed by Achen et al. (1998), with a high degree of variability between birds. This variability is better captured by the stochastic model, as shown in **Figure 6**. Indeed, many birds in Achen et al.'s study are shown to clear *Campylobacter* successfully from their system, a result rarely observed on commercial broiler farms. Likewise this result was only observed in the model case of individual, isolated broilers (see **Figure 6D**).

Most important is the mechanism observed in **Figure 7**, where the broad spectrum of oscillatory behavior observed within a broiler is greatly reduced in a large flock of birds. Indeed the vast examples of individual dynamics observed in **Figure 6**, large

TABLE 2 | Sensitivity analysis of parameters in a stochastic model for one *Campylobacter* strain in a flock of broilers.

S_i	Parameter	S_{T_i}	Parameter
−0.0112	η_3	−0.0031	σ_C
−0.0054	η_{BC}	−0.0026	η_4
−0.0051	a	−0.0016	η_5
−0.0017	η_C	−0.0011	d_4
−0.0011	η_2	0.0056	b
0.0009	η_5	0.0065	a
0.0020	d_4	0.0114	γ_C
0.0025	b	0.0122	ξ
0.0038	η_4	0.0224	η_C
0.0054	ξ	0.0228	η_{BC}
0.0062	σ_C	0.0378	η_3
0.0077	γ_C	0.0389	Ω
0.0085	Ω	0.0417	η_2
0.0096	d_5	0.0543	d_5
0.0125	β_C	0.1380	d_C
0.0441	d_C	0.1925	β_C
0.0459	r_C	0.2088	r_C
0.1131	d_3	0.3117	d_3
0.1622	r_2	0.4418	d_2
0.1900	d_2	0.4789	r_2

The first-order sensitivity index and total effect index is given for a sensitivity analysis of 2,000 runs for 20 parameters. The output function considered is the probability of Campylobacter going extinct within the flock based on the given parameter set.

oscillations and perhaps extinctions, are completely replaced by the same, homogenized dynamics seen within flock-reared birds in **Figures 7A,B**, as the populations of *Campylobacter* within each bird are consistently reinforced by the amount of *Campylobacter* in the environment. The wealth of experiments in monitoring flock *Campylobacter* expression for varying flock sizes means this effect can be observed taking place across multiple experiments of different flock magnitudes and densities. Morishita et al. (1997) measured the amount of *Campylobacter* in a flock of thirty birds in a sizeable pen. This flock was small enough to observe oscillating behavior in the prevalence of *Campylobacter*, and yet there do not appear to be any clear cases of birds being able to clear the bacteria from their system. Stern N.J. et al. (2001) experimented with flocks of 70 birds at a density of 15.4 birds/m^2. A small cyclic pattern is observable in their results but there are clearly far higher incidence rates. Lastly, Van Gerwe et al. (2005) studied flocks of 400 birds housed at 20 birds/m^2 (the same density considered in the above flock modeling), where now no cyclic patterns can be observed, and all birds quickly reach a constant state of *Campylobacter* expression. This effect is seen in **Figure 7**, and almost always observed in commercial farms (Stern et al., 1995; Evans and Sayers, 2000). Our work presented here is the first, to our knowledge, to be able to propose a mechanistic explanation for this observed effect, namely that the housing density of reared flocks is correlated to *Campylobacter* prevalence.

This dynamic, whereby broilers are consistently infected with *Campylobacter* due to highly contaminated living space, can also explain the observed phenomena whereby broiler breeder flocks (flocks kept for the breeding of meat birds) display a consistently lower *Campylobacter* prevalence rate than commercial broiler flocks (Colles et al., 2011). Breeder birds will regularly move between periods of testing positive and negative for *Campylobacter*, inconsistently with the state of other birds in the flock, unlike the much younger birds grown for meat which remain consistently positive. Our case studies suggest that this may be due to the lower stocking density afforded to breeder birds, as it would appear the route of infection between breeder birds is weaker than that between broilers. Our sensitivity analysis however also highlighted that the gut flora can have a strong impact on the survival of *Campylobacter*. The differences in diet and management practice for breeder birds likely results in a different variety of bacterial colonies to broilers, which could also be a cause of the differences seen between breeders and broilers in *Campylobacter* expression.

Additionally, we note that over these case studies we have seen that the outbreak dynamics are unaffected by the initial method of inoculation. There is no clear consensus yet on whether flocks are initially infected through horizontal or vertical transmission, and our model predictions show that this may not be possible to determine from flock infection dynamics. Case studies showed no difference between initialization with an infected environment, or an infected number of broilers. More specifically, **Figure 7** shows clearly how the dynamic profile of a broiler that is initialized with *Campylobacter*, is not significantly different from that of a bird which is infected through the environment, representing the effect of vertical and horizontal transmission respectively.

Over time, our model shows strains of equal fitness will tend to settle at equal levels of prevalence on average across a flock (**Figure 8E**), a result that has also been shown experimentally (Colles and Maiden, 2014; Colles et al., 2015). However, it is very common for an individual broiler to have only one or two dominant strains against far smaller proportions of other strains (Colles et al., 2019), as our model represents in **Figures 8A–D**, **9**. Our results show that this effect is most prominently seen early on in the chicken's lifespan, where usually only one strain will be present during the initial population surge of *Campylobacter*. Colles et al. (2019) shows that a greater diversity of strains are observable later on in a broiler's lifespan, but usually at a far lower prevalence compared to a dominating strain. Evidently, when one strain is already well-established within a chicken's gut, it is difficult for new competing strains to grow. This is due to the broiler already having a heightened level of immune response (P) due to the currently present strain. In the deterministic case, later strains would never be able to establish themselves as much as strains that were earlier to arrive (**Figure 5B**). However, in the stochastic model, there is the potential for a stochastic event to reduce the population of the currently dominating strain, and increase the population of a less-established strain.

Across the whole flock, weaker strains can be quickly outcompeted by other strains. **Figure 10** shows two weaker strains (strains with lower growth rates) attempting to survive within a flock, even having a slight population peak at the optimal point of strain introduction, before eventually being forced to

extinction by the other three strains. Parameter variation showed that reducing a strain's demographic parameters by a very small amount can allow it to persist still in the flock at a smaller average population than the others, but the majority of realizations would always end with weaker strains becoming extinct. Clearly this shows an environment where genetic dominance is very quickly selected for.

These results have considerable implications for biosecurity. While smaller flocks may have a very real opportunity to be protected from *Campylobacter* invasions, *Campylobacter* prevalence is far more stable in larger commercial flocks, and our model shows it to be exceptionally difficult to remove. Efforts can be made to prevent initial inoculations, but once a bacterial presence is established, it may be all but impossible to remove from a flock. Considerable improvements to biosecurity have been made in recent years, but very little impact has been observed in this having reduced *Campylobacter* incidence (Hermans et al., 2011). These measures do not reduce the speed of proliferation of the bacteria, and our results suggest that better attention to bird health is likely to have a greater effect on preventing flock infection.

This model's greatest strength is its lack of overarching assumptions. We model only the most basic bacterial interactions, all supported and verified through experimental work. Our stochastic system is capable of exhibiting a plethora of interesting dynamical interactions based on just a few known biological interactions. In moving forward with this work, the model can be used to theorize optimal methods by which to decrease the likelihood of *Campylobacter* outbreaks, and begin collaborative efforts in better explaining the evolving genetic diversity of this bacteria.

One area in which the model is admittedly lacking currently, is that it does not represent the physiological changes that occur as a bird grows. Broilers have been genetically selected over the many decades to grow excessively fast, which has been shown to have numerous concerning implications for their health (Buzała et al., 2015). This is likely to then result in differences to their auto-immune capabilities over time. More pertinently, the gut flora of a chicken is known to change and develop as the birds age (Lu et al., 2003), suggesting varying degrees of inter-bacterial uncertainty.

Our sensitivity analysis gives great insight into the optimal routes of infection prevention. **Table 2** clearly shows that bolstering the growth rate and inhibition capabilities of the other bacteria populating a broiler's gut is the best way to force extinction of *Campylobacter*, primarily through suppressing *Campylobacter* at its initial appearance in a system, before it has the opportunity to propagate. As such, the sensitivity analysis suggests further exploration and experimentation into the impact of factors which would affect the gut flora of a broiler. Probiotics are a clear way of impacting the microflora (Mountzouris et al., 2007) and have shown some effect in studies into their impact on *Campylobacter* expression (Santini et al., 2010). Equally, the stressors linked with stocking density have been shown to affect the gut microflora by Guardia et al. (2011). Burkholder et al. (2008) have shown that feed withdrawal and heat stress can considerably alter and limit the gut microflora. These highlight that general bird health and welfare can be equally strong factors in determining the values of r_2, d_2, and β_C; some of the parameters highlighted as most "important" by the sensitivity analysis. **Table 2** also however highlights the importance of parameter d_3, the death rate of host defense peptides. This parameter has been shown to be strongly affected by stressors such as overcrowding (Gomes et al., 2014). As such, this result would lend further support to giving greater care to the health and welfare of broilers, as the resulting improvement to host defense peptide production would have a positive impact on helping prevent *Campylobacter* outbreaks.

These caveats notwithstanding, the model presented is capable of explaining a wealth of experimentally observed *Campylobacter* population dynamics, further elucidating an urgent public health risk. We have used our framework to investigate multiple strain interactions, to understand better the spread of genotypes across a flock. Finally, we were able to use the model to highlight the factors most responsible for causing outbreaks of infection. Looking forward, this work can be used to understand better observed differences in outbreak dynamics between different farms and indeed countries, and further our goal of minimizing public exposure to this dangerous pathogen.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

TR designed the model and performed the presented case studies. TR wrote the manuscript. MB and MD supervised the project and proof-read the manuscript.

FUNDING

This work was supported through an Engineering and Physical Sciences Research Council (EPSRC) (https://epsrc.ukri.org/) Systems Biology studentship award (EP/G03706X/1 to TR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Frances Colles, Adrian Smith, and Martin Maiden for their insight and contribution in discussing the model and our findings. This manuscript has been released as a preprint at https://www.biorxiv.org/content/10.1101/476119v2 (Rawson et al., 2019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01940/full#supplementary-material

REFERENCES

EFSA Panel on Biological Hazards (BIOHAZ) (2011). Scientific opinion on campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA J.* 9:2105. doi: 10.2903/j.efsa.2011.2105

Achen, M., Morishita, T. Y., and Ley, E. C. (1998). Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Dis.* 42, 732–737. doi: 10.2307/1592708

Acke, E., McGill, K., Golden, O., Jones, B., Fanning, S., and Whyte, P. (2009). A comparison of different culture methods for the recovery of campylobacter species from pets. *Zoonoses Public Health* 56, 490–495. doi: 10.1111/j.1863-2378.2008.01205.x

Adkin, A., Hartnett, E., Jordan, L., Newell, D., and Davison, H. (2006). Use of a systematic review to assist the development of campylobacter control strategies in broilers. *J. Appl. Microbiol.* 100, 306–315. doi: 10.1111/j.1365-2672.2005.02781.x

Burkholder, K., Thompson, K., Einstein, M., Applegate, T., and Patterson, J. (2008). Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to salmonella enteritidis colonization in broilers. *Poult. Sci.* 87, 1734–1741. doi: 10.3382/ps.2008-00107

Buzała, M., Janicki, B., and Czarnecki, R. (2015). Consequences of different growth rates in broiler breeder and layer hens on embryogenesis, metabolism and metabolic rate: a review. *Poult. Sci.* 94, 728–733. doi: 10.3382/ps/pev015

Cawthraw, S., Ayling, R., Nuijten, P., Wassenaar, T., and Newell, D. (1994). Isotype, specificity, and kinetics of systemic and mucosal antibodies to *Campylobacter jejuni* antigens, including flagellin, during experimental oral infections of chickens. *Avian Dis.* 38, 341–349. doi: 10.2307/1591960

Colles, F. M., and Maiden, M. C. (2014). "Exploring the population dynamics of multiple campylobacter strains in poultry," in *Campylobacter Ecology and Evolution*, ed S. K. Sheppard (Norfolk: Caister Academic Press), 213.

Colles, F. M., McCarthy, N. D., Bliss, C. M., Layton, R., and Maiden, M. C. (2015). The long-term dynamics of campylobacter colonizing a free-range broiler breeder flock: an observational study. *Environ. Microbiol.* 17, 938–946. doi: 10.1111/1462-2920.12415

Colles, F. M., McCarthy, N. D., Layton, R., and Maiden, M. C. (2011). The prevalence of campylobacter amongst a free-range broiler breeder flock was primarily affected by flock age. *PLoS ONE* 6:e22825. doi: 10.1371/journal.pone.0022825

Colles, F. M., Preston, S. G., Barfod, K. K., Flammer, P. G., Maiden, M. C., and Smith, A. L. (2019). Parallel sequencing of pora reveals a complex pattern of campylobacter genotypes that differs between broiler and broiler breeder chickens. *Sci. Rep.* 9:6204. doi: 10.1038/s41598-019-42207-9

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'Kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome* 6:88. doi: 10.1186/s40168-018-0477-5

Council of European Union (2007). *Council Directive 2007/43/ec of 28 June 2007 Laying Down Minimum Rules for the Protection of Chickens Kept for Meat Production [2007] oj l182/19.* Avaialble online at: https://eur-lex.europa.eu/eli/dir/2007/43/oj

Evans, S., and Sayers, A. (2000). A longitudinal study of campylobacter infection of broiler flocks in great britain. *Prevent. Vet. Med.* 46, 209–223. doi: 10.1016/S0167-5877(00)00143-4

Ge, B., White, D. G., McDermott, P. F., Girard, W., Zhao, S., Hubert, S., et al. (2003). Antimicrobial-resistant campylobacter species from retail raw meats. *Appl. Environ. Microbiol.* 69, 3005–3007. doi: 10.1128/AEM.69.5.3005-3007.2003

Ghareeb, K., Awad, W., Mohnl, M., Schatzmayr, G., and Boehm, J. (2013). Control strategies for campylobacter infection in poultry production. *World's Poult. Sci. J.* 69, 57–76. doi: 10.1111/jam.12986

Gielda, L. M., and DiRita, V. J. (2012). Zinc competition among the intestinal microbiota. *MBio* 3, e00171–12. doi: 10.1017/S0043933913000068

Gomes, A., Quinteiro-Filho, W. M., Ribeiro, A., Ferraz-de Paula, V., Pinheiro, M., Baskeville, E., et al. (2014). Overcrowding stress decreases macrophage activity and increases salmonella enteritidis invasion in broiler chickens. *Avian Pathol.* 43, 82–90. doi: 10.1080/03079457.2013.874006

Guardia, S., Konsak, B., Combes, S., Levenez, F., Cauquil, L., Guillot, J.-F., et al. (2011). Effects of stocking density on the growth performance and digestive microbiota of broiler chickens. *Poult. Sci.* 90, 1878–1889. doi: 10.3382/ps.2010-01311

Hartnett, E., Kelly, L., Newell, D., Wooldridge, M., and Gettinby, G. (2001). A quantitative risk assessment for the occurrence of campylobacter in chickens at the point of slaughter. *Epidemiol. Infect.* 127, 195–206. doi: 10.1017/S0950268801005866

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., et al. (2011). Campylobacter control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol.* 152, 219–228. doi: 10.1016/j.vetmic.2011.03.010

Higham, D. J. (2001). An algorithmic introduction to numerical simulation of stochastic differential equations. *SIAM Rev.* 43, 525–546. doi: 10.1137/S0036144500378302

Jorgensen, F., Madden, R. H., Arnold, E., Charlett, A., and Elviss, N. C. (2015). *FSA Project fs241044 - Survey Report - A Microbiological Survey of Campylobacter Contamination in Fresh Whole UK Produced Chilled Chickens at Retail Sale (2014-15)*. London, UK: Food Standards Agency.

Khachatourians, G. G. (1998). Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Can. Med. Assoc. J.* 159, 1129–1136.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., and Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl. Environ. Microbiol.* 69, 6816–6824. doi: 10.1128/AEM.69.11.6816-6824.2003

McCarthy, N., and Giesecke, J. (2001). Incidence of guillain-barré syndrome following infection with *Campylobacter jejuni*. *Am. J. Epidemiol.* 153, 610–614. doi: 10.1093/aje/153.6.610

Morishita, T. Y., Aye, P. P., Harr, B. S., Cobb, C. W., and Clifford, J. R. (1997). Evaluation of an avian-specific probiotic to reduce the colonization and shedding of *Campylobacter jejuni* in broilers. *Avian Dis.* 41, 850–855. doi: 10.2307/1592338

Mountzouris, K., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., and Fegeros, K. (2007). Evaluation of the efficacy of a probiotic containing lactobacillus, bifidobacterium, enterococcus, and pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult. Sci.* 86, 309–317. doi: 10.1093/ps/86.2.309

Murphy, C., Carroll, C., and Jordan, K. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Nauta, M., Jacobs-Reitsma, W. F., Evers, E. G., Van Pelt, W., and Havelaar, A. H. (2005). *Risk Assessment of Campylobacter in the Netherlands via Broiler Meat and Other Routes.* RIVM Rapport 250911006.

Newell, D., and Fearnley, C. (2003). Sources of campylobacter colonization in broiler chickens. *Appl. Environ. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Newell, D., and Wagenaar, J. (2000). *Poultry Infections and Their Control at the Farm Level, 2nd Edn*. Washington DC: ASM Press.

Pan, D., and Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* 5, 108–119. doi: 10.4161/gmic.26945

Park, S. F. (2002). The physiology of campylobacter species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol.* 74, 177–188. doi: 10.1016/S0168-1605(01)00678-X

Raines, D. J., Moroz, O. V., Blagova, E. V., Turkenburg, J. P., Wilson, K. S., and Duhme-Klair, A.-K. (2016). Bacteria in an intense competition for iron: key component of the *Campylobacter jejuni* iron uptake system scavenges enterobactin hydrolysis product. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5850–5855. doi: 10.1073/pnas.1520829113

Rawson, T., Dawkins, M. S., and Bonsall, M. B. (2019). A mathematical model of Campylobacter dynamics within a broiler flock. *bioRxiv 574301*. doi: 10.1101/574301

Sahin, O., Kassem, I. I., Shen, Z., Lin, J., Rajashekara, G., and Zhang, Q. (2015). Campylobacter in poultry: ecology and potential interventions. *Avian Dis.* 59, 185–200. doi: 10.1637/11072-032315-Review

Sahin, O., Zhang, Q., Meitzler, J. C., Harr, B. S., Morishita, T. Y., and Mohan, R. (2001). Prevalence, antigenic specificity, and bactericidal activity of poultry anti-campylobacter maternal antibodies. *Appl. Environ. Microbiol.* 67, 3951–3957. doi: 10.1128/AEM.67.9.3951-3957.2001

Saltelli, A., Ratto, M., Andres, T., Campolongo, F., Cariboni, J., Gatelli, D., et al. (2008). *Global Sensitivity Analysis: The Primer*. Chichester: John Wiley & Sons.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Schoeni, J. L., and Doyle, M. P. (1992). Reduction of *Campylobacter jejuni* colonization of chicks by cecum-colonizing bacteria producing anti-c. jejuni metabolites. *Appl. Environ. Microbiol.* 58, 664–670.

Shanker, S., Lee, A., and Sorrell, T. (1990). Horizontal transmission of *Campylobacter jejuni* amongst broiler chicks: experimental studies. *Epidemiol. Infect.* 104, 101–110. doi: 10.1017/S0950268800054571

Stahl, M., Butcher, J., and Stintzi, A. (2012). Nutrient acquisition and metabolism by *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.* 2:5. doi: 10.3389/fcimb.2012.00005

Stern, N., Clavero, M., Bailey, J., Cox, N., and Robach, M. (1995). *Campylobacter* spp. in broilers on the farm and after transport. *Poult. Sci.* 74, 937–941. doi: 10.3382/ps.0740937

Stern, N., Cox, N., Bailey, J., Berrang, M., and Musgrove, M. (2001). Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. *Poult. Sci.* 80, 156–160. doi: 10.1093/ps/80.2.156

Stern, N. J., Cox, N. A., Musgrove, M. T., and Park, C. (2001). Incidence and levels of campylobacter in broilers after exposure to an inoculated seeder bird. *J. Appl. Poult. Res.* 10, 315–318. doi: 10.1093/japr/10.4.315

Strachan, N. J., and Forbes, K. J. (2010). The growing UK epidemic of human campylobacteriosis. *Lancet* 376, 665–667. doi: 10.1016/S0140-6736(10)60708-8

Travers, K., and Michael, B. (2002). Morbidity of infections caused by antimicrobial-resistant bacteria. *Clin. Infect. Dis.* 34(Suppl. 3), S131–S134. doi: 10.1086/340251

Tresse, O., Alvarez-Ordóñez, A., and Connerton, I. F. (2017). About the foodborne pathogen campylobacter. *Front. Microbiol.* 8:1908. doi: 10.3389/fmicb.2017.01908

Van Gerwe, T., Bouma, A., Jacobs-Reitsma, W., Van den Broek, J., Klinkenberg, D., Stegeman, J., et al. (2005). Quantifying transmission of *Campylobacter* spp. among broilers. *Appl. Environ. Microbiol.* 71, 5765–5770. doi: 10.1128/AEM.71.10.5765-5770.2005

Welkos, S. (1984). Experimental gastroenteritis in newly-hatched chicks infected with *Campylobacter jejuni*. *J. Med. Microbiol.* 18, 233–248. doi: 10.1099/00222615-18-2-233

Wiles, T. J., Jemielita, M., Baker, R. P., Schlomann, B. H., Logan, S. L., Ganz, J., et al. (2016). Host gut motility promotes competitive exclusion within a model intestinal microbiota. *PLoS Biol.* 14:e1002517. doi: 10.1371/journal.pbio.1002517

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 October 2019
doi: 10.3389/fmicb.2019.02303

Caecal Microbiota of Experimentally *Campylobacter jejuni*-Infected Chickens at Different Ages

Julia Hankel[1], Klaus Jung[2], Henrike Kuder[1], Birgit Keller[1], Christoph Keller[3], Eric Galvez[4], Till Strowig[4] and Christian Visscher[1]*

[1] Institute for Animal Nutrition, University of Veterinary Medicine Hannover, Hanover, Germany, [2] Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Hanover, Germany, [3] Boehringer Ingelheim Veterinary Research Center GmbH & Co. KG, Hanover, Germany, [4] Helmholtz Center for Infection Research, Braunschweig, Germany

Campylobacter jejuni is the most common bacterial cause of foodborne zoonosis in the European Union. Infections are often linked to the consumption and handling of poultry meat. The aim of the present study was to investigate the caecal microbiota of birds infected with *C. jejuni* at different ages. Therefore, a total of 180 birds of the laying hybrid Lohmann Brown-Classic were housed in 12 subgroups of 15 animals each in three performed repetitions. Three birds per subgroup were experimentally infected with *C. jejuni* at an age of about 21 days and about 78 days (4.46 ± 0.35 $\log_{10}$ CFU/bird). Twenty-one days after experimental infection, microbiome studies were performed on 72 caecal samples of dissected birds (three primary infected and three further birds/subgroup). Amplification within the hypervariable region V 4 of the 16S rRNA gene was performed and sequenced with the Illumina MiSeq platform. Statistical analyses were performed using SAS®Enterprise Guide®(version 7.1) and R (version 3.5.2). Both factors, the experimental replication ($p < 0.001$) and the chickens' age at infection ($p < 0.001$) contributed significantly to the differences in microbial composition of the caecal samples. The factor experimental replication explained 24% of the sample's variability, whereas the factor age at infection explained 14% thereof. Twelve of 32 families showed a significantly different count profile between the two age groups, whereby strongest differences were seen for seven families, among them the family *Campylobacteraceae* (adjusted $p = 0.003$). The strongest difference between age groups was seen for a bacterial species that is assigned to the genus *Turicibacter* which in turn belongs to the family *Erysipelotrichaceae* (adjusted $p < 0.0001$). Correlation analyses revealed a common relationship in both chicken ages at infection between the absolute abundance of *Campylobacteraceae* and *Alcaligenaceae,* which consists of the genus *Parasutterella.* In general, concentrations of particular volatile fatty acids (VFA) demonstrated a negative correlation to absolute abundance of *Campylobacteraceae*, whereby the strongest link was seen for n-butyrate (−0.51141; $p < 0.0001$). Despite performing consecutive repetitions, the factor experimental replication contributed more to the differences of microbial composition in comparison to the factor age at infection.

Keywords: foodborne pathogen, *Campylobacter jejuni*, 16S rRNA, gut microbiota, volatile fatty acids

OPEN ACCESS

Edited by:
Michael J. Rothrock, United States Department of Agriculture (USDA), United States

Reviewed by:
Alexandre Thibodeau, Université de Montréal, Canada
Odile Tresse, INRA Centre Angers-Nantes Pays de la Loire, France

****Correspondence:***
Julia Hankel
julia.hankel@tiho-hannover.de

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

Received: *31 May 2019*
Accepted: *20 September 2019*
Published: *10 October 2019*

Citation:
Hankel J, Jung K, Kuder H, Keller B, Keller C, Galvez E, Strowig T and Visscher C (2019) Caecal Microbiota of Experimentally Campylobacter jejuni-Infected Chickens at Different Ages. Front. Microbiol. 10:2303. doi: 10.3389/fmicb.2019.02303

INTRODUCTION

More than 100 years ago, chickens were already recognised as an important source of zoonotic infection (Higgins, 1898). Nowadays, the zoonotic pathogen *Campylobacter jejuni* is the most common bacterial cause of foodborne zoonosis in the European Union, with infections often being linked to the consumption and handling of poultry meat (Efsa, 2017). Intestinal colonisation of chickens results in faecal contamination of the carcasses during the slaughtering process (Rosenquist et al., 2006; Guerin et al., 2010). One approach to eliminating *C. jejuni* from the food chain is to prevent the colonisation of broiler chickens (Newell and Fearnley, 2003). The extent of contamination shows a positive correlation between the number of *Campylobacter* present in the caecal content and the number of bacteria on the carcasses and cut products (Rosenquist et al., 2006; Reich et al., 2008). Therefore, reduced prevalence and *Campylobacter* load are strived for at production level prior to slaughter (Hermans et al., 2011).

Investigations have already demonstrated that with advanced age of the birds, the levels of *Campylobacter* excretion are lower compared to younger birds (Glünder, 1995; Colles et al., 2009; Hankel et al., 2018). Hankel et al. (2018) observed higher *C. jejuni* counts in caecal contents of young chickens 21 days after experimental infection with *C. jejuni* in comparison to those in old ones. Of the investigated influencing factors genetics, age and diet composition, the factor age was identified as the most influential variable and the only variable affecting caecal *Campylobacter* counts, with significant differences of almost two log steps between young and old birds ($\log_{10}$ CFU/g: 8.57 ± 0.46 vs. 6.66 ± 1.43). These findings might lead to the assumption that a higher age at infection is one of the greatest influencing factors on *C. jejuni* load in the chicken intestine. It is of great interest to make further investigations concerning the characteristics of the environment in which the bacterium lives in order to detect possible influences. Despite the fact that the chicken gut microbiome is being increasingly characterised by means of modern sequencing approaches, little is known about the factors influencing its modulation (Thibodeau et al., 2015) or the abilities of most species present in the caecum (Qu et al., 2008). Commensal bacteria benefit the host by their ability to competitively exclude other bacteria from colonising the intestine (Brisbin et al., 2008; Buffie and Pamer, 2013). Investigations with laying hybrid lines offer the opportunity to examine a possible influence of the chicken's age on *Campylobacter*, as they are also marketed with advanced age. Regarding the marketing standards for poultry meat (COMMISSION REGULATION (EC) No. 543/2008), male chickens of laying strains have to be slaughtered at an age of at least 90 days to be marketed as young cocks. Alternatively, chickens of less than 650 g carcass weight (expressed without giblets, head, and feet) can be marketed as poussin, also called coquelet. The aim of the present study was to examine under standardised conditions the bacterial composition in the caecum of *C. jejuni*-infected chickens at different ages in three consecutive trials. We hypothesised that possible large variations in microbiota due to the chickens' age would affect the abundance of *Campylobacteraceae* in caecal contents of chickens that were experimentally infected with *C. jejuni* under equal standardised procedures. Deeper knowledge concerning the susceptibility of chickens to members of the *Campylobacteraceae* family are of great interest to take preventive measures against this zoonotic pathogen in order to increase food safety in the poultry industry.

MATERIALS AND METHODS

The study was carried out with the aim of clarifying the question as to why a *Campylobacter* infection, susceptibility and excretion are age dependent. Against this background, caecal microbiota and the concentrations of bacterial fermentation products of experimentally *C. jejuni*-infected chickens at different ages were examined.

Experimental Design, Animals Housing and Sampling

The birds of the breed Lohmann Brown-Classic were supplied as day-old chicks from the same hatchery (Lohmann Tierzucht GmbH, Cuxhaven, Germany). Only male birds were used for the experiments.

The investigations took place in three independent repetitions (experiments 1–3, $r = 3$) where in total 180 birds were reared under the same conditions (**Figure 1**).

During the rearing phase, all birds were reared in identical floor pens. Pens were littered with wood shavings. The duration of the rearing phase was variable due to the study's objective to infect a part of the birds at an advanced age. For this reason, half of the birds were reared for about 2 weeks (LBC-21/22), whereas the other half of the birds were reared for 9 weeks in experiment 1 and 10 weeks in experiments 2 and 3 (LBC-70/78) before being transferred from the rearing to the infection unit of a level 2 animal facility. The birds were kept for a further 4 weeks on solid floor pens. Pens were littered with wood shavings (1 kg/m^2). Stocking density amounted to a maximum of 30 kg per square metre. The birds were maintained on a 16 L:8 D light schedule during the whole experiment. Each age group was randomly assigned to two subgroups per experiment, each made up of 15 birds, so that each age group consisted of 30 birds per experiment. One week after transferring the animals to the infection unit, an experimental challenge with *C. jejuni* took place in all subgroups. In accordance with Hankel et al. (2018), three of 15 broilers of each subgroup (seeder birds) were administered orally with a 1 mL inoculum of *C. jejuni* (4.46 ± 0.35 $\log_{10}$ CFU/mL). Qualitative detection of *C. jejuni* in cloacal swabs of all birds (days 2, 4, 7, 14, and 21 after inoculation) and quantitative detection of *C. jejuni* in excreta samples of seeder birds (days 2, 11, and 17 after inoculation) and in caecal samples of all birds at dissection were performed during the experimental period in order to examine prevalence and excretion of *C. jejuni*. The results were already published in Hankel et al. (2018). Three weeks after the experimental challenge with *C. jejuni*, all chickens were dissected (LBC-21/22: at an age of 42 days in experiments 1 and 3 and 43 days in experiment 2; LBC-70/78: at an age of 91 days in experiment 1 and 98 days

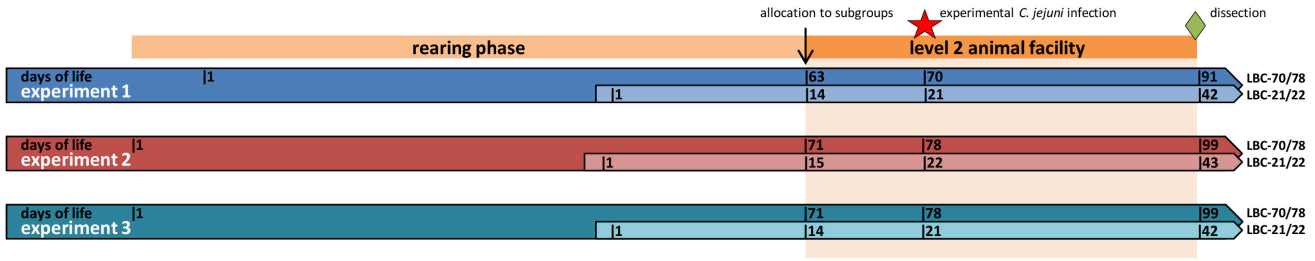

FIGURE 1 | Schedule of the experiments with actions in chickens of different ages at infection (LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days).

in experiments 2 and 3). Anaesthesia and killing of birds were carried out in accordance with Annex 2 [to paragraph 2 (2)] of the Regulations on the Welfare of Animals Used for Experiments or for Other Scientific Purposes (TierSchVersV). Anaesthesia was performed by head stroke. After bleeding, caecal contents were removed under sterile conditions and placed in reaction vessels. In addition, caecal contents of the seeder birds and three further birds/subgroup were immediately frozen and stored at −80°C for further microbiota analyses. Unfortunately, no caecal chyme was available from one bird in the LBC-21/22 group. One further caecal sample of one bird in the LBC-70/78 group was stored instead.

Feeding Regime, Diet, and Performance Parameters

The birds were given conventional complete diets based on wheat, soybean meal, corn and rape cake, which were supplemented by whole wheat, coccidiostats and NSP-degrading enzymes. The diets were produced and delivered by Best 3 Geflügelernährung GmbH (Twistringen, Germany). The diets were designed in accordance with the recommendations for energy and nutrient supply of the laying hens and fowls (broilers) of the Committee for Needs Standards of the Society of Nutrition Physiology (GfE, 1999). Energy content and nutrient composition of the diets can be taken from Hankel et al. (2018).

The rearing phase was divided into a 1-week starter phase during which a conventional pelleted starter diet was offered (starter) and a subsequent 7-day (LBC-21/22), or 56/64-day phase (LBC-70/78) with a commercially available pelleted grower diet (grower). The finisher diet was offered beginning from week 3, or beginning from week 10/11 until dissection. All diets were offered *ad libitum*. At level 2 animal facility water was offered *ad libitum* in drinking lines equipped with Top Nipples and drinking cups (Big Dutchman International GmbH, Vechta-Calveslage, Germany) The water was treated with 0.3 mg free chlorine/L (Virbac Clean Pipe, VIRBAC Tierarzneimittel GmbH, Bad Oldesloe, Germany).

At the day the animals were transferred to the infection unit and at the end of the trial the individual body weight of birds was determined as well as the feed intake being measured at subgroup level. The average daily feed intake/bird and the feed conversion ratio (FCR) at subgroup level could be calculated.

Analyses

Chemical Analyses

Caecal chyme was homogenised and the volatile fatty acids (VFA) concentration was measured by gas chromatography (610 Series, Unicam, Kassel, Germany). The samples were mixed with an internal standard (10 mL of formic acid [89%] and 0.1 mL of 4-methylvaleric acid). The mixture was centrifuged and afterward subjected to gas chromatography with a column temperature of 155°C (injector: 175°C, detector: 180°C).

Bacteriological Analyses

Qualitative and quantitative detection of *C. jejuni* was done in caecal samples of all 180 birds at dissection.

The qualitative bacteriological examination was based on the DIN EN ISO 10272–1:2006 in accordance with § 64 LFBG. Pre-enrichment was performed in Bolton Boullion, a liquid selective nutrient medium. Samples that were to be examined were incubated in a one-to-nine ratio (sample:Bolton Boullion) in sterile 5 mL tubes mounted with a vent cap (Sarstedt AG & Co., Nuembrecht, Germany) for 4 h at 37°C followed by 44 ± 4 h at 41.5°C in a microaerobic atmosphere (oxygen content of 5 ± 2%, carbon dioxide content of 10± 3%). Microaerobic atmosphere was created in a CO_2 incubator with O_2 control (CB 160, BINDER GmbH, Tuttlingen, Germany). After enrichment, sterile 10 µL inoculation loops were used to streak the samples onto two solid selective culture media (mCCD agar and Karmali agar; Oxoid Germany GmbH, Wesel, Germany) and afterward incubated again for 44 ± 4 h at 41.5°C in a microaerophilic atmosphere. To confirm the presence of *Campylobacter* individual colonies were analysed by phase contrast microscopy (Distelkamp-Electronic, Kaiserslautern, Germany) and biochemical methods (API Campy, bioMérieux SA, Marcy- l'Etoile, France).

For quantitative bacteriological examination 0.5 g sample material was diluted. A ten-fold dilution series was made with phosphate buffered saline (PBS, Phosphate Buffered Saline, Oxoid Germany GmbH, Wesel, Germany). In duplicate, 100 µL of each dilution was plated onto mCCD agar (Oxoid Germany GmbH, Wesel, Germany). After incubation in a microaerophilic atmosphere for 44 ± 4 h at 41.5°C, the colonies were counted and an average value from the two duplicate experiments was taken for calculating the CFU/g intestinal content.

16S rRNA Analyses

A total of 72 samples were included in the study. All caecal samples of the 72 birds included in the study were qualitatively detected as *C. jejuni* positive. Samples were stored at −80°C until simultaneous analysis. Mixer mill (Retsch MM 400, Haan, Germany) was used to homogenise the chyme for 1 min before DNA-extraction was done on an automated liquid handler (Microlab Star, Hamilton Germany GmbH, Gräfelfing, Germany) based on the DNeasy Blood&Tissue Kit (Qiagen, Hilden, Germany). An additional purification step (Kit: BS 365, BioBasic, Ontario, Canada) was performed before the hypervariable region V 4 of the 16S rRNA gene was amplified using primer F515/R806 in accordance with previously described protocols (Caporaso et al., 2011). Sequencing the amplicons was done on the Illumina MiSeq platform (PE250) and the Usearch8.1 software package[1] was used to assemble, quality control and cluster obtained reads. Reads were merged using -fastq_mergepairs –with fastq_maxdiffs 30. Chimeric sequences were identified and removed using cluster_otus (-otu_radius_pct 3) and the Uchime command included in the Usearch8.1 workflow. Quality filtering was set up with fastq_filter (-fastq_maxee 1); minimum read length, 200 bp. Reads were clustered into 97% ID operational taxonomic units (OTUs). The OTU clusters and representative sequences were determined using the UPARSE algorithm (Edgar, 2013). Taxonomy assignment was done with the help of Silva database v128 (Quast et al., 2013) and the RDP Classifier (Wang et al., 2007) with a bootstrap confidence cutoff of 70%.

Statistical Analyses

For the statistical evaluation of counts of *C. jejuni* in caecal content samples determined via quantitative bacteriological examination, the data were logarithmised. Statistical analyses were performed using SAS (version 7.1, SAS Institute Inc., Cary, NC, United States). The performance data and counts of *C. jejuni* in the caecal content were analysed with respect to the factor age at infection by one-way analysis of variance (ANOVA) for independent samples. All statements of statistical significance were based upon p-values smaller than 0.05.

Statistical analyses of microbiota were performed using R (version 3.5)[2] with the R-package "phyloseq" (version 1.24.4) (McMurdie and Holmes, 2013). Permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distances was used to identify factors contributing to the differences in microbial composition of the samples. The Bray-Curtis dissimilarity matrix was used to compare community dissimilarity based on abundance of OTUs, whereas the Jaccard distance was additionally used to compare community dissimilarity based on presence/absence of OTUs. Sample diversity was measured with the species richness estimators Observed Species and Chao 1 index, whereas the Shannon index characterises species diversity accounting for abundance and evenness of the species. To find taxa with significantly different abundance between age groups, counts were normalised and compared using the R-package DESeq (version 1.24) which uses tests based on the negative binomial distribution (Love et al., 2014). To identify phyla with significantly different abundance profile the rotation test implemented in the R-package "limma" was used which acts in a similar manner to a permutation test (Wu et al., 2010). In the taxa and phylum specific analyses, experiment was included in the models as independent factor. P-values from these tests were either adjusted by the Bonferroni-Holm (phyla) or Benjamini and Hochberg ("BH", taxa) method to control either for a family-wise error rate or the false discovery rate of 5%, respectively. The Spearman's rank correlation was performed to measure the strength of association between absolute abundance data of *Campylobacteraceae* to other bacterial families within the caecal microbiota as well as to the concentrations of VFAs in the caecal content.

[1] http://www.drive5.com/usearch/

[2] www.r-project.org

RESULTS

The general health of each animal was checked at least twice a day. The experiments ran without complications. No animal losses occurred after the rearing phase.

Performance Data

At dissection, significant differences in bodyweight as a function of age were present (LBC-70/78: 1834 g ± 137 > LBC-21/22: 595 g ± 59.0, $p < 0.0001$). The FCR (kg/kg, in the last 4 weeks before dissection) was significantly higher for LBC-70/78 (3.67 ± 0.28) in comparison to LBC-21/22 (2.12 ± 0.07, $p < 0.0001$).

Caecal Campylobacter Counts

Except for one bird (LBC-70/78), qualitative bacteriological examination revealed all caecal samples as *C. jejuni* positive.

Campylobacter counts in the caecum differed significantly depending on the age of the chickens ($p < 0.0001$). The LBC-70/78 chickens had significantly lower numbers of *C. jejuni* in the caecum (6.66 ± 1.43 $\log_{10}$ CFU/g) in comparison to the LBC-21/22 chickens. Both groups differed by almost two log steps (LBC-21/22: 8.57 ± 0.46 $\log_{10}$ CFU/g).

Intestinal Microbiota

The dataset contained 1,034,213 reads (average number of reads: 14,364; range: 4,170–87,441) mapped to 216 OTUs.

Alpha and Beta Diversity

Despite carrying out the experiments under the same conditions, the PERMANOVA test on Bray-Curtis dissimilarities indicated that both the experimental replication ($p < 0.001$) and the age at infection ($p < 0.001$) contributed significantly to the differences in microbial composition of the caecal samples. The factor experimental replication explained 24% of the sample's variability, whereas the factor age at infection explained 14% thereof. The fact that birds were primary infected (seeder birds) or became infected during *C. jejuni* spread did not contribute to the differences in microbial composition of the samples, neither in LBC-21/22 samples ($p = 0.790$) nor in LBC-70/78 samples ($p = 0.804$).

Ordination was performed using Bray-Curtis dissimilarity-based principal coordinate analysis (PCoA, **Figure 2**) also provided in the R-package "phyloseq". Dissimilarity matrix (**Table 1**) showed the highest community dissimilarity between Exp 1 and Exp 3. Jaccard dissimilarity indicated that for all experiments, both age groups shared half of the OTUs (50.2%, **Table 2**).

Alpha diversity of microbiota in caecal content of chickens of different ages at infection in the three consecutive experiments are pictured as box-plots using the indices Observed Species, Chao1, and Shannon (**Figure 3**). **Table 3** shows statistical analyses of alpha diversity indices Observed Species, Chao 1 and Shannon for the factors chickens' ages at infection and experiment. Irrespective of the factor experimental replication, no significant differences were found for all measured alpha diversity indices between chickens of different ages at infection.

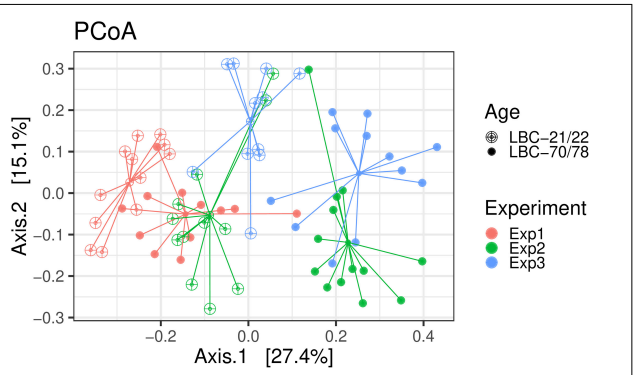

FIGURE 2 | Bray-Curtis dissimilarity-based principal coordinate analysis (PCoA) was performed on caecal samples collected in experiments 1–3. Each point represents a different bird infected at different age [LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days (open points); LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days (filled points)]; coloured lines connect birds of one experiment (Exp 1, Experiment 1; Exp 2, Experiment 2; Exp3, Experiment 3).

TABLE 1 | Community dissimilarity of microbiota in caecal contents of birds collected in three consecutive and similarly performed trials based on Bray-Curtis and Jaccard distance.

Bray-Curtis		Exp1	Exp2
	Exp2	0.3658507	
	Exp3	0.4537453	0.2774941
Jaccard		Exp1	Exp2
	Exp2	0.5357112	
	Exp3	0.6242432	0.4344350

Community dissimilarity with a range from 0 (similar) to 1 (dissimilar); Exp1, Experiment 1; Exp2, Experiment 2; Exp3, Experiment 3.

TABLE 2 | Community dissimilarity of microbiota in caecal contents of birds infected at different ages based on Bray-Curtis and Jaccard distance.

Bray-Curtis		LBC-21/22
	LBC-70/78	0.3317109
Jaccard		LBC-21/22
	LBC-70/78	0.4981725

Community dissimilarity with a range from 0 (similar) to 1 (dissimilar); Exp1, Experiment 1; Exp2, Experiment 2; Exp3, Experiment 3; LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days.

Relative Abundance per Sample and Age Effects on the Phylum and OTU Level

Independent of the chickens' age at infection and experiment, the caecal microbiota was dominated at phylum level by *Firmicutes* (91.1%) and *Proteobacteria* (5.02%), followed by *Tenericutes* (2.25%) and *Bacteroides* (1.00%). Relative abundance of bacterial phyla within all samples is shown in **Supplementary Figure S1**. Older animals showed consistently higher values for relative abundance of *Firmicutes* in all experiments. Relative abundance of *Firmicutes* reached highest values in experiment 2 (LBC-21/22: 94.7%; LBC-70/78: 97.6% of total microbiota), while lowest relative abundance of *Firmicutes* was found in experiment 1 (LBC-21/22: 82.9%; LBC-70/78: 87.4% of total microbiota). *Campylobacteraceae* could be found within the most abundant eleven OTUs in experiment 1. For this reason, the default presentation of the most abundant ten OTUs was extended by one OTU (**Figure 4**). Experiment 1 showed a conspicuously higher relative abundance of *Proteobacteria* in comparison to the other experiments. *Proteobacteria* mainly consisted of the families *Alcaligenaceae* and *Campylobacteraceae* in experiment 1 and *Alcaligenaceae* in experiment 3, while in experiment 2, this family was not present among the 11 most abundant OTUs (**Figure 4**). The 11 most abundant OTUs in experiment 2 all belonged within the phylum *Firmicutes* to the families *Erysipelotrichaceae, Lachnospiraceae*, *Peptostreptococcaceae,* and *Ruminococcaceae*. OTUs belonging to these four families were found among the 11 most abundant ones in all experiments.

Global tests on normalised counts on each phylum yielded five (of seven) phyla that showed a significantly different count profile between the two age groups (**Supplementary Table S1**). The strongest difference between age groups was seen between *Firmicutes* (adjusted p = 0.0007), whereas *Cyanobacteria* and *Verrucomicrobia* were not significant. Global tests on normalised counts yielded twelve (of 32) families that showed a significantly different count profile between the two age groups (**Supplementary Table S2**). The strongest difference between age groups was seen between *Campylobacteraceae*, *Enterococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Peptostreptococcaceae,* and *Ruminococcaceae* (adjusted p = 0.00259974). At the species level, 28 of 216 OTUs showed a significantly (plus an additional log fold change criterion of ± 2) different abundance between the two age groups, where 11 of these OTUs were more enriched in the older animals and 17 more enriched in the younger animals. Log fold changes for these 28 OTUs are shown in **Supplementary Table S3** and plotted in **Figure 5**. OTU_3 had the smallest p-value, followed by OTU_14.

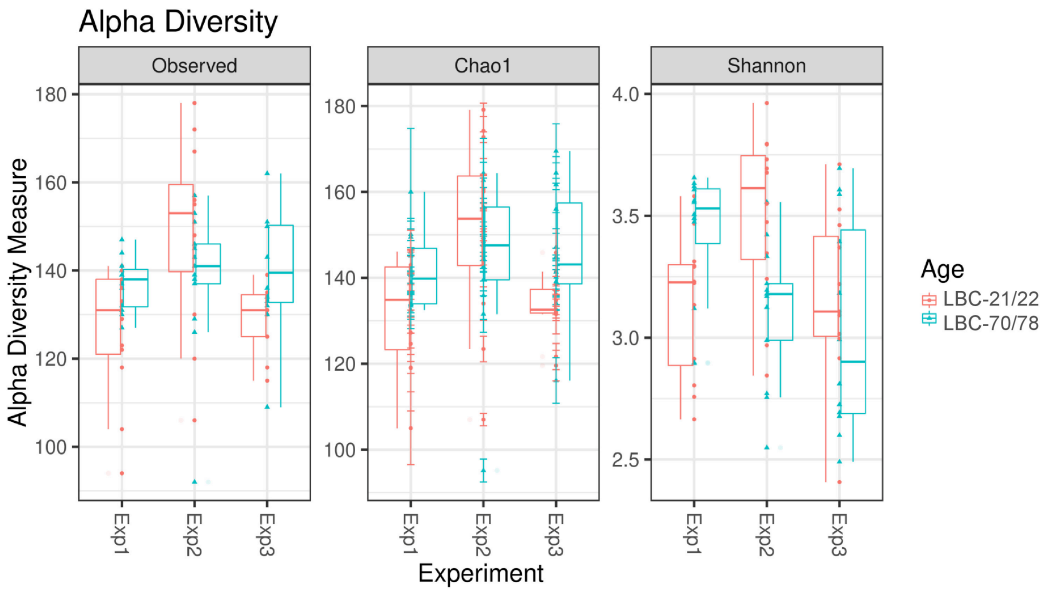

FIGURE 3 | Alpha diversity in samples from caecal content depending on experiment and age at infection. Box-plots showing alpha diversity in samples using the Observed Species index, the Chao1 index and Shannon index (Exp1, Experiment 1; Exp2, Experiment 2; Exp3, Experiment 3; LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days).

Correlation Analysis

Spearman's rank correlation coefficient was used to quantify the strength of association and the direction of the relationship between *Campylobacteraceae* and other taxa at the taxonomic level family (**Table 4**). There was a positive Spearman's rank correlation between absolute abundances of *Campylobacteraceae* and *Alcaligenaceae* in caecal contents of young (Spearman's rank correlation coefficient: 0.437; $p < 0.01$) and old chickens (Spearman's rank correlation coefficient: 0.434; $p < 0.01$). Additionally, in caecal samples of older chickens, a positive relationship between *Campylobacteraceae* and *Bacteroidaceae* could be observed (Spearman's rank correlation coefficient: 0.408; $p < 0.05$), while in younger chickens, a negative Spearman's rank correlation was found for *Campylobacteraceae* and *Comamonadaceae* (Spearman's rank correlation coefficient: 0.501) at a significance level of $p < 0.01$. There was a negative Spearman's rank correlation between *Campylobacteraceae* and *Erysipelotrichaceae* in samples of the young chickens, while Spearman's rank correlation in samples of the older chickens was positive. Spearman's rank correlation coefficients between both taxa remained low (Spearman's rank correlation coefficient: on average ± 0.151) and insignificant independent of the chickens' age at infection.

TABLE 3 | Comparison of alpha-diversity indices of the microflora in caecal contents of chickens.

Index	Factor	*p*-value
Observed	Age at infection	0.28014
	Experimental replication	0.02147
	Age at infection: Experimental replication	0.01617
Chao 1	Age at infection	0.14196
	Experimental replication	0.04055
	Age at infection: Experimental replication	0.04862
Shannon	Age at infection	0.27704
	Experimental replication	0.08584
	Age at infection: Experimental replication	0.00326

Fermentation Pattern in Caecal Chyme

The concentration of the bacterial fermentation products n-butyrate, acetate and propionate were significantly higher in older chickens compared to younger birds (**Table 5**). Differences in VFA concentrations between the experiments were demonstrated as being insignificant both in samples of younger chickens and in older birds (**Table 6**). Spearman's rank correlation analysis between bacterial families and concentrations of VFAs demonstrated a negative relationship between *Campylobacteraceae* and concentrations of n-butyrate, acetate and propionate. The highest coefficient within bacterial families and concentrations of VFAs was seen for *Campylobacteraceae* and n-butyrate (−0.51141; $p < 0.0001$; **Table 7**).

DISCUSSION

In spite of the fact that it has already be proven in chickens that microbial richness and diversity increase with age (Awad et al., 2016) with them gaining maturation (Lu et al., 2003), the microbial richness in samples of older chickens in the present study showed, contrary to expectations, no statistically significant differences compared to younger chickens.

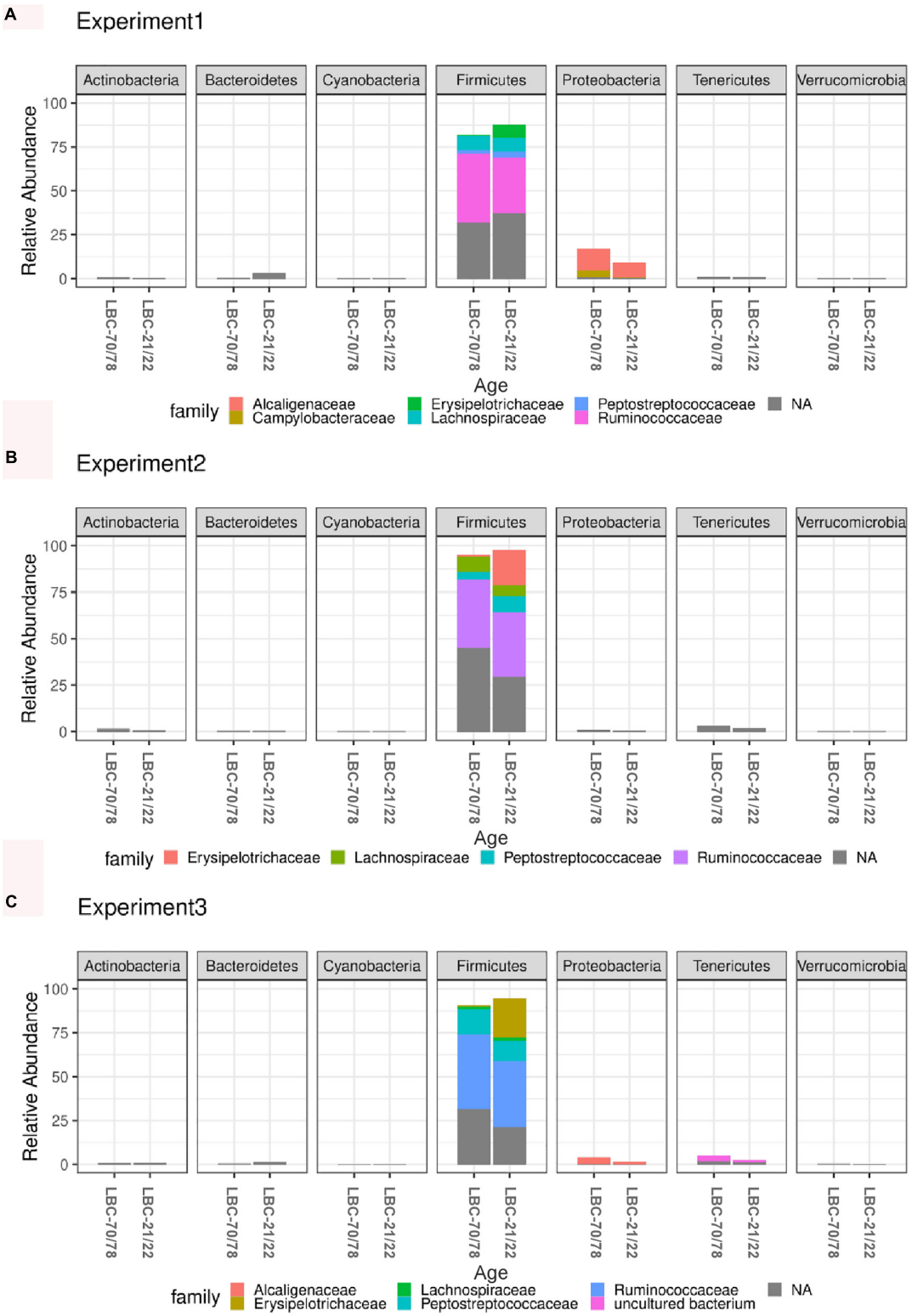

FIGURE 4 | Microbiota composition analysis using 16S rRNA sequencing in caecal contents of different aged chickens at time of infection. Relative abundance of the 11 most abundant OTUs belonging to bacterial families within different phyla in experiment 1 **(A)**. Relative abundance of the 11 most abundant OTUs belonging to bacterial families within different phyla in experiment 2 **(B)**. Relative abundance of the 11 most abundant OTUs belonging to bacterial families within different phyla in experiment 3 **(C)**. LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days; NA, OTUs without genus-level taxonomic assignment.

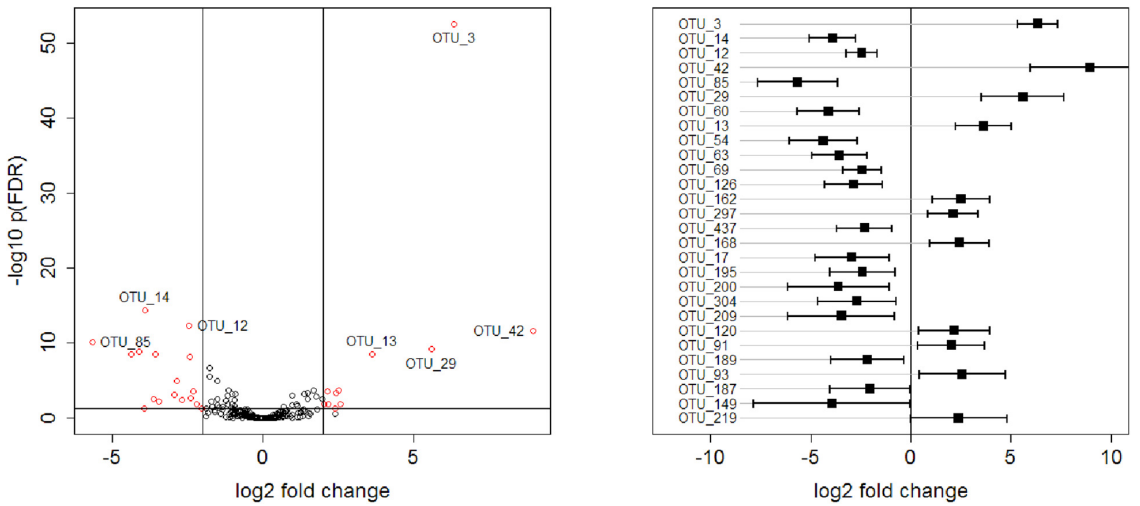

FIGURE 5 | Left: Volcano plot representing –log10 FDR-adjusted *p*-values versus log2 fold changes for all 216 OTUs. **Right**: log2 fold changes of 28 OTUs, selected with a criterion of FDR-adjusted *p*-values < 0.05 and absolute log2 fold change >2. OTUs are ordered according to *p*-values, with the OTU_3 having the smallest *p*-value. Confidence intervals were calculated according to Jung et al. (2011).

TABLE 4 | Spearman's rank correlation analysis of absolute abundance of *Campylobacteraceae* at the taxonomic level; family.

Taxa	LBC-21/22 (*n* = 35)	LBC-70/78 (*n* = 37)
	Campylobacteraceae	Campylobacteraceae
	Spearman's rank correlation coefficient	
Comamonadaceae	−0.50098**	−0.00922
Alcaligenaceae	0.43710**	0.43407**
uncultured rumen bacterium	−0.38954*	−0.06161
Desulfovibrionaceae	−0.35297*	0.12081
Clostridiaceae 1	0.33853*	
Peptostreptococcaceae	−0.33651*	−0.00529
Sphingomonadaceae	−0.27200	0.35269*
Ambiguous_taxa	−0.15390	0.33483*
Bacteroidaceae	−0.01011	0.40751*

*Only significant correlations were plotted; *Correlation is significant at p < 0.05; **Correlation is significant at p < 0.01; LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days.*

TABLE 5 | Characterisation of fermentation products (mmol/kg fresh matter) in caecal contents of chickens of different ages at time of infection.

Group	*N*	Acetic acid		Propionic acid		n-Butyric acid	
		Mean	SD	Mean	SD	Mean	SD
LBC-21/22	35	58.7^{B}	28.6	3.34^{B}	1.79	14.3^{B}	14.6
LBC-70/78	37	92.4^{A}	23.2	5.50^{A}	2.72	34.7^{A}	11.0

LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days; ^{A,B}values within a column with different superscripts differ significantly at p < 0.001.

When assessing the ratio of the concentrations of acetate to butyrate in caecal contents, noticeable differences can be seen between the chickens' ages at infection [higher acetate:butyrate-ratio in younger chickens (2.71:1), lower ratio in older chickens (1.82:1)]. Spearman's rank correlation analysis between *Campylobacteraceae* and the concentrations of VFA demonstrated an overall negative relationship. The highest coefficient within bacterial families and the concentrations of VFA was seen for *Campylobacteraceae* and n-butyrate. *In vitro* obtained results of a study conducted by Van Deun et al. (2008a) identified butyrate as the most successful of the examined short-chain fatty acids in being bactericidal (12.5 mM at pH 6.0) for *C. jejuni* while propionate and acetate had a bacteriostatic effect (50 mM). Nevertheless, adding of butyrate-coated micro-beads to the diet of 2-week-old broilers was unsuccessful in reducing *C. jejuni* caecal colonisation. The authors suspect behind the unsuccessful protection *in vivo* protective effects of mucous and the rapid absorption of butyrate by the enterocytes (Van Deun et al., 2008a). Different scientific working groups used Caco-2 model to investigate whether butyrate could play a protective role during *C. jejuni* infection by decreasing bacterial paracellular translocation across intestinal cell layers. Butyrate was able to protect Caco-2 cells from two major virulence mechanisms of *C. jejuni*, invasion and translocation in studies of Van Deun et al. (2008b). Additionally, Cresci et al. (2017) found that *C. jejuni* adhesion was reduced with butyrate pretreatment of Caco-2 cells, therefore showing protective effects of butyrate during *C. jejuni* infection. The authors postulated that an optimal gut microbiota composition and/or a dietary formulation with a view to enhancing butyrate levels and maintaining gut microbiota balance may influence the prevalence, incidence and outcome of *Campylobacteriosis*. Further *in vivo* investigations on potential protective effects of dietary supplemented butyrate against *Campylobacter* infection showed divergent results. Dietary supplementation with coated calcium butyrate (0.1% of the diet) had no effect on *C. jejuni* colonisation or shedding levels in experimentally infected broiler chickens (Ocejo et al., 2017). A coated butyrate-based product

TABLE 6 | Characterisation of the fermentation products (volatile fatty acid [VFA]) in the caecal contents of each experimental replicate.

LBC-21/22		**Exp 1 (*n* = 12)**		**Exp 2 (*n* = 12)**		**Exp 3 (*n* = 11)**	
VFA		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Acetic acid	[mmol/kg fresh matter]	62.3	34.8	54.7	21.1	59.3	30.4
Propionic acid		2.96	1.18	2.95	1.36	4.17	2.50
n-Butyric acid		15.9	17.1	10.6	9.95	16.4	16.5
LBC-70/78		**Exp 1 (*n* = 12)**		**Exp 2 (*n* = 13)**		**Exp 3 (*n* = 12)**	
VFA		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Acetic acid	[mmol/kg fresh matter]	85.9	27.2	98.9	21.3	91.6	20.6
Propionic acid		5.6	3.99	5.34	1.44	5.54	2.42
n-Butyric acid		32.5	13.5	34.9	9.26	36.6	10.6

LBC-21/22, Lohmann Brown-Classic, infection at the age of 21/22 days; LBC-70/78, Lohmann Brown-Classic, infection at the age of 70/78 days; no statistically significant differences could be found between the experiments.

TABLE 7 | Spearman's correlation analysis of absolute abundance of bacterial families and fermentation products in all samples.

	Acetic acid	**Propionic acid**	**n-Butyric acid**
Taxa	**Spearman's rank correlation coefficient**		
Bacteroidaceae	0.22014	0.35281**	0.24147*
Campylobacteraceae	−0.45982***	−0.40915**	−0.51141***
Enterococcaceae	−0.26985*	−0.21472	−0.30396**
Erysipelotrichaceae	0.44652***	0.45589***	0.47003***
Peptostreptococcaceae	0.23533*	0.21979	0.26781*
Rikenellaceae	0.22481	0.14929	0.29722*
Streptococcaceae	−0.20648	−0.22982	−0.23192*

*Only significant correlations were plotted; *Correlation is significant at $p < 0.05$; **Correlation is significant at $p < 0.01$; ***Correlation is significant at $p < 0.0001$.*

in a higher dosage (0.3% of the diet) was able to reduce caecal *Campylobacter* counts significantly compared to the control group during the whole fattening period. However, the observed reduction was not homogenous among the birds; some remained contaminated with caecal *Campylobacter* counts of 7 $\log_{10}$ CFU/g (Guyard-Nicodème et al., 2015).

Statistical analyses of all 216 OTUs for differences in bacterial abundance between the two age groups revealed smallest FDR-adjusted *p*-values for OTU_3 and OTU_14. OTU_ 3 represents a bacterial species that is assigned to the genus *Turicibacter* which belongs to the family *Erysipelotrichaceae.* In the present study, eight OTUs could be assigned to the family *Erysipelotrichaceae* but the family was mainly formed by the genus *Turicibacter*. *Turicibacter* is a well-known coloniser of livestock animals including chickens. However, little is known about this genus and its members, which may have an unknown potential to positively influence animal health (Abudabos et al., 2017). OTU_14 represents a bacterial species that is assigned to the genus *Campylobacter* which belongs to the family *Campylobacteraceae.* OTU_3 was more enriched in the older birds while OTU_14 was more enriched in the younger birds. This was consistent with the results of the quantitative bacteriological examination.

Independent of the chickens' age at infection, Spearman's rank correlation coefficients between both taxa remained low and insignificant, thereby indicating no relationship between the two bacterial families.

Correlation analyses of *Campylobacteraceae* with other bacterial families demonstrated no strong link in this study. A similar pattern was seen in microbiota studies of egg-laying hens (Videnska et al., 2014). In the present study, one positive association was found for the *Alcaligenaceae* family at both ages at infection. In our findings, the family *Alcaligenaceae* was formed to 100% by the genus *Parasuterella*. *Parasutterella* is a non-motile, strictly anaerobic genus which is proposed to accommodate a novel family named *Sutterellaceae* (Morotomi et al., 2011). This genus was defined as a core component of the human and mouse gut microbiota, producing succinate as a fermentative end-product while relying, as asaccharolytic genus, on amino acids such as asparagine, aspartate and serine (Ju et al., 2019). Those amino acids support its metabolic activities and physiological functions, indicating an adaptation to the gut environment, which contains readily available non-essential amino acids (Ju et al., 2019). Amino acids, in particular aspartate, glutamate, proline and serine are key carbon and energy sources for the asaccharolytic zoonotic pathogen *C. jejuni* as well (Guccione et al., 2008). Varying amino-acid concentrations in the diet of broiler chickens preferentially utilised by *C. jejuni*, such as aspartate, glutamate, proline and serine are able to influence the spread and the shedding of *C. jejuni* (Visscher et al., 2018). It can be hypothesised that an environment favouring *C. jejuni* metabolism should also favour growing conditions of *Parasutterella*, as their metabolism relies on similar amino acids. This would explain the positive relationship between these bacterial families.

Contrary to expectations, the factor experimental replication in the present study was discovered to contribute more to the differences of microbial composition of the samples in comparison to the factor age at infection. The dissimilarity matrix revealed higher dissimilarity of samples between experiment 1 and the other experiments. Of special interest is the higher occurrence of *Proteobacteria* in experiment 1, independent of the age of the chickens at infection, mainly consisting of the families *Campylobacteraceae* and *Alcaligenaceae* compared to the other experiments. Interestingly, the differences in VFA concentrations (n-butyrate, acetate and propionate) between the experiments were demonstrated as not being significant. This could indicate that the function of the microbiota was kept even if the microbial composition was variable between experimental replicates. The gut microbiota are immensely divers and vary interpersonal (Lozupone et al., 2012). However, the basics of microbial metabolism remain stable among the group of studied individuals because many biochemical pathways varied little among members of the microbiome (Abubucker et al., 2012). Stanley et al. (2013) pointed out that most published studies investigate changes in the microbiota of chickens in a single trial; only rarely have results been reported for replicated trials

and scientific rigour requires a hypothesis to be supported by replicated results. In a previous study, they investigated microbiota changes associated with a *Clostridium perfringens* infection and noted an unexpected but important finding: there were large differences in the microbiota in the control groups between trials that were designed to be as similar as possible (same line of commercial chickens from the same hatchery, fed the same diet formulation and housed in the same facility) (Stanley et al., 2012). Therefore Stanley et al. (2013) investigated batch-to-batch variations in chicken caecal microbiota in three similar trials and equally showed that even under carefully controlled conditions, large variations in microbiota composition still occur. The authors hypothesised that this large variability is due to the colonisation by bacteria originating from the wider environment rather than predominantly from maternally derived bacteria. Thibodeau et al. (2017) examined the influence of selenium-yeast on chicken caecal microbiota in the context of colonisation by *C. jejuni* in two experimental replicates with two independent batches of chickens. They saw that bacterial community structure was significantly different between the experimental replicates and concluded that there is a need for true biological replication when studying the chicken intestinal microbiota, especially when the observed changes are subtle.

CONCLUSION

Relative abundance of the families *Campylobacteraceae* and *Erysipelotrichaceae* were significantly different between younger and older chickens in all experiments without showing a link between one another. In general, correlation analyses of absolute abundance of *Campylobacteraceae* with other bacterial families revealed the strongest link to the genus *Parasutterella* and the strongest negative relationship to butyrate concentrations in caecal contents. Finally, despite performing three consecutive repetitions, the factor experimental replication was discovered to contribute more to the differences of microbial composition of the samples in comparison to the factor age at infection.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the BioProject ID PRJNA557374.

ETHICS STATEMENT

Animal experiments were performed in accordance with the German rules and regulations. The experiments were approved by the Ethics Committee of Lower Saxony for Care and Use of Laboratory Animals (LAVES) (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit; reference: 33.19-42502-05-15A500).

AUTHOR CONTRIBUTIONS

CV was the initiator of the idea and revised the manuscript. CV and JH designed the study and interpreted the data. JH carried out the experiments, collected the data with the help of CV, and wrote the manuscript. HK, BK, and CK performed the DNA extraction of the samples and prepared the samples for microbiome analyses. EG and TS carried out the microbiome analyses. KJ and JH statistically analysed the data, and compiled the figures and tables. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by Ahrberg-Foundation, Germany. The role of the funding body was merely financial. It did not play any role in the study design, collection, interpretation, and analysis of the data, or preparation of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Frances Sherwood-Brock for proofreading the manuscript to ensure correct English. Thanks also go to the Ahrberg-Foundation for financially supporting the investigations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02303/full#supplementary-material

REFERENCES

Abubucker, S., Segata, N., Goll, J., Schubert, A. M., Izard, J., Cantarel, B. L., et al. (2012). Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput. Biol.* 8:e1002358. doi: 10.1371/journal.pcbi.1002358

Abudabos, A. M., Al-Atiyat, R. M., Albatshan, H. A., Aljassim, R., Aljumaah, M. R., Alkhulaifi, M. M., et al. (2017). Effects of concentration of corn distillers dried grains with solubles and enzyme supplementation on cecal microbiota and performance in broiler chickens. *Appl. Environ. Microbiol.* 101, 7017–7026. doi: 10.1007/s00253-017-8448-5

Awad, W. A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M., et al. (2016). Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with *Campylobacter jejuni* infection. *Front. Cell. Infect. Microbiol.* 6:154. doi: 10.3389/fcimb.2016.00154

Brisbin, J. T., Gong, J., and Sharif, S. (2008). Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Anim. Health Res. Rev.* 9, 101–110. doi: 10.1017/S146625230800145X

Buffie, C. G., and Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nat. Rev. Immunol.* 13, 790–801. doi: 10.1038/nri3535

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Colles, F., McCarthy, N., Howe, J., Devereux, C., Gosler, A., and Maiden, M. (2009). Dynamics of *Campylobacter* colonization of a natural host, *Sturnus vulgaris* (European starling). *Environ. Microbiol.* 11, 258–267. doi: 10.1111/j.1462-2920.2008.01773.x

Cresci, G. A. M., Mayor, P. C., and Thompson, S. A. (2017). Effect of butyrate and *Lactobacillus GG* on a butyrate receptor and transporter during *Campylobacter jejuni* exposure. *FEMS Microbiol. Lett.* 364:fnx046. doi: 10.1093/femsle/fnx046

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods.* 10, 996–998. doi: 10.1038/nmeth.2604

Efsa, (2017). The european union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* 15:5077. doi: 10.2903/j.efsa.2017.5077

GfE, (1999). *Recommendations for the Energy and Nutrient Supply of Laying Hens and Broilers*. Frankfurt am Main: DLG-Verlag.

Glünder, G. (1995). Zur Infektiosität von *Campylobacter jejuni* und *Campylobacter coli* bei Hühnern. *Berl. Münch. Tierarztl. Wochenschr.* 108, 101–104.

Guccione, E., Del Rocio Leon-Kempis, M., Pearson, B. M., Hitchin, E., Mulholland, F., Van Diemen, P. M., et al. (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol. Microbiol.* 69, 77–93. doi: 10.1111/j.1365-2958.2008.06263.x

Guerin, M., Sir, C., Sargeant, J., Waddell, L., O'Connor, A., Wills, R., et al. (2010). The change in prevalence of *Campylobacter* on chicken carcasses during processing: a systematic review. *Poult. Sci.* 89, 1070–1084. doi: 10.3382/ps.2009-00213

Guyard-Nicodème, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., et al. (2015). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult. Sci.* 95, 298–305. doi: 10.3382/ps/pev303

Hankel, J., Popp, J., Meemken, D., Zeiger, K., Beyerbach, M., Taube, V., et al. (2018). Influence of lauric acid on the susceptibility of chickens to an experimental *Campylobacter jejuni* colonisation. *PLoS One* 13:e0204483. doi: 10.1371/journal.pone.0204483

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., et al. (2011). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol.* 152, 219–228. doi: 10.1016/j.vetmic.2011.03.010

Higgins, C. H. (1898). Notes upon an epidemic of fowl cholera and upon the comparative production of acid by allied bacteria. *J. Exp. Med.* 3, 651–658. doi: 10.1084/jem.3.6.651

Ju, T., Kong, J. Y., Stothard, P., and Willing, B. P. (2019). Defining the role of *Parasutterella*, a previously uncharacterized member of the core gut microbiota. *ISME J.* 13, 1520–1534. doi: 10.1038/s41396-019-0364-5

Jung, K., Friede, T., and Beißbarth, T. (2011). Reporting FDR analogous confidence intervals for the log fold change of differentially expressed genes. *BMC Bioinformatics* 12:288. doi: 10.1186/1471-2105-12-288

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., and Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl. Environ. Microbiol.* 69, 6816–6824. doi: 10.1128/AEM.69.11.6816-6824.2003

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Morotomi, M., Nagai, F., and Watanabe, Y. (2011). *Parasutterella secunda* sp. nov., isolated from human faeces and proposal of Sutterellaceae fam. nov. in the order Burkholderiales. *Int. J. Syst. Evol. Microbiol.* 61, 637–643. doi: 10.1099/ijs.0.023556-0

Newell, D., and Fearnley, C. (2003). Sources of *Campylobacter* colonization in broiler chickens. *Appl. Environ. Microbiol.* 69, 4343–4351. doi: 10.1128/AEM.69.8.4343-4351.2003

Ocejo, M., Oporto, B., Juste, R. A., and Hurtado, A. (2017). Effects of dry whey powder and calcium butyrate supplementation of corn/soybean-based diets on productive performance, duodenal histological integrity, and *Campylobacter* colonization in broilers. *BMC Vet. Res.* 13:199. doi: 10.1186/s12917-017-1121-5

Qu, A., Brulc, J. M., Wilson, M. K., Law, B. F., Theoret, J. R., Joens, L. A., et al. (2008). Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One* 3:e2945. doi: 10.1371/journal.pone.0002945

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Reich, F., Atanassova, V., Haunhorst, E., and Klein, G. (2008). The effects of *Campylobacter* numbers in caeca on the contamination of broiler carcasses with *Campylobacter*. *Int. J. Food Microbiol.* 127, 116–120. doi: 10.1016/j.ijfoodmicro.2008.06.018

Rosenquist, H., Sommer, H. M., Nielsen, N. L., and Christensen, B. B. (2006). The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *Int. J. Food Microbiol.* 108, 226–232. doi: 10.1016/j.ijfoodmicro.2005.12.007

Stanley, D., Geier, M. S., Hughes, R. J., Denman, S. E., and Moore, R. J. (2013). Highly variable microbiota development in the chicken gastrointestinal tract. *PLoS One* 8:e84290. doi: 10.1371/journal.pone.0084290

Stanley, D., Keyburn, A. L., Denman, S. E., and Moore, R. J. (2012). Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. *Vet. Microbiol.* 159, 155–162. doi: 10.1016/j.vetmic.2012.03.032

Thibodeau, A., Fravalo, P., Yergeau, É., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS One* 10:e0131978. doi: 10.1371/journal.pone.0131978

Thibodeau, A., Letellier, A., Yergeau, É., Larrivière-Gauthier, G., and Fravalo, P. (2017). Lack of evidence that selenium-yeast improves chicken health and modulates the caecal microbiota in the context of colonization by *Campylobacter jejuni*. *Front. Microbiol.* 8:451. doi: 10.3389/fmicb.2017.00451

Van Deun, K., Haesebrouk, F., Van Immerseel, F., Ducatelle, R., and Pasmans, F. (2008a). Short-chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. *Avian Pathol.* 37, 379–383. doi: 10.1080/03079450802216603

Van Deun, K., Pasmans, F., Van Immerseel, F., Ducatelle, R., and Haesebrouck, F. (2008b). Buytrate protects Caco-2 cells from *Campylobacter jejuni* invasion and translocation. *Br. J. Nutr.* 100, 480–484. doi: 10.1017/S0007114508921693

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., et al. (2014). Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS One* 9:e115142. doi: 10.1371/journal.pone.0115142

Visscher, C., Klingenberg, L., Hankel, J., Brehm, R., Langeheine, M., and Helmbrecht, A. (2018). Influence of a specific amino acid pattern in the diet on the course of an experimental *Campylobacter jejuni* infection in broilers. *Poult. Sci.* 97, 4020–4030. doi: 10.3382/ps/pey276

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wu, D., Lim, E., Francois Vaillant, F., Asselin-Labat, M.-L., Visvader, J. E., and Smyth, G. K. (2010). ROAST: rotation gene set tests for complex microarray experiments. *Bioinformatics* 26, 2176–2182. doi: 10.1093/bioinformatics/btq401

Conflict of Interest: CK was employed by company Boehringer Ingelheim Veterinary Research Center GmbH & Co. KG.

The remaining 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

METHODS
published: 22 October 2019
doi: 10.3389/fmicb.2019.02292

A *Gallus gallus* Model for Determining Infectivity of Zoonotic *Campylobacter*

*Dennis Lye[1], Ian Struewing[2], Theresa M. Gruber[1], Kevin Oshima[1], Eric N. Villegas[1] and Jingrang Lu[1]**

[1] Office of Research and Development, USEPA, Cincinnati, OH, United States, [2] Pegasus Technical Services, Inc., Cincinnati, OH, United States

To better understand public health implications of waterfowl as reservoirs for zoonotic sources of *Campylobacter* in recreational waters, we developed a *Gallus gallus* (chick) model of infection to assess the pathogenicity of environmental isolates of *Campylobacter*. This method involved exposure of 1-day-old chicks through ingestion of water, the natural route of infection. Viable *Campylobacter* from laboratory-infected animals were monitored by using a modified non-invasive sampling of fresh chick excreta followed by a passive polycarbonate-filter migration culture assay. The method was used to evaluate the infectivities of three laboratory strains of *Campylobacter* spp. (*Campylobacter coli, Campylobacter jejuni*, and *Campylobacter lari*), three clinical isolates of *C. jejuni*, and four environmental *Campylobacter* spp. isolated from California gulls (*Larus californicus*). The results revealed that chicks were successfully infected with all laboratory and clinical isolates of *Campylobacter* spp. through ingestion of *Campylobacter*-spiked water, with infection rates ranging from <10 to $>90\%$ in a dose-dependent manner. More importantly, exposure of chicks with *Campylobacter* spp. isolated from *Gallus gallus* excreta also resulted in successful establishment of infection ($\leq 90\%$). Each monitored *Campylobacter* spp. contained $\geq 7.5 \times 10^4$ CFU·g^{-1} of feces 7 days post-exposure. These results suggest that a *G. gallus* model can be used to assess infectivity of *Campylobacter* isolates, including gull and human clinical isolates. Use of an avian animal model can be applied to assess the importance of birds, such as the *G. gallus*, as potential contributors of waterborne-associated outbreaks of campylobacteriosis.

Keywords: *Campylobacter*, colonization, chick model, infectivity, gull, avian

OPEN ACCESS

Edited by:
Steven C. Ricke,
University of Arkansas, United States

Reviewed by:
Jose Manuel Silván Jiménez,
Institute of Food Science Research (CIAL), Spain
Pallavi Singh,
Northern Illinois University, United States

***Correspondence:**
Jingrang Lu
lu.jingrang@epa.gov

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

Received: *22 May 2019*
Accepted: *19 September 2019*
Published: *22 October 2019*

Citation:
Lye D, Struewing I, Gruber TM, Oshima K, Villegas EN and Lu J (2019) A Gallus gallus Model for Determining Infectivity of Zoonotic Campylobacter. Front. Microbiol. 10:2292. doi: 10.3389/fmicb.2019.02292

INTRODUCTION

Campylobacter spp. bacteria are a major cause of zoonotic human enteric infections commonly transmitted by ingestion of contaminated food or water (Pitkänen, 2013). These bacteria, which are commensal organisms within the gastrointestinal tract of various animals, including birds, have also been isolated from contaminated fresh and marine recreational beach sites (Savill et al., 2001; Stoddard et al., 2005). Recent studies have suggested that *Campylobacter* spp. present in a

water source often reflect the source(s) and/or type of fecal pollution at that site. For example, *Campylobacter jejuni* is most associated with sewage discharges, whereas *Campylobacter coli* and *Campylobacter lari* are associated more with agricultural runoff and/or the presence of abundant waterfowl fecal contamination (Pitkänen, 2013). Because *Campylobacter* spp. require specific fastidious environmental parameters for growth, they are unable to multiply and persist in most surface waters (Obiri-Danso et al., 2001); thus, detection of culturable *Campylobacter* spp. in surface waters is usually an indication of recent fecal contamination.

Amid increasing public concerns about transmission of enteric disease from waters harboring large bird populations, fecal releases from wild birds have been reported to have a significant role in water quality impairment of recreational waters (Lévesque et al., 2000). Additionally, seagulls and ducks have been reported to be major contributors of *Salmonella* and *Campylobacter* bacteria via release and dispersal of their feces in recreational waters (Kapperud and Rosef, 1983; Quessy and Messier, 1992). The extent of colonization and persistence of various *Campylobacter* spp. within wild birds remains largely undetermined. Ramos et al. (2010) reported a direct relationship between *Campylobacter* infection of fledgling gull chicks with exposure/consumption diets within human-altered environments (particularly related to garbage and sewage). The colonized gulls showed no adverse health impacts, which could lead to the potential dispersal of *Campylobacter* over large geographical areas (Bingham-Ramos and Hendrixson, 2008).

Understanding the role of waterfowl as reservoirs for zoonotic *Campylobacter* spp. in recreational waters has important public health implications. The animal models (piglet, mouse, and rabbit) to assess the infectivity and pathogenicity of different strains of *Campylobacter* have been used (Field et al., 1981; Babakhani et al., 1993; Hodgson et al., 1998; Stahl and Vallance, 2015; Giallourou et al., 2018; Hartley-Tassell et al., 2018). Considering that *Campylobacter* spp., especially *C. jejuni*, have evolved to preferentially colonize the avian gut, the chick model was developed and is the more relevant animal model for investigating bacterial colonization factors (Newell, 2001; Müller et al., 2006; Manes-Lazaro et al., 2017; Sweeney et al., 2017; Salaheen et al., 2018). Although animal models have also been shown to be useful for investigating *Campylobacter* virulence factors involved during infection, these approaches have inherent limitations, specifically the use of invasive procedures and surgical intervention and/or atypical administration of bacterial inoculums (via oral inoculation or gavage) that do not mimic natural infections with *Campylobacter* (Ringoir et al., 2007; Stern, 2008). Invasive sampling procedures, such as terminal surgical tissue (Stern, 2008; Clavijo and Flórez, 2017) or cecal swab sampling (Cawthraw et al., 1996; Ringoir et al., 2007), have also been used. Together, these approaches may alter animal behavior and susceptibility to *Campylobacter* infection and potentially affect accurate assessment of susceptibility to and persistence of *Campylobacter* infections.

Detection and isolation of *Campylobacter* spp. from the environment are laborious and often based on enrichment procedures, selective media, and antibiotic resistance. Moreover, many environmental *Campylobacter* isolates are sensitive to antibiotics, which makes it difficult to use selective culture-based detection (Steele and McDermott, 1984). Nevertheless, an improved method has been reported that uses a passive-filtration plating technique for isolation of *Campylobacter* spp. from environmental waters and animal samples; the method uses size exclusion filters to select for "smaller" highly motile *Campylobacter* from most other "larger" less motile bacteria that may be found in environmental samples (Steele and McDermott, 1984; Jokinen et al., 2012). In a subsequent study, the use of polycarbonate filters gave increased recovery of *Campylobacter* spp. from stool samples when using the same passive-filtration plating procedure (Nielsen et al., 2013). This passive-filtration technique can be valuable for determining the viability of environmental *Campylobacter* isolates. Thus, this filter method should be a suitable approach for this study.

The aim of this study was twofold: (1) develop a non-invasive chick model of infection that mimics the natural route of infection to assess the infectivity of environmental *Campylobacter* isolates, and (2) use the passive-filtration plating technique to assess the bacterial burden in animals following infection, including the persistence of various *Campylobacter* spp. in a natural host. More broadly, since the prevalence and zoonotic potential of *Campylobacter* spp. found in wild avian species and recreational waters remains poorly understood (Weis et al., 2014, 2016) the techniques described herein will be useful toward developing more accurate risk assessment models of waterfowl-derived *Campylobacter* spp. human infections in a recreational water exposure scenario.

MATERIALS AND METHODS

Animals

Specific pathogen-free fertilized chicken layer eggs (*Gallus gallus*) were obtained from Charles River Laboratories (North Franklin, CT, United States) and were incubated upon receipt (37–38°C, at 45–55% relative humidity) for 21 days, with occasional rotation. On day 18, the eggs were placed on hatching trays at a temperature range of 31–32°C and a relative humidity of 60–65% and allowed to hatch with no additional rotation. Once the chicks hatched, were fully dry, and were able to walk, they were transferred from the hatchery and placed in an individually ventilated cage (IVC) system with the temperature maintained between 32 and 38°C throughout the experiment. The chicks were then randomly assigned to one of the three dose groups (A, B, or C; **Supplementary Table S1**) per *Campylobacter* isolate (with 17–23 chicks per group, n = 630). There is no "intermixing" between or among the strains with this system. Once randomly assigned to a dose group and placed into the IVC system, they were given the infected water. Both the chamber and cage system were sterilized prior to placing the eggs and chicks. All animal experiments were approved by the US Environmental Protection Agency (USEPA) Animal Facility Oversight of Institutional Animal Care and Use Committee.

Bacterial Isolates

Ten *Campylobacter* species/isolates were analyzed in this study: three laboratory, three clinical, and four environmental isolates. The laboratory isolates were *C. jejuni* (ATCC 29428), *C. coli* (ATCC 33559), and *C. lari* (ATCC 35221) (American Tissue Culture Collection, Manassas, VA, United States). The clinical isolates were cultured specimens taken from human fecal samples of diagnosed *C. jejuni* infections (kindly provided by a local hospital doctor). The environmental isolates (58BB: *C. lari*; 63A: *C. jejuni*; 64BB: *C. lari*; 70BB: *Campylobacter volucre*) (**Supplementary Table S2**) were obtained from California gull (*Larus californicus*) fecal samples collected from Southern California Hobie Beach as previously described (Lu et al., 2011). All isolates were grown and maintained on 5% sheep blood agar plates, as described below. Their physiological and biochemical characteristics were tested and summarized in **Supplementary Table S3**. For long-term storage, frozen (−80°C) glycerol stocks were made for each clone used in this study, as previously described (Han et al., 1995).

Inoculum Preparation of *Campylobacter* spp.

Campylobacter spp. were cultured by using sheep blood agar plates (SBAP) containing 5% sheep blood (VWR International Inc., Radnor, PA, United States) incubated for 48–72 h at 37°C, which is considered as optimal growth temperature (Hsieh et al., 2018), in microaerophilic chambers (Mitsubishi AnaeroPack System, Fisher Scientific, Hanover Park, IL, United States). Single *Campylobacter* colonies were transferred by swab onto four fresh SBAPs and incubated for 48 h at 37°C in a microaerophilic chamber until a full bacterial lawn on each plate was achieved. Lawns from these plates were then harvested by flooding each plate with 20.0 mL of sterile water and then pooled and diluted until an optical density$_{640}$ = 0.14 was achieved. This initial suspension of *Campylobacter* was then diluted 10-fold with sterile water, which typically resulted in titers of about 1×10^7 CFU mL^{-1}. To make sure that animals would show some infection (colonization) under the lowest dose, two additional 10-fold serial dilutions were prepared in sterile water to give a total of three different inoculum doses of culturable *Campylobacter* used throughout the study (**Supplementary Table S1**). The volumes prepared for each inoculum dose were sufficient to provide 100 mL for each chick for 24 h use. Since *Campylobacter* spp. have been shown to be sensitive to light and temperature (Obiri-Danso et al., 2001) and can become non-culturable within 30 min of exposure to artificial light according to our test (data not shown), fresh *Campylobacter* suspensions were made for each experiment and protected from light in 15-mL conical tubes wrapped with aluminum foil and kept on ice (or refrigerated) until used.

Infection Through Natural Ingestion of *Campylobacter*-Containing Drinking Water

Fifty milliliters of freshly prepared *Campylobacter* suspensions, as described above, were dispensed into sterile isolator containers (Bio Serv, Flemington, NJ, United States) and placed into individual chick cages. An initial 50-mL inoculum solution was placed in the cages for 8 h and then replaced with a new 50-mL inoculum solution of the same suspension dilution for an additional 16 h before replacing with sterile drinking water. The viability of inoculum after 8 h in the cage only had slight decrease. The total volume of inoculum solution ingested by each chick was monitored and calculated for the entire 24-h exposure period. Total bacterial numbers ingested were calculated on the basis of the volume ingested and densities of *Campylobacter* suspension (**Supplementary Table S1**). Uninfected control chicks (n = 22) just received the same sterile water and were monitored in a manner similar to that for the infected group. Six hundred and thirty 1-day-old chicks were exposed to three different concentrations of laboratory, gull, and human clinical isolates of *Campylobacter* spp. and monitored over a 7-day period.

Assessing Bacterial Burden in Chick Feces by Using the Passive-Filtration Culture Method

Fresh fecal materials collected at two time points (2 and 7 days) were assayed by using a modified passive-filtration culture method (Steele and McDermott, 1984; Jokinen et al., 2012). Briefly, individual chicks were placed in a cage containing sterile paper bottoms and allowed to defecate. Within 10–15 min, most chicks had defecated onto the sterile paper, and samples were taken by swiping a sterile swab through the freshly deposited feces. Each fecal swab sample with an average of 390 mg was placed into a tube containing 3.0 mL of sterile water (held on ice) and agitated to dislodge and disperse the fecal matter. After agitation, 0.1 mL, which contained an average of 13 mg of the fecal specimens, was plated directly onto a SBAP plate (100 mm in diameter) with two 47-mm diameter polycarbonate filters of 0.6-μm pore size (GE Water & Process Technologies, Addison, IL, United States) placed side by side and on top. The filter pore and diameter size provided optimal results for culturing *Campylobacter* spp. for this study (data not shown). Samples were then spread gently and evenly across the two filters. Finally, the SBAPs were incubated at 37°C for 45 min (no microaerophilic chamber). After 45 min, the filters were removed from the surface by using a sterile forceps, and the SBAPs (without filters) were placed in a microaerophilic chamber and incubated at 37°C. *Campylobacter* colonies were enumerated after 48 h of incubation. On the basis of the processed 14–27 replicates with no-dilution, the Most Probable Number (MPN) calculation program (version 5) (Jarvis et al., 2010) was used to perform MPN analyses. A number of colonies were randomly tested and confirmed for the targeted strains using qPCR methods (**Supplementary Table S4**) mentioned in previous study (Lu et al., 2013).

Data Analysis

To check for normality of the data, the Shapiro–Wilk test was performed. As the data were not normally distributed, the non-parametric Kruskal–Wallis One Way Analysis of Variance on Ranks was used to determine if the differences in the

median values among treatment groups were greater than would be expected by chance. If a statistically significant difference ($p \leq 0.05$) was found, Dunn's test of multiple comparisons was conducted to isolate the group or groups that differed from the others. All statistical tests among different strains, different doses, were done via SigmaPlot 13.0.

RESULTS

Comparison Using Spread Plate vs. Passive-Filter Culture Assays of *Campylobacter* spp. Recoveries

We compared the traditional spread plate with the passive-filter technique for culturing of three laboratory strains (*C. jejuni, C. coli,* and *C. lari*), three clinical strains (C1–3), and four gull isolated strains of *Campylobacter* species (**Table 1**). The results revealed differences in total colony counts between the spread plate vs. passive-filter culture techniques. Total colony counts in cultures grown by using the spread plating were higher ($2 \times 10^6 - 1 \times 10^8$ CFU mL^{-1}) than those grown by using passive-filter plating ($2 \times 10^5 - 5 \times 10^6$ CFU mL^{-1}). The colony ratio detected between the two plating types ranged from 6 to 30 (mean = 16). In addition, the passive-filter technique proved to be less labor intensive and did not require additional sample dilution to avoid overcrowding of the plate (data not shown). On the basis of the added technical advantages and a built-in enrichment step provided by the passive-filter culture technique in this study, as well as on reports by Nielsen et al. (2013), all subsequent experiments described below used this bacterial culture procedure.

TABLE 1 | Comparisons of spread plating vs. passive filter platings to assess total viability of various laboratory, clinical, and environmental *Campylobacter* strains (CFU mL^{-1}).

Isolate	Titer from spread plating[a]	Titer from passive -filter plating[b]	Ratio of spread count vs. filter count cells[c]
Laboratory			
C. coli	3×10^7	1×10^6	30:1
C. jejuni	4×10^7	5×10^6	8:1
C. lari	1×10^7	1×10^6	10:1
Environmental			
64BB	3×10^7	2×10^6	15:1
58BB	8×10^6	6×10^5	14:1
70BB	3×10^7	5×10^6	6:1
63A	5×10^7	2×10^6	25:1
Clinical			
C1	2×10^6	2×10^5	10:1
C2	1×10^8	5×10^6	20:1
C3	1×10^7	4×10^5	25:1

[a] *Titers calculated from 0.1 mL of bacterial suspension dilutions plated directly onto BAP plates;* [b] *Titers calculated from 0.1 mL of the same bacterial suspension dilutions plated onto BAP-containing polycarbonate filters as described in section "Materials and Methods";* [c] *Ratio of culturable cells calculated from spread plates vs. passive-filter plates.*

Infection of Chicks With *Campylobacter* Laboratory Isolates

The ability of three laboratory isolates of *C. coli, C. jejuni,* and *C. lari* to successfully infect 1-day-old chicks was evaluated. As shown in **Figure 1**, chicks exposed to various concentrations of *Campylobacter* spp. (**Supplementary Table S1**) via a natural ingestion route, as described above, were successfully infected. By contrast, uninfected control animals remained negative for *Campylobacter*. The three different laboratory strains showed significantly different patterns of infection rates ($p < 0.05$, compared at 3-doses and at 2-day time points), which were detected up to 7 days post-infection (**Figure 1**). *C. jejuni* had the most consistent colonization rates ranging from 71.4 to 85.7% of animals that were positive at both time points evaluated and at all three doses. *C. lari* had the highest infection rates at all three doses at 2 days post-infection (87.5–93.3%) but had slightly lower infection rates than those of *C. jejuni* by 7 days post-infection (66.7–75.0 vs. 72.2–85.7%, respectively). By contrast, *C. coli* had the lowest infection rates among the three strains.

When fecal bacterial burden following infection was examined by using MPN, the chicks infected with *C. lari* had the highest detectable bacterial load followed by those infected by *C. jejuni* and *C. coli* for each dose at 2 days post-infection (**Figure 2**). Both *C. jejuni* and *C. lari* infections were more persistent than those of *C. coli*. We found that *C. jejuni* easily infected 1-day-old chicks even at the lowest inoculum dose and had the most consistent rate and persistence of infection among the laboratory strains. When inoculated with the same dose as *C. jejuni,* we found that *C. coli* infected fewer chicks at the same inoculum dosages (**Supplementary Table S1**). No overt disease symptoms were observed in the chicks infected by the three *Campylobacter* species. The natural ingestion exposure route developed in our study enabled successful infection of the young chicks with both the laboratory and clinical strains of *Campylobacter (C. jejuni, C. lari,* and *C. coli*) with minimal physiological distress.

Infection of Chicks With *Campylobacter* Clinical Isolates

One-day-old chicks were inoculated with three *C. jejuni* isolates collected from patients with campylobacteriosis to determine if human *Campylobacter* isolates are also capable of establishing infection in this chick model of infection. All three isolates successfully established infection and persisted to 7 days post-infection (**Figure 3**). Although C1 showed a consistent infection rate (81.8%) at both time points, C2 and C3 were more variable. C2 had a lower infection rate at 2 days post-infection (54.4%, $p \leq 0.019$) than at 7 days post-infection (72.7%), whereas C3 had the reverse trend (91.7 and 25.0%, respectively).

Infection of Environmental *Campylobacter* spp. Isolates

Four California gull isolates (58BB, 63A, 64BB, and 70BB), which were homologous to *C. lari* (≥94% homology), *C. jejuni* (≥97% homology), *C. lari* (≥97% homology), and *C. volucri* (≥97% homology), respectively, were capable of infecting chicks via this chick model. *C. volucri,* a new *Campylobacter* species isolated

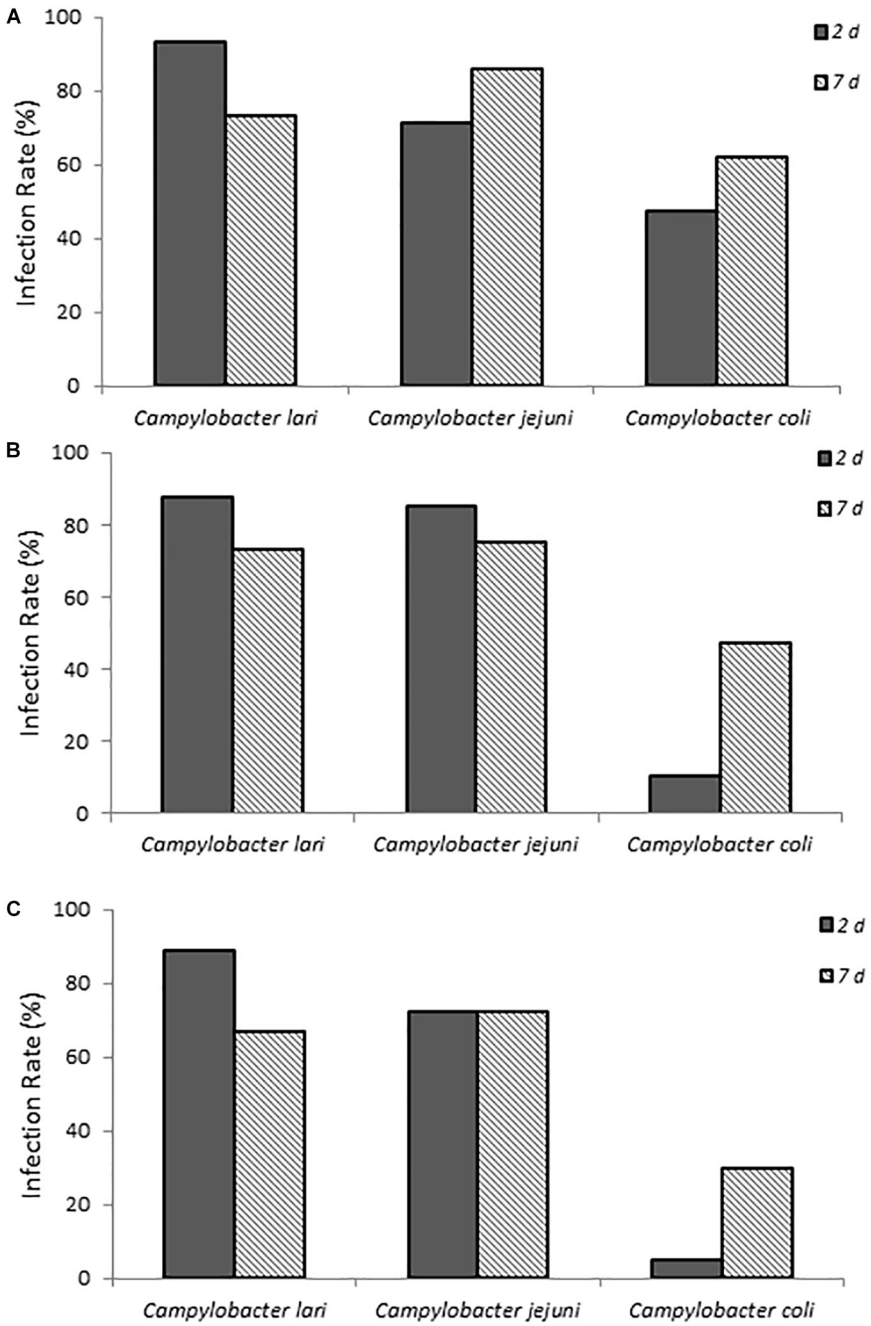

FIGURE 1 | Infection rates of laboratory isolates of *Campylobacter* spp. **(A–C)** show chicks infected with dose A, B, and C *Campylobacter* spp., respectively. Percent infected was determined by dividing the number of infected chicks by the total number of chicks inoculated with the various *Campylobacter* species. Chicks were considered infected if fecal samples collected at the various times were positive for *Campylobacter* spp. as determined by using the passive-filter plating described in section "Materials and Methods."

from black-headed gulls (*Larus ridibundus*) (Debruyne et al., 2010) is more closely related to *C. jejuni* than to *C. lari* according to phylogenetic analysis of 16S rRNA and hsp60 gene sequences (Debruyne et al., 2010).

The two *C. lari*-like isolates (58BB and 64BB) showed the highest infection rates at the two highest doses, with >80% of the animals infected at 2 days post-infection and the infection persisting to 7 days post-infection (**Figure 4**). Even at the lowest dose, 64BB continued this trend, with >80% of the animals infected on both days. At the lowest dose, 58BB was less infective and infected <32% of the animals on each day. By contrast, chicks inoculated with 70BB *C. volucri*-like or 63A *C. jejuni*-like isolates had consistently lower rates of infection than those of both *C. lari*-like isolates, with more variations in their infection

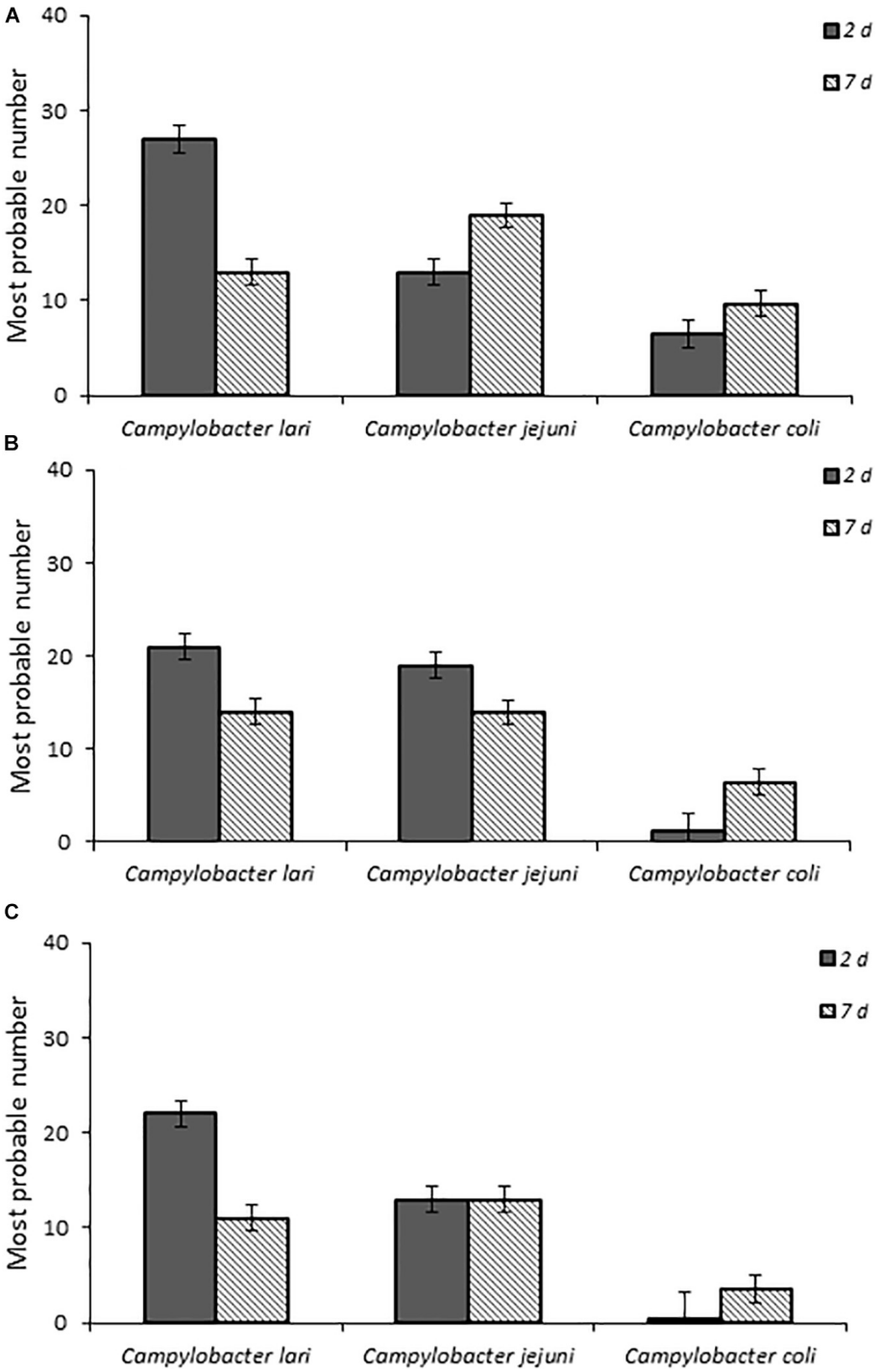

FIGURE 2 | Fecal bacterial burden in infected chicks with *Campylobacter* spp. laboratory isolates at doses **(A–C)**, respectively, as determined by the most probable number (MPN) calculations as described in section "Materials and Methods" and in **Supplementary Table S1**.

rates. The *C. volucri*-like isolate had a high infection rate of 72.7% on 2 days post-infection for dose B, which dropped to 13.6% by 7 days post-infection. Similarly, the *C. jejuni*-like isolate had a high infection rate of 70.0% at 2 days post-infection for dose A, which dropped to 15.0% at 7 days post-infection (**Figure 4**).

Chicks that received the highest inoculum dose of 64BB and 58BB also had the highest *Campylobacter* burden, as indicated by MPN, compared with those of 63A and 70BB at both time points tested (**Figure 5**). The overall *Campylobacter* burden observed from infection with 63A and 70BB, although similar to each other,

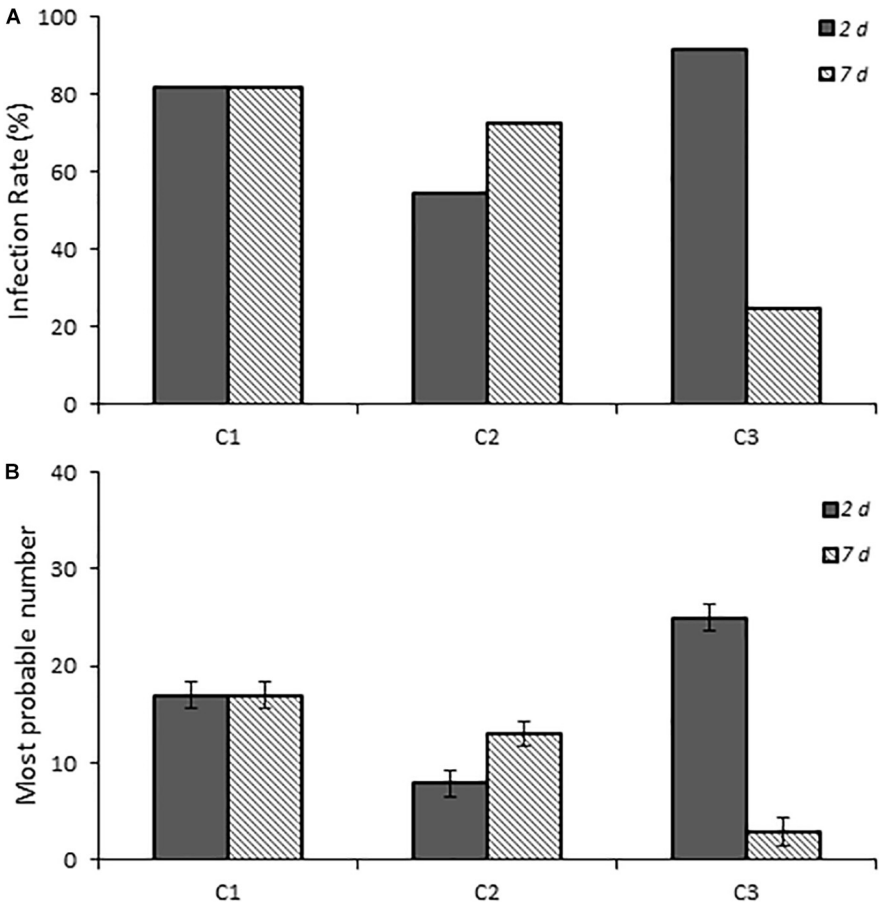

FIGURE 3 | Infection rates and fecal burden levels of *Campylobacter* from chicks infected with clinical isolates of *Campylobacter* spp. **(A)** shows chicks infected with dose B *Campylobacter* spp. Percent infected was determined by dividing the number of infected chicks by the total number of chicks inoculated with the various *Campylobacter* species. Chicks were considered infected if fecal samples collected at the various times were positive for *Campylobacter* spp. as determined by using the passive-filter plating as described in the section "Materials and Methods." **(B)** shows the fecal burden as measured by using the most probable number calculator.

was lower than the bacterial fecal burdens from animals infected with the 64BB and 58BB isolates ($p \leq 0.009$).

DISCUSSION

The *G. gallus* chick species has long been used as an important model for investigation of bacterial colonization factors, especially for *Campylobacter* infection, because of their preferential colonization of avian guts under optimal growth conditions (Wassenaar et al., 1993; Cawthraw et al., 1996; Hendrixson and DiRita, 2004; Stern, 2008). In the previous studies, bacterial strains, including laboratory-adapted *C. jejuni* isolates and *C. jejuni* isolates from chicken, patients, and waterborne outbreaks, were administered via oral gavage to chickens of various ages, tissue or cecal samples were collected, and samples were enumerated by plating of serial dilutions of samples. In those experiments, the cecum was found to be the main site of colonization, although organisms were also recovered throughout the gastrointestinal tract as well as the spleen and liver. Unlike the previous chick models of infection, the model in our studies was one we developed to be less invasive than others by infecting animals with *Campylobacter* through their drinking water, which mimics the natural route of infection, uses a sampling procedure that measures bacterial burden from freshly excreted fecal samples, and uses a passive filtration *in vitro* culture procedure as a more feasible model to assess *Campylobacter* infectivity. Especially, the developed model was further used to evaluate the infectivities of different *Campylobacter* species and *Campylobacter* isolates from wild fowl, which have not been documented previously.

This study also demonstrated successful infection and persistence (≤21 days post-infection) (data not shown) of *Campylobacter* spp. from three different groups (laboratory-maintained isolates, clinical isolates from human specimens, and environmental isolates from fresh fecal samples of California gull) in newly hatched chicks. This approach also provided the sensitivity to reveal variations of *Campylobacter* infectivity (e.g., virulence) among the different species, isolates, and environmental genotypes evaluated. For example, the laboratory

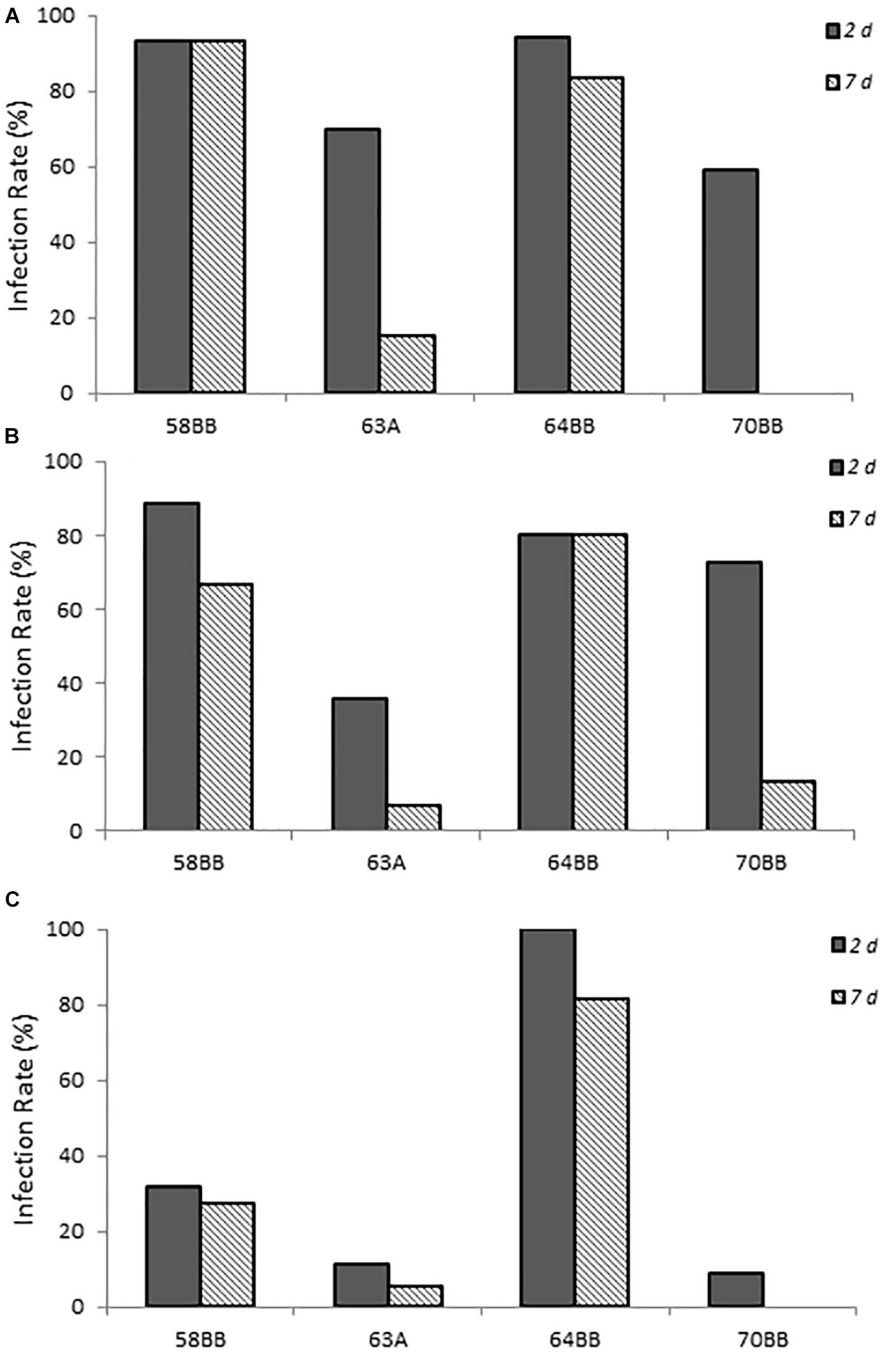

FIGURE 4 | Infection rates of environmental isolates of *Campylobacter* spp. **(A–C)** show chicks infected with *Campylobacter* spp. environmental isolates at doses A–C, respectively, as described in **Supplementary Table S1**. Percent infected was determined by dividing the number of infected chicks by the total number of chicks inoculated with the various *Campylobacter* species. Chicks were considered infected if fecal samples collected at the various times were positive for *Campylobacter* spp. as determined by using the passive-filter plating as described in section "Materials and Methods."

isolates (*C. lari* and *C. jujuni)*, which had been maintained under *in vitro* laboratory growth conditions for an unknown extended period of time, resulted in the highest infection rates and fecal bacterial burdens, whereas *C. coli* resulted in the lowest (**Figures 1**, **2**). More importantly, the results from the California gull isolates revealed that environmental isolates released from avian reservoirs can infect chicks. Among the gull isolates, 64BB (*C. lari*-like genotype) exhibited infectivity and persistence rates comparable to those of the *C. jejuni* clinical isolate. Isolate 58BB (*C. lari*-like genotype) exhibited infectivity and persistence rates comparable to those of the *C. lari* laboratory isolate (**Figures 4**, **5**). The observed differences in infectivity between

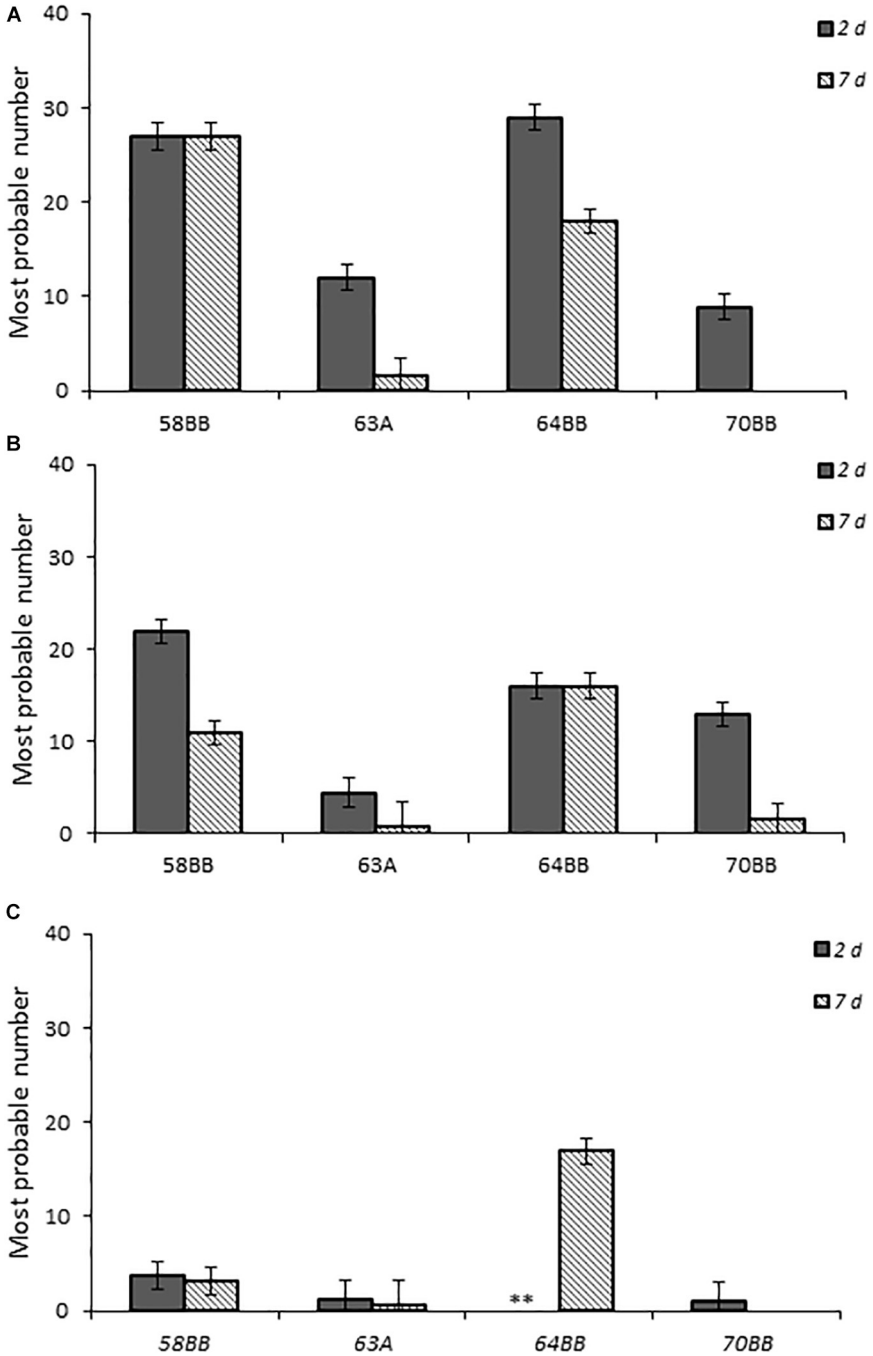

FIGURE 5 | Fecal bacterial burden in chicks infected with *Campylobacter jejuni*. Environmental isolates at doses **(A–C)**, respectively, as determined by the most probable number (MPN) calculations as described in section "Materials and Methods."

the environmental, clinical, and laboratory isolates could be attributed to differential expression of bacterial virulence factors and/or host immune responses during infection.

Furthermore, this model may help us to understand the bacterial burden and its release through defecation. Previously, it has been shown that 1-day-old chicks, orally challenged with a 10^4 CFU *C. jejuni* isolate or as few as 30 CFUs, which experienced a single passage of model chicks, achieved maximal cecal colonization within 3 days at levels of $\leq 1 \times 10^{10}$ CFU g^{-1} cecal contents (Cawthraw et al., 1996). In the other studies for challenge of 2-day-old chicks, *C. jejuni* has been observed at up to $\sim 10^8$ CFU g^{-1} of cecal contents (Wassenaar et al., 1993; Cawthraw et al., 1996). Once *Campylobacter* levels are established, they tend to remain at high levels throughout

the life of the chick (Stern, 2008). In this study, a high bacterial cecal burden was stable at 10^6 to 10^8 CFU g^{-1} of cecal material throughout the duration of this experiment. The majority (57%)of positive samples from all isolates contained $\geq 7.5 \times 10^4$ CFU g^{-1} of feces. Assuming an average release of 1.5 g of fecal material for each defecation, 29.94 g of total solids per hen-day, and voids 20 times a day (Huttly et al., 1998), each individual host is capable of releasing $>3.0 \times 10^5$ culturable bacteria into the environment with each defecation event or 6.0×10^6 culturable bacteria into the environment per day.

In conclusion, this new method provides not only the ability to monitor infection through time course but also to assay virulence and other pathogen factors with relative ease. Although the relationship between the colonization in the chick ceca and the mammalian gut is unknown, the chick model still provides a relevant and natural host for *Campylobacter* infection. This new approach can also lend itself to identifying novel *Campylobacter* virulence factors, understanding host immune responses following infection with *Campylobacter*, screening for potential therapeutic agents, and developing vaccines relevant to the poultry industry and human health (Nedrud, 1999; Nassar, 2018). Lastly, the levels and persistence of infectious bacteria released in feces within the chick gastrointestinal tract monitored over time are important parameters when assessing the importance of waterfowl in transmission and exposure to zoonotic *Campylobacter* spp. in beach sites used for human recreation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DL and JL designed and supervised all experiments, did some data analysis, performed some experiments, and prepared the manuscript. IS did qPCR and summarized qPCR data. TG did some data analysis. KO helped to initiate the experiments and made a critical review. EV coordinated the experiments, meeting, and data analysis, and helped some manuscript preparation.

FUNDING

The United States Environmental Protection Agency (USEPA), through its Office of Research and Development, funded and managed the research described here (SSWR3.02A).

ACKNOWLEDGMENTS

We would like to thank Sharon Detmer, Diana Miller, Katrina Pratt, and MPF Federal for Animal Husbandry and to Rich Haugland for technical review of the manuscript. The study has been subjected to the USEPA's administrative review and was approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation by the USEPA for use. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the views of the USEPA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02292/full#supplementary-material

REFERENCES

Babakhani, F. K., Bradley, G. A., and Joens, L. A. (1993). Newborn piglet model for campylobacteriosis. *Infect. Immun.* 61, 3466–3475.

Bingham-Ramos, L. K., and Hendrixson, D. R. (2008). Characterization of two putative cytochrome c peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. *Infect. Immun.* 76, 1105–1114. doi: 10.1128/iai.01430-07

Cawthraw, S., Wassenaar, T., Ayling, R., and Newell, D. (1996). Increased colonization potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol. Infect.* 117, 213–215. doi: 10.1017/s0950268800001333

Clavijo, V., and Flórez, M. J. V. (2017). The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. *Poult. Sci.* 97, 1006–1021. doi: 10.3382/ps/pex359

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S. L., and Vandamme, P. (2010). *Campylobacter volucris* sp. nov., isolated from black-headed gulls (*Larus ridibundus*). *Int. J. Syst. Evol. Microbiol.* 60, 1870–1875. doi: 10.1099/ijs.0.013748-0

Field, L. H., Underwood, J. L., Pope, L. M., and Berry, L. (1981). Intestinal colonization of neonatal animals by *Campylobacter fetus* subsp. *jejuni*. *Infect. Immun.* 33, 884–892.

Giallourou, N., Medlock, G. L., Bolick, D. T., Medeiros, P. H., Ledwaba, S. E., Kolling, G. L., et al. (2018). A novel mouse model of *Campylobacter jejuni* enteropathy and diarrhea. *PLoS Pathog.* 14:e1007083. doi: 10.1371/journal.ppat.1007083

Han, S., Flamm, R., Hachem, C., Kim, H., Clarridge, J., Evans, D., et al. (1995). Transport and storage of *Helicobacter pylori* from gastric mucosal biopsies and clinical isolates. *Eur. J. Clin. Microbiol. Infect. Dis.* 14, 349–352. doi: 10.1007/bf02116531

Hartley-Tassell, L., Day, C., Semchenko, E., Tram, G., Calderon-Gomez, L., Klipic, Z., et al. (2018). A peculiar case of *Campylobacter jejuni* attenuated aspartate chemosensory mutant, able to cause pathology and inflammation in avian and murine model animals. *Sci. Rep.* 8:12594. doi: 10.1038/s41598-018-30604-5

Hendrixson, D. R., and DiRita, V. J. (2004). Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. *Mol. Microbiol.* 52, 471–484. doi: 10.1111/j.1365-2958.2004.03988.x

Hodgson, A., McBride, B., Hudson, M., Hall, G., and Leach, S. (1998). Experimental *Campylobacter* infection and diarrhoea in immunodeficient mice. *J. Med. Microbiol.* 47, 799–809. doi: 10.1099/00222615-47-9-799

Hsieh, Y.-H., Simpson, S., Kerdahi, K., and Sulaiman, I. M. (2018). A comparative evaluation study of growth conditions for culturing the isolates of *Campylobacter* spp. *Curr. Microbiol.* 75, 71–78. doi: 10.1007/s00284-017-1351-6

Huttly, S. R., Lanata, C. F., Yeager, B. A., Fukumoto, M., Del Aguila, R., and Kendall, C. (1998). Feces, flies, and fetor: findings from a Peruvian shantytown. *Rev. Panam. Salud Pública* 4, 75–79. doi: 10.1590/s1020-49891998000800001

Jarvis, B., Wilrich, C., and Wilrich, P. T. (2010). Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. *J. Appl. Microbiol.* 109, 1660–1667. doi: 10.1111/j.1365-2672.2010.04792.x

Jokinen, C. C., Koot, J. M., Carrillo, C. D., Gannon, V. P., Jardine, C. M., Mutschall, S. K., et al. (2012). An enhanced technique combining pre-enrichment and passive filtration increases the isolation efficiency of *Campylobacter jejuni* and *Campylobacter coli* from water and animal fecal samples. *J. Microbiol. Methods* 91, 506–513. doi: 10.1016/j.mimet.2012.09.005

Kapperud, G., and Rosef, O. (1983). Avian wildlife reservoir of *Campylobacter* fetus subsp. *jejuni*, yersinia spp., and salmonella spp. in Norway. *Appl. Environ. Microbiol.* 45, 375–380.

Lévesque, B. T., Brousseau, P., Bernier, F., Dewailly, É, and Joly, J. (2000). Study of the bacterial content of ring-billed gull droppings in relation to recreational water quality. *Water Res.* 34, 1089–1096. doi: 10.1016/s0043-1354(99)00266-3

Lu, J., Ryu, H., Santo Domingo, J. W., Griffith, J. F., and Ashbolt, N. (2011). Molecular detection of *Campylobacter* spp. in California gull (*Larus californicus*) excreta. *Appl. Environ. Microbiol.* 77, 5034–5039. doi: 10.1128/AEM.00018-11

Lu, J., Ryu, H., Vogel, J., Santo Domingo, J., and Ashbolt, N. J. (2013). Molecular detection of *Campylobacter* and fecal indicators during the northern migration of sandhill cranes (*Grus canadensis*) at the central platte river. *Appl. Environ. Microbiol.* 79, 3762–37629.

Manes-Lazaro, R., Van Diemen, P., Pin, C., Mayer, M., Stevens, M., and Narbad, A. (2017). Administration of *Lactobacillus johnsonii* FI9785 to chickens affects colonisation by *Campylobacter jejuni* and the intestinal microbiota. *Br. Poult. Sci.* 58, 373–381. doi: 10.1080/00071668.2017.1307322

Müller, J., Schulze, F., Müller, W., and Hänel, I. (2006). PCR detection of virulence-associated genes in *Campylobacter jejuni* strains with differential ability to invade Caco-2 cells and to colonize the chick gut. *Vet. Microbiol.* 113, 123–129. doi: 10.1016/j.vetmic.2005.10.029

Nassar, F. S. (2018). Poultry as an experimental animal model in medical research and pharmaceutical industry. *Biomed. J.* 2:4.

Nedrud, J. G. (1999). Animal models for gastric *Helicobacter* immunology and vaccine studies. *Pathog. Dis.* 24, 243–250. doi: 10.1111/j.1574-695x.1999.tb01290.x

Newell, D. (2001). Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. *J. Appl. Microbiol.* 90, 57S–67S. doi: 10.1046/j.1365-2672.2001.01354.x

Nielsen, H. L., Ejlertsen, T., Engberg, J., and Nielsen, H. (2013). High incidence of *Campylobacter concisus* in gastroenteritis in North Jutland, Denmark: a population−based study. *Clin. Microbiol. Infect.* 19, 445–450. doi: 10.1111/j.1469-0691.2012.03852.x

Obiri-Danso, K., Paul, N., and Jones, K. (2001). The effects of UVB and temperature on the survival of natural populations and pure cultures of *Campylobacter jejuni*, *Camp. coli*, *Camp. lari* and urease−positive thermophilic *Campylobacters* (UPTC) in surface waters. *J. Appl. Microbiol.* 90, 256–267. doi: 10.1046/j.1365-2672.2001.01239.x

Pitkänen, T. (2013). Review of *Campylobacter* spp. in drinking and environmental waters. *J. Microbiol. Methods* 95, 39–47. doi: 10.1016/j.mimet.2013.06.008

Quessy, S., and Messier, S. (1992). Prevalence of *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. in ring-billed gulls (*Larus delawarensis*). *J. Wildlife Dis.* 28, 526–531. doi: 10.7589/0090-3558-28.4.526

Ramos, R., Cerdà-Cuéllar, M., Ramírez, F., Jover, L., and Ruiz, X. (2010). Influence of refuse sites on the prevalence of *Campylobacter* spp. and *Salmonella* serovars in seagulls. *Appl. Environ. Microbiol.* 76, 3052–3056. doi: 10.1128/AEM.02524-09

Ringoir, D. D., Szylo, D., and Korolik, V. (2007). Comparison of 2-day-old and 14-day-old chicken colonization models for *Campylobacter jejuni*. *FEMS Immunol. Med. Microbiol.* 49, 155–158. doi: 10.1111/j.1574-695x.2006.00181.x

Salaheen, S., Tabashsum, Z., Gaspard, S., Dattilio, A., Tran, T. H., and Biswas, D. (2018). Reduced *Campylobacter jejuni* colonization in poultry gut with bioactive phenolics. *Food Control* 84, 1–7. doi: 10.1016/j.foodcont.2017.07.021

Savill, M., Hudson, J., Ball, A., Klena, J., Scholes, P., Whyte, R., et al. (2001). Enumeration of *Campylobacter* in New Zealand recreational and drinking waters. *J. Appl. Microbiol.* 91, 38–46. doi: 10.1046/j.1365-2672.2001.01337.x

Stahl, M., and Vallance, B. A. (2015). Insights into *Campylobacter jejuni* colonization of the mammalian intestinal tract using a novel mouse model of infection. *Gut Microb.* 6, 143–148. doi: 10.1080/19490976.2015.1016691

Steele, T. W., and McDermott, S. (1984). The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. *Pathology* 16, 263–265. doi: 10.3109/00313028409068535

Stern, N. (2008). *Salmonella* species and *Campylobacter jejuni* cecal colonization model in broilers. *Poult. Sci.* 87, 2399–2403. doi: 10.3382/ps.2008-00140

Stoddard, R. A., Gulland, F. M., Atwill, E. R., Lawrence, J., Jang, S., and Conrad, P. A. (2005). *Salmonella* and *Campylobacter* spp. in northern elephant seals, California. *Emerg. Infect. Dis.* 11, 1967–1969.

Sweeney, T., Meredith, H., Vigors, S., McDonnell, M. J., Ryan, M., Thornton, K., et al. (2017). Extracts of laminarin and laminarin/fucoidan from the marine macroalgal species *Laminaria digitata* improved growth rate and intestinal structure in young chicks, but does not influence *Campylobacter jejuni* colonisation. *Anim. Feed Sci. Technol.* 232, 71–79. doi: 10.1016/j.anifeedsci.2017.08.001

Wassenaar, T. M., van der Zeijst, B. A., Ayling, R., and Newell, D. G. (1993). Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *Microbiology* 139, 1171–1175. doi: 10.1099/00221287-139-6-1171

Weis, A. M., Miller, W. A., Byrne, B. A., Chouicha, N., Boyce, W. M., and Townsend, A. K. (2014). Prevalence and pathogenic potential of *Campylobacter* isolates from free-living, human-commensal American crows. *Appl. Environ. Microbiol. Mar* 1, 1639–1644. doi: 10.1128/AEM.03393-13

Weis, A. M., Storey, D. B., Taff, C. C., Townsend, A. K., Huang, B. C., Kong, N. T., et al. (2016). Genomic comparison of *Campylobacter* spp. and their potential for zoonotic transmission between birds, primates, and livestock. *Appl. Environ. Microbiol.* 15, 7165–7175. doi: 10.1128/aem.01746-16

Conflict of Interest: IS was employed by Pegasus Technical Services, Inc.

The remaining 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 January 2020
doi: 10.3389/fmicb.2019.03062

The Preliminary Development of an *in vitro* Poultry Cecal Culture Model to Evaluate the Effects of Original XPC™ for the Reduction of *Campylobacter jejuni* and Its Potential Effects on the Microbiota

*Kristina M. Feye[1†], Peter M. Rubinelli[2†], William Evan Chaney[3], Hilary O. Pavlidis[3], Michael H. Kogut[1] and Steven C. Ricke[2]**

[1] Southern Plains Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, College Station, TX, United States, [2] Department of Food Science, Center for Food Safety, University of Arkansas, Fayetteville, AR, United States, [3] Diamond V, Cedar Rapids, IA, United States

OPEN ACCESS

Edited by:
Adolfo J. Martinez-Rodriguez, Institute of Food Science Research (CIAL), Spain

Reviewed by:
Komala Arsi, University of Arkansas, United States
Shaun Cawthraw, Animal and Plant Health Agency, United Kingdom

***Correspondence:**
Steven C. Ricke
sricke@uark.edu

[†] These authors have contributed equally to this work

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

Received: 13 September 2019
Accepted: 18 December 2019
Published: 23 January 2020

Citation:
Feye KM, Rubinelli PM, Chaney WE, Pavlidis HO, Kogut MH and Ricke SC (2020) The Preliminary Development of an in vitro Poultry Cecal Culture Model to Evaluate the Effects of Original XPC™ for the Reduction of Campylobacter jejuni and Its Potential Effects on the Microbiota. Front. Microbiol. 10:3062. doi: 10.3389/fmicb.2019.03062

Poultry is a major reservoir for the pathogen *Campylobacter jejuni*. *C. jejuni* inhabits the poultry gastrointestinal tract as a part of the gut microbiota. The objective of this study was to evaluate both the survival of *C. jejuni* and the changes in the population dynamics of the cecal microbiome during an *in vitro C. jejuni* inoculation in the presence or absence of the functional metabolites of Diamond V Original XPC™ (XPC). Two independent trials were conducted. Broiler chickens (n = 6 per Trial 1 and n = 3 per Trial 2) were raised according to standard industry guidelines and euthanized on Day 41. The ceca were collected aseptically, their contents removed independently and then used in an *in vitro* microaerobic model with 0.1% cecal contents + *Campylobacter* with or without 1% XPC (w/v). Before the inoculation with a chloramphenicol resistant marker strain of *C. jejuni*, the cecal contents were pre-incubated with XPC at 42°C for 24 h, in a shaking incubator (200 rpm) under microaerobic conditions, then experimentally inoculated with 10^8/ml of *C. jejuni* into the appropriate treatment groups. At 0 and 24 h for Trial 1, and 48 h for Trial 2, sub-samples of the culture (n = 3 ceca, two technical replicates per ceca, XPC alone or ceca culture alone) were enumerated using a Petroff–Hausser counter, and the DNA was extracted for microbiome analysis. DNA was isolated using the Qiagen QIAamp Fast Stool DNA Mini Kit and sequenced using the Illumina MiSeq platform. The reads were filtered, normalized, and assigned taxonomical identities using the QIIME2 pipeline. The relative microbiota populations were identified via ANCOM. Altogether, evidence suggests that XPC alters the microbiome, and in turn reduces *Campylobacter* survival.

Keywords: *Campylobacter*, microbiota, XPC, competitive exclusion hypothesis, yeast fermentate

INTRODUCTION

Arguably, very few foodborne bacterial pathogens exhibit as much metabolic and genomic plasticity and adaptability in the face of environmental stressors as *Campylobacter* spp. As a result, *Campylobacter* are difficult to control and may require multi-modal approaches to successfully reduce the pathogen in commercial poultry production globally. While other food animals are known carriers of *Campylobacter*, specific nuances of avian physiology enable *Campylobacter* colonization to be both successful and clinically undetectable in most cases (Sibanda et al., 2018). As a result, *Campylobacter* are ubiquitous on the farm, with prevalence upward of 100% in many instances (Beery et al., 1988; Stern, 2008; Meade et al., 2009; Sibanda et al., 2018). Human exposure occurs via the fecal oral route along the farm to fork continuum as gastrointestinal contents are often disrupted and expressed during evisceration, contaminating the carcass. Contaminated poultry can result in significant morbidity and mortality in humans worldwide (Kirk et al., 2015).

Unlike some foodborne diseases, such as *Salmonella*, *Campylobacter* infections are known to be associated with several autoimmune sequela, such as Guillain–Barré Syndrome and reactive arthritis (Janssen et al., 2008), with the latter resulting in life-threatening paralysis. Therefore, for many reasons, *Campylobacter* is a significant threat to human health and is an extraordinarily difficult challenge to mitigate in commercial poultry production.

As public scrutiny and government regulations increase, significant innovations in pre-harvest control measures are required to help reduce the risk of *Campylobacter* in commercial poultry production. Historically, the use of in-feed antibiotics has been used in food animal production, with limited effectiveness at reducing *Campylobacter*. While debates as to whether or not the agricultural sector contributes to the world-wide rise in antimicrobial resistance, any measurable increase in publicly described antibiotic resistant reservoirs is concerning. As public pressure increases, the production of natural, antibiotic-free products is increasing.

One approach to controlling *Campylobacter* pre-harvest is by influencing the gut microbiome with various probiotic, prebiotic, enzyme, and fermentate compounds to promote the competitive exclusion of *Campylobacter* and the fortification of the host immunobiology-microbiome axis (Baffoni et al., 2012; Guyard-Nicodème et al., 2016; Schneitz and Hakkinen, 2016). Specifically, yeast fermentates likely promote changes to the microbiome that are theorized to aid in the stability of the host-immunobiology axis (Possemiers et al., 2013; Han et al., 2017). Evidence also suggests that the microbiota dictates both the window of opportunity for *Campylobacter* colonization in poultry, while also aiding in the TH_1 immune response polarization to control *Campylobacter* colonization (Han et al., 2017). Previous research by our group and others demonstrated that the *Saccharomyces cerevisiae* fermentate Original XPC^{TM} (XPC; Diamond V, Cedar Rapids, IA, United States) inhibits *Salmonella* Typhimurium, both in an *in vitro* anaerobic mixed culture assay and *in vivo* (Feye et al., 2016; Rubinelli et al., 2016; Roto et al., 2017).

Based on the results from studies evaluating the anti-*Salmonella* effects of XPC, it is hypothesized that XPC might also be effective in the control of *Campylobacter*. Furthermore, it is hypothesized that the use of XPC beneficially modulates the microbiome and promotes populations of bacteria that are potentially antagonistic to *Campylobacter jejuni*. In doing so, the use of XPC may result in a multi-modal mechanism to reduce *Campylobacter* fitness in chicken ceca. As an initial step in testing this hypothesis, a study was conducted where XPC was applied in an *in vitro* cecal model inoculated with a marker strain of *C. jejuni*. Those results indicate that inclusion of XPC in this model system can significantly reduce *C. jejuni* survival and results in significant changes to the cecal microbial ecology.

MATERIALS AND METHODS

Ceca Acquisition

Cobb 500 broiler ceca were collected during standard poultry processing at a commercial plant by Diamond V for the study. Animals processed in that commercial plant were done in accordance with standard industry guidelines and ethical practices. Therefore, the University of Arkansas and the Center for Food Safety was outside of the prevue of IACUC as the animal work conducted throughout this study was in accordance with industry guidelines. These birds were not raised for the study, but instead were raised for consumption in accordance with standard industry guidelines and practices.

Bacterial Cultures

A *C. jejuni* marker strain was graciously donated by Dr. Young-Min Kwon, from the Department of Poultry Science at the University of Arkansas in Fayetteville. This strain of *Campylobacter* substitutes the chloramphenicol exporter gene for the non-essential gene hippicurate biosynthesis, Δ*hipO,* thus conferring chloramphenicol resistance. The ΔhipO strain, henceforth known as *C. jejuni*, was grown for 24 h in Mueller-Hinton broth (MHB; BD, Sparks, MD, United States) and cell density was quantified using a Petroff–Hausser Counter (Hausser Scientific, Horsham, PA, United States). This starter culture containing 10^8 cells/ml *C. jejuni* was subsequently added to 20 mL Bolton Enrichment Broth (Neogen, Lansing, MI, United States). The Bolton Enrichment Broth contained 20 μg/ml cefoperazone, 50 μg/mL cycloheximide, 20 μg/mL trimethoprim, 20 μg/mL vancomycin, and 5% defibrinated horse blood, which further selected and enriched for *Campylobacter* populations. Aliquots from the Bolton enrichment broth cultures were serially diluted with tryptone salt broth and spread-plated on Cefex agar (Neogen, Lansing, MI, United States) containing 33 μg/mL cefoperazone, 5% defibrinated horse blood, and 10 μg/ml chloramphenicol to empirically verify the inoculum concentration.

In vitro Campylobacter Survival Assay

Two independent trials were conducted throughout the course of this *in vitro* experiment using the ceca collected from

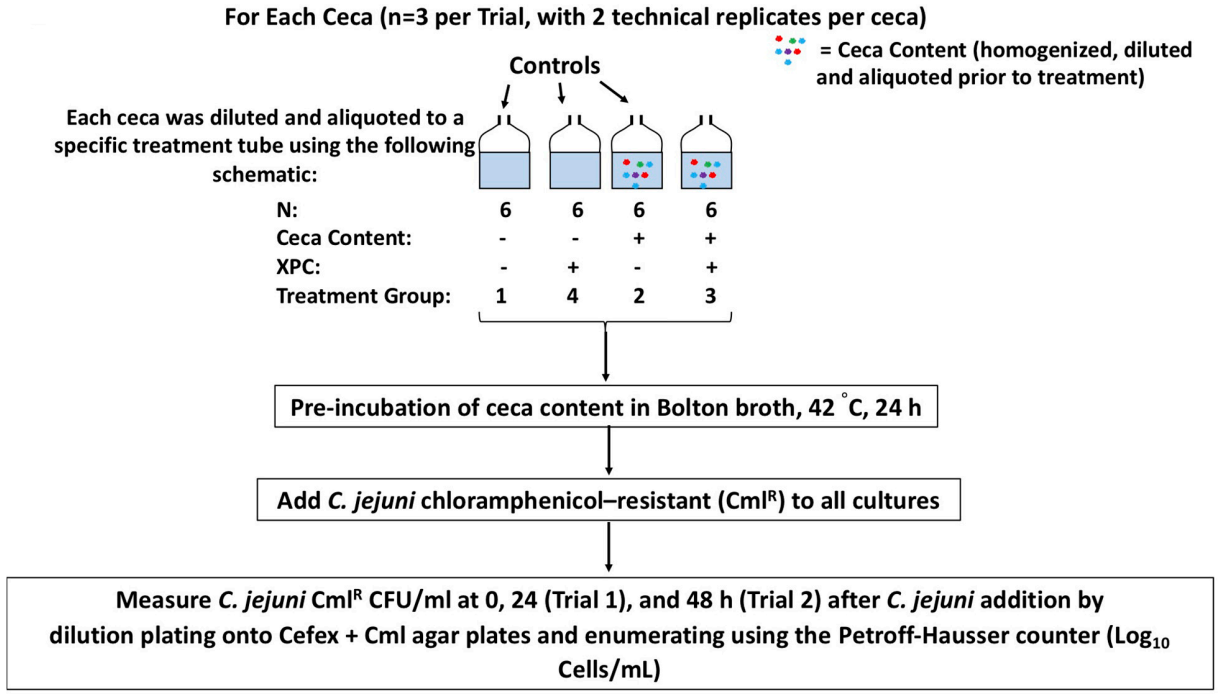

FIGURE 1 | A schematic of the experimental design. Experiment 1 was two independent trials (n = 3 ceca each trial, with two technical replicates per cecum), with sample collection at 0 and 24 h. Experiment 2 carried the study out through 48 h.

commercial broilers by Diamond V. At the processing facility 41-day-old Cobb 500 broiler male ceca were collected, chilled, and shipped overnight from Diamond V (Cedar Rapids, IA, United States). Both trials are delineated in **Figure 1**. Per treatment in Trial 1, three biological replicates of ceca from three different chickens were used, with two technical replicates executed per ceca and two independent trials conducted in total, resulting in a total of 12 replications per treatment. The aliquots collected at each time point were not sequenced in Trial 1 as specific changes to the microbiota became the focus during the latter half of the study. Per treatment in Trial 2, three ceca were used, with two technical replicates per ceca, with a total of six replicates per treatment. The ceca contents from Trial 2 were sequenced to evaluate the stability of the microbiome after exposure to XPC and *Campylobacter* using our ceca culture model.

As outlined in **Figure 1**, Trial 1, four treatment groups were tested: (1) *C. jejuni* alone; (2) *C. jejuni* + 0.1% cecal content; (3) *C. jejuni* + cecal + 1% XPC (w/v); (4) *C. jejuni* + 1% XPC (no cecal content). The controls of XPC alone, and *C. jejuni* and XPC alone were added to rule out the direct effects of XPC on *C. jejuni* survival.

In Trial 2, the same treatment groups were followed, except that the following groups were omitted: *C. jejuni* alone and *C. jejuni* + 1% XPC (no cecal content). The adjustment to Trial 2 was made because there were no detectible differences in pathogen load over time in the controls from previous studies (ceca alone, XPC alone). For Trial 2, 0, 24, and 48 h post-inoculation samples were collected.

Irrespective of the trial, the contribution to the ceca with each culture method was standardized, with cecal contents being weighed out to ensure homogeneity between the biological and technical replicates, and then added to 20 mL of Bolton Broth at a final concentration of 0.1% (w/v; ceca to serum bottle volume). Bolton's Broth was selected as it has been historically used in our laboratory for the long-term maintenance of viable, log-phase *Campylobacter*. Additionally, the antibiotics were included as the background microbiota made *Campylobacter* recovery difficult. Other media were evaluated; however, the successful recovery of *Campylobacter* was not consistent nor accurate using other methods. For each trial, the experimental units were pre-incubated in sterile serum vials with a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 42°C, 150 RPM for 24 h (Trial 1) and through 48 h (Trial 2). *C. jejuni* was added to each culture at a final concentration of 1×10^8 cells/mL after the 24 h pre-incubation time (Rubinelli et al., 2016). A 1 mL aliquot was removed from each culture at the start of the experiment and at each time point to count the *C. jejuni* per mL using the Petroff Hausser counter (Ziprin et al., 2003). Petroff-Hausser count data was reported for each trial as it closely paralleled microscopy confirmations of the cell morphology and structure and can be used to detect viable but non-cultivable *Campylobacter* (Ziprin et al., 2003). Because of the difficulty associated with accurately quantifying the *Campylobacter* in microaerobic stasis as identified by pilot studies, using the Petroff-Hausser counter ensured that we also did not report solely prevalence data for *Campylobacter*. As a result, data presented herein is strictly from cell counts.

Microbiome Sequencing

Throughout Trial 2, DNA was extracted from the aseptically collected aliquots of the microaerobic cultures taken at the indicated time points (0, 24, and 48 h) using the Qiagen Qiamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA purity was assessed, and then the DNA samples were diluted to 10 ng/mL. The paired-end sequencing libraries were prepared by targeting the hypervariable region 4 of the 16S ribosomal RNA with PCR primers containing the linker and adapter sequence. The libraries were assessed for qualitative and quantitative homogeneity, and then sequenced using the Illumina MiSeq platform as previously described (Kozich et al., 2013).

Microbiome Bioinformatic Analysis

Data sequences were uploaded onto the BaseSpace Website[1] (Illumina, San Diego, CA, United States), where sequence run quality and run completion were determined. De-multiplexed data was then downloaded locally and uploaded onto QIIME2-2018.8 via the Casava1.8 paired-end pipeline. All data analysis on QIIME2 were conducted using the q2cli interface. Data were visualized and then trimmed in DADA2 using the chimera consensus pipeline. Alpha and beta diversity were computed via the QIIME phylogeny align-to-tree-mafft-fasttree methodology, then analyzed for all available metrics of alpha and beta diversity via QIIME diversity core-metrics-phylogenetic, with a sampling depth of 14,000 reads for both diversity and alpha-rarefaction analysis. Taxonomic assignment of the operational taxonomical units was conducted using the QIIME feature-classifier classify-sklearn Bayesian methodology with the QIIME2-2018.8 SILVA database. Statistically significant differences in alpha and beta diversity were computed via PERMANOVA. Compositional differential abundance via ANCOM (Analysis of Composition) was conducted and unique phylogenetic features in the relative abundance tables were exported into Microsoft Excel (Microsoft, Redmond, Washington, United States), sorted for the top nine features that were statistically significant ($Q < 0.05$), and visualized.

Statistical Analysis

The cell counts from the Petroff–Hausser counter were recorded, $\log_{10}$ transformed, then inputted into SAS JMP 14.0 (SAS, Cary, NC, United States) where they were evaluated for the main effects of treatment (XPC or CON) and time and the interaction thereof as well as the random effects of experiment. The random effects of trial date were not considered statistically significant. Dunnett corrections for multiple comparisons were implemented *post hoc* to correct for errors in pair-wise comparisons, which were made across all treatment groups relative to the most basic reduced control as identified in the section "Results." Significance was defined at a $P \leq 0.05$. The statistical analyses for the microbiome were computed as a component of QIIME2 using standard pipelines. With the microbiome data, when considering alpha and beta diversity as well as the ANCOM analyses, a $P \leq 0.05$ was considered statistically significant for the overall effect, with $Q \leq 0.05$ being significant for individual contrasts in order to incorporate a stringent false discovery rate (ANCOM).

[1] www.basespace.illumina.com

RESULTS

Campylobacter Survival in Cultures in the Presence and Absence of XPC

The effects of XPC has on broilers reqiures the metabolization of the product by the microbiota for its anti-*Salmonella* effects (Rubinelli et al., 2016). *Campylobacter* is a significant foodborne pathogen; therefore, as the poultry industry is constantly trying to evaluate new tools to address this issue, it became important to see if XPC had *in vitro* anti-*Campylobacter* effects. In order to initially evaluate the potential anti-*Campylobacter* effects of XPC, determining whether or not the metabolites produced by XPC *in vitro* reduced *Campylobacter* were essential. Because working with *Campylobacter* is difficult, we modified our traditional *in vitro* model system piloted by Rubinelli et al. (2016) to function for *Campylobacter*. The anerobic model was ineffective; thus, modifications using established media in microaerobic conditions were used after numerous attempts. Antibiotics were included to enhance the recovery of *Campylobacter*. While artificial, it does enable researchers to evaluate trends.

Irrespective of the trial, 0 h pre-inoculation samples were collected and plated and evaluated with the Petroff–Hausser counter, with no growth observed. The first experiment set out to evaluate whether or not XPC impacted *Campylobacter* directly and whether or not it required the ceca to reduce *C. jejuni* and was repeated two times. The results of Experiment 1 are shown in **Figure 2A**. All groups were compared to the *C. jejuni* only group using the Dunnet's test. There was no significant reduction in *C. jejuni* recovered from groups containing XPC alone. However, when XPC was added to cecal contents, there was a 1.57 log reduction in recovered *C. jejuni* ($P < 0.0001$). That reduction was greater the cecal content alone, where the *C. jejuni* recovered from the cecal contents only demonstrated a 0.86 log reduction as compared to the *C. jejuni* group alone ($P < 0.001$). There was no difference between *C. jejuni* + XPC and *C. jejuni* alone throughout the trial, meaning cecal contents are required in order for XPC to produce anti-*C. jejuni* effects. This requirement coincides with observations made in previous studies with a similar cecal *in vitro* system and *Salmonella* (Rubinelli et al., 2016). Comparing the treatments with *C. jejuni* alone, a significant decrease in log-phase *C. jejuni* was observed in the 24 h mixed cultures with XPC compared to 24 h mixed cultures without XPC (**Figure 2A**).

Trial 2 was conducted to evaluate whether or not the reduction in *C. jejuni* was sustained over time, and if there were substantial shifts in the microbiome. The 48 h time point was added to the trial to determine if the XPC-mediated reduction demonstrated in the first trial changed over time. As characteristic of this specific *in vitro* model (data not shown), there is a bloom of *Campylobacter* from 0 to 24 h (**Figure 2B**). This is likely due to the abundance of nutrients available in Bolton's broth and the ceca. There was an effect of treatment (XPC vs. CON) and an effect of time ($P < 0.001$) and there was a trend toward the

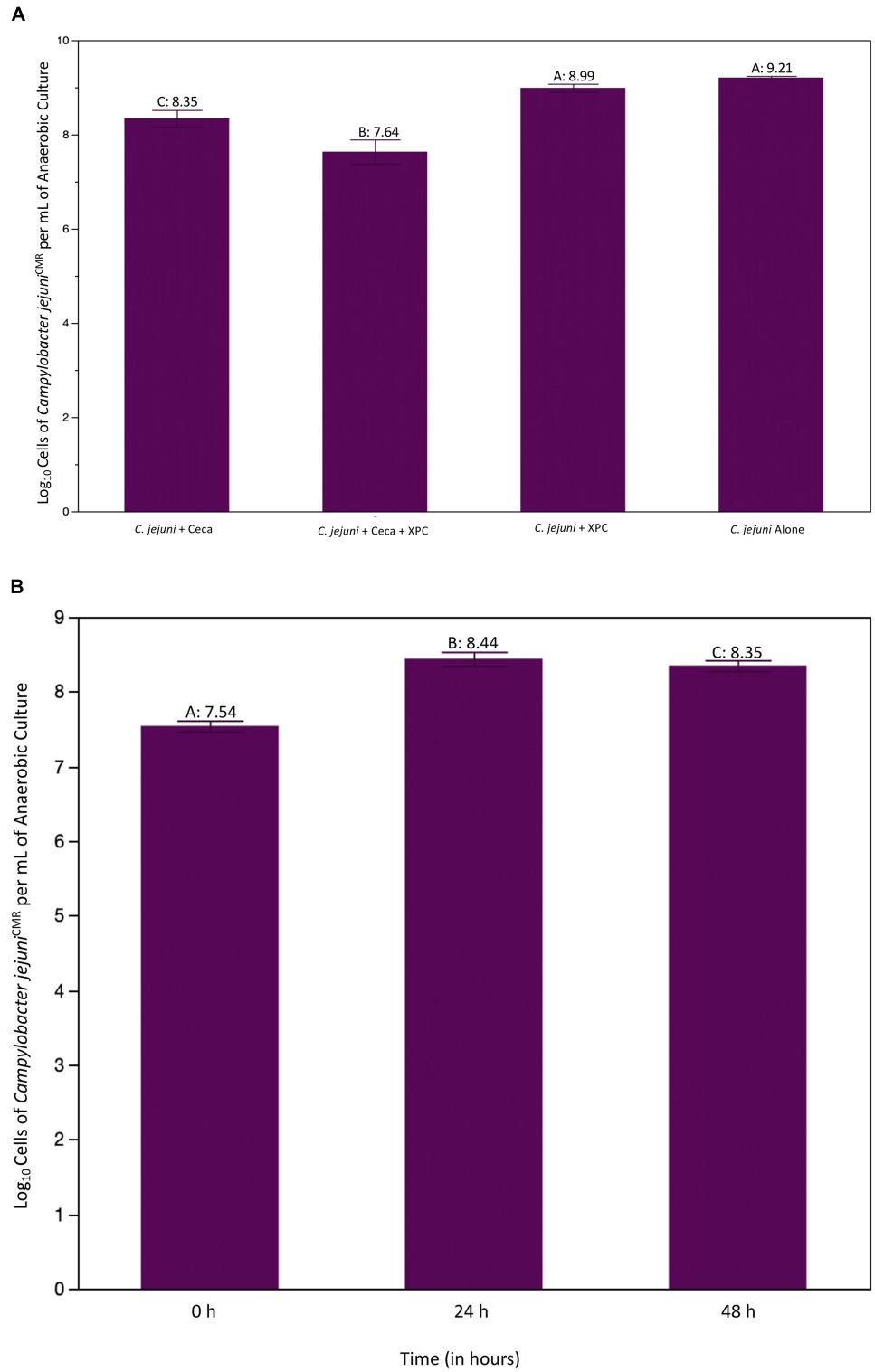

FIGURE 2 | (A) Recovered log cells of *Campylobacter jejuni* per mL of ceca culture at 24 h. Ceca were aseptically harvested from 41 day old Cobb 500 male broilers for Trial 1. All comparisons were made against *C. jejuni* in Bolton's broth only using Dunnet's Test for multiple comparisons. The bars represent the standard error of the sample mean. Each different letter indicates statistical significance and each number represents the mean Log_{10} CFU recovery of *Campylobacter* ($P < 0.05$). **(B)** Log cells of *C. jejuni* per mL of cecal culture recovered over time. *C. jejuni* were isolate at 0, 24, and 48 h and quantified using a Petroff–Hausser counter. The purple line is the CON group, the aqua line is the XPC treated group. There were significant effects of treatment and time as compared to the CON at 0 h group. There were no differences in XPC and CON at 0 h, therefore all differences are theorized to emerge due to treatment effects.

interaction of time and treatment being important ($P = 0.063$). Using Dunnet's test setting 0 h as the comparison, 24 h and 48 h were significantly different than 0 h ($P < 0.001$; **Figure 2B**). Additionally, the main effect of treatment was also significant ($P = 0.0124$) when comparing the CON group to the XPC group (**Figure 2**). The lack of numerical differences between 24 and 48 h in the XPC + *C. jejuni* group is likely driving the lack of significance in the interaction when compared to the *C. jejuni* + 0.1% ceca alone ($P = 0.063$).

Regarding the effect of treatment, there was a significant reduction in log cells/mL of *C. jejuni* recovered in XPC treatment groups vs. CON ($P = 0.0124$). When separating out the means using Dunnet's multiple comparisons setting CON as the contrast, there is a sustained reduction in *C. jejuni* in XPC as compared to the CON group. Therefore, the stability of *C. jejuni* populations in both groups are numerically different from one another, though lack statistical significance as the rate of change is not different from 24 to 48 h.

Microbiome Analysis: Alpha and Beta Diversity Analyses

Alpha and beta diversity analysis is an essential first step in understanding how the XPC impacts the microbiome, and, in turn, is mechanistically tied to the reduction of *Campylobacter*. Alpha diversity focuses on the richness of the sample, which is defined as unique operational taxonomical units [OTU(s)] per sample as well as how even that count is between samples or treatment groups. Whereas beta diversity focuses on the dissimilarity of OTUs between samples or treatment groups with or without phylogenetic alignments playing a factor.

All available indices for alpha diversity were conducted in QIIME2. The alpha diversity results are shown in **Table 1**. Pielou's Evenness and Shannon Diversity indices were statistically significant throughout the course of the study ($P < 0.05$). Pielou's evenness speaks to how consistent the unique OTUs were between samples, whereas Shannon's Diversity focuses on the number of unique samples per sample. Together, both indices provide a complete description of the alpha diversity in this study. Differences between time and treatment were observed with both metrics (Pileou Evenness; **Figure 3A**, Shannon Diversity Index; **Figure 3B** and **Table 1**). There was no significant difference in the alpha diversity using either indices at 0 h between XPC and CON. There were significant differences in diversity over time as compared to 0 h, with an increase in the *H*-score in control and CON independently over time. This indicated that time drove many of the differences observed, which parallels the *C. jejuni* microbiology data. When comparing treatments, there were no significant differences at the same time points. However, there were significant differences in alpha diversity as compared to 0 h for all treatment groups. Therefore, the number of unique OTUs and the evenness between samples is not significantly different over comparable time points by treatment. Any differences in the microbiota are due to microbial compositional diversity, not individual OTU count.

However, unlike the alpha diversity analyses, beta diversity began to exhibit significant differences between groups at specific time points. All of the available analytics were conducted when evaluating whether or not specific metrics for beta diversity were statistically important. Both Bray–Curtis (**Figure 3A**), and the weighted unifrac distance matrix (**Figure 3B**) indices, were statistically significant between treatments ($P < 0.05$; **Table 2**). The plots are visualized in **Figures 4A,B**, and the statistical analysis indicated profound differences in beta diversity.

Bray–Curtis (BC) Beta Diversity evaluates the quantitative dissimilarity of OTUs between samples. There was no difference in BC at 0 h when directly comparing XPC vs. the CON. There was also no difference between 0 h CON and XPC. However, there are statistically significant differences when comparing CON at 0 h vs. 24 and 48 h. Conversely, there is no difference in BC diversity between CON at 24 and 48 h, indicating that the fluctuations in beta diversity occurred within the control group after 24 h. When comparing CON vs. XPC at 24 h, there was a statistically significant change in beta diversity between the groups ($Q = 0.0075$). At 48 h, there were significant changes in beta diversity between XPC and the control group ($Q = 0.03$). When comparing beta diversity across time in the XPC treatment group, there were significant changes in diversity from 0 h and 24 and 48 h. There was no significant change in BC diversity between 24 and 48 h in the XPC treated groups.

Weighed Unifrac (WU) Beta Diversity Matrices quantitatively measures dissimilarity between samples and considers the phylogenetic relationships. There was no difference in WU diversity between CON and XPC at 0 h. Therefore, any differences occurring thereafter were due to time and treatment. Comparing the CON group across all time points, there were differences at 0 vs. 24 h, and 0 vs. 48 h. When comparing XPC and CON at the same time points, there were significant differences in WU diversity at 24 h but not 48 h. Finally, differences in WU diversity in XPC groups was significantly different at 0 vs. 24 h and 0 and 48 h.

In order to fully visualize whether or not the effect of treatment was due to the continuous variable of time, principle coordinate analysis (PCoA) plots were constructed to evaluate the contribution that time contributed to the data variability between treatment groups for both BC (**Figure 4A**) and WU (**Figure 4B**) analyses. The further down the axis the data point was, the more that axis contributed to the variability of the data. The greater the distance down the axis there was between groups, the greater the effect of time had on the microbiome. It is clearly demonstrated in both plots that when defining time as a contributing variable, significant differences in beta diversity demonstrated in **Table 2** were readily visualized. Therefore, there were significant divergences between different treatment groups, which is not only driven by treatment, but is also heavily influenced by time, specifically 0–24 h.

Microbiome Analysis: Taxonomy Visualization and ANCOM Analysis of Compositional Diversity Differences

After the alignment, taxonomical bar plots were created to give a complete visualization of the microbial diversity as supported by ANCOM analysis (species epithet; **Figure 5**). The declaration of

TABLE 1 | Changes in Shannon Diversity Index and Pilou's Evenness.

Kruskal–Wallis pairwise comparisions				Shannon Diversity Index			Evenness Index		
Group	***n***	**Group**	***n***	***H***	***P*-value**	***Q*-value**	***H***	***P*-value**	***Q*-value**
0 h CON	(*n* = 12)	0 h CON	(*n* = 12)	1.92	0.17	0.25	2.43	0.12	0.15
0 h CON	**(*n* = 12)**	**24 h CON**	**(*n* = 12)**	**5.88**	**0.02**	**0.04**	**8.0033**	**0.00**	**0.02**
0 h CON	(*n* = 12)	24 h XPC	(*n* = 12)	4.56	0.03	0.06	3.8533	0.05	0.07
0 h CON	**(*n* = 12)**	**48 h CON**	**(*n* = 12)**	**7.05**	**0.01**	**0.04**	**9.0133**	**0.00**	**0.01**
0 h CON	**(*n* = 12)**	**48 h CON**	**(*n* = 12)**	**5.33**	**0.02**	**0.04**	3.8533	0.05	0.07
0 h XPC	**(*n* = 12)**	**24 h CON**	**(*n* = 12)**	**7.68**	**0.01**	**0.04**	**10.083**	**0.00**	**0.01**
0 h XPC	**(*n* = 12)**	**24 h XPC**	**(*n* = 12)**	**6.16**	**0.01**	**0.04**	**6.1633**	**0.01**	**0.04**
0 h XPC	**(*n* = 12)**	**48 h CON**	**(*n* = 12)**	**8.67**	**0.00**	**0.04**	**10.083**	**0.00**	**0.01**
0 h XPC	**(*n* = 12)**	**48 h XPC**	**(*n* = 12)**	**6.45**	**0.01**	**0.04**	**5.88**	**0.02**	**0.04**
24 h CON	(*n* = 12)	24 h XPC	(*n* = 12)	0.96	0.33	0.44	3	0.08	0.11
24 h CON	(*n* = 12)	48 h CON	(*n* = 12)	0.56	0.45	0.52	0.6533	0.42	0.45
24 h CON	(*n* = 12)	48 h XPC	(*n* = 12)	0.48	0.49	0.52	2.2533	0.13	0.15
24 h XPC	(*n* = 12)	48 h CON	(*n* = 12)	2.25	0.13	0.22	4.32	0.04	0.07
24 h XPC	(*n* = 12)	48 h XPC	(*n* = 12)	0.05	0.82	0.82	0.2133	0.64	0.64
24 h XPC	(*n* = 12)	48 h XPC	(*n* = 12)	0.85	0.36	0.44	4.32	0.04	0.07
Evenness									
Group	**1**	**Group**	**2**						
0exp3Sham	(*n* = 12)	0exp3XPC	(*n* = 12)						
0exp3Sham	**(*n* = 12)**	**24exp3Sham**	**(*n* = 12)**						
0exp3Sham	(*n* = 12)	24exp3XPC	(*n* = 12)						
0exp3Sham	**(*n* = 12)**	**48exp3Sham**	**(*n* = 12)**						
0exp3Sham	(*n* = 12)	48exp3XPC	(*n* = 12)						
0exp3XPC	**(*n* = 12)**	**24exp3Sham**	**(*n* = 12)**						
0exp3XPC	**(*n* = 12)**	**24exp3XPC**	**(*n* = 12)**						
0exp3XPC	**(*n* = 12)**	**48exp3Sham**	**(*n* = 12)**						
0exp3XPC	**(*n* = 12)**	**48exp3XPC**	**(*n* = 12)**						
24exp3Sham	(*n* = 12)	24exp3XPC	(*n* = 12)						
24exp3Sham	(*n* = 12)	48exp3Sham	(*n* = 12)						
24exp3Sham	(*n* = 12)	48exp3XPC	(*n* = 12)						
24exp3XPC	(*n* = 12)	48exp3Sham	(*n* = 12)						
24exp3XPC	(*n* = 12)	48exp3XPC	(*n* = 12)						
48exp3Sham	(*n* = 12)	48exp3XPC	(*n* = 12)						

The significant pair-wise differences as determined by Kruskal–Wallis Pairwise Comparisons are bolded, with a Q < 0.05 being considered significant.

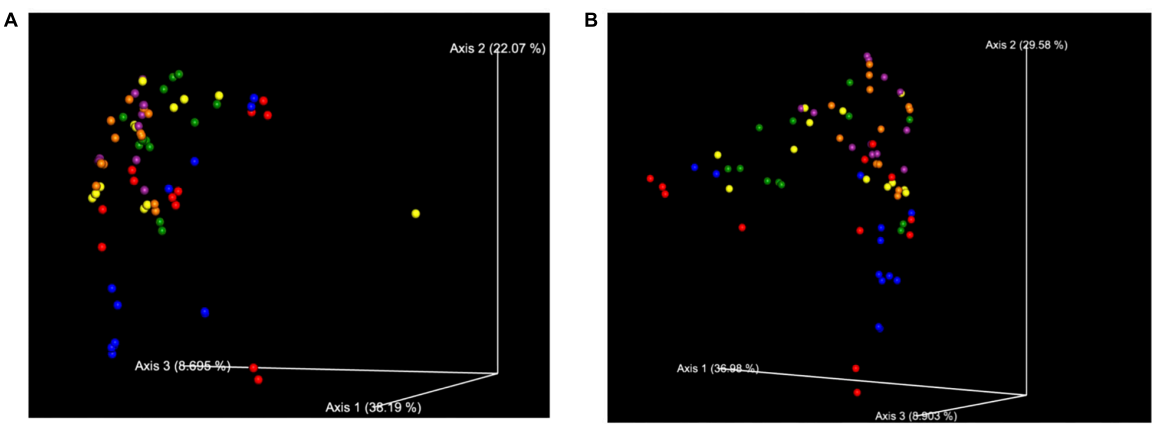

FIGURE 3 | (A) Bray Curtis Diversity Plot. The PCoA of the Bray–Curtis Diversity Plot. Key: Red: 0 h; CON: Blue; 0 h XPC: Orange: 24 h CON; Green: 24 h XPC; Purple: 48 h CON; Yellow 48 h XPC. **(B)** Weighted Unifrac Diversity Plot. The PCoA of the Weighted Unifrac Diversity Plot. Key: Red: 0 h; CON: Blue; 0 h XPC: Orange: 24 h CON; Green: 24 h XPC; Purple: 48 h CON; Yellow 48 h XPC.

TABLE 2 | Changes in Bray–Curtis and Weighted Unifrac Diversity Indices.

ANISOM comparisons				Bray–Curtis Diversity Index			Weighted Unifrac Diversity Index		
Group	**Group**	**Sample**	**Permutations**	**pseudo-*F***	***P*-value**	***Q*-value**	**pseudo-*F***	***P*-value**	***Q*-value**
0 h CON	0 h XPC	24	999	1.73	0.18	0.21	1.40	0.23	0.27
0 h CON	24 h CON	**24**	**999**	**6.31**	**0.00**	**0.01**	**6.68**	**0.00**	**0.00**
0 h CON	24 h XPC	24	999	1.75	0.15	0.19	1.97	0.14	0.18
0 h CON	48 h CON	**24**	**999**	**6.36**	**0.00**	**0.01**	**7.13**	**0.00**	**0.01**
0 h CON	48 h XPC	24	999	2.08	0.11	0.15	2.33	0.07	0.10
0 h XPC	24 h CON	**24**	**999**	**9.96**	**0.00**	**0.01**	**11.02**	**0.00**	**0.00**
0 h XPC	24 h XPC	**24**	**999**	**4.90**	**0.00**	**0.01**	**5.04**	**0.01**	**0.01**
0 h XPC	48 h CON	**24**	**999**	**10.55**	**0.00**	**0.01**	**12.29**	**0.00**	**0.00**
0 h XPC	48 h XPC	**24**	**999**	**3.79**	**0.01**	**0.01**	**4.22**	**0.00**	**0.00**
24 h CON	24 h XPC	**24**	**999**	**6.36**	**0.00**	**0.01**	**4.88**	**0.01**	**0.01**
24 h CON	48 h CON	24	999	0.32	0.85	0.85	0.42	0.69	0.69
24 h CON	48 h XPC	24	999	2.23	0.01	0.01	2.42	0.03	0.05
24 h XPC	48 h CON	**24**	**999**	**5.56**	**0.01**	**0.02**	**4.49**	**0.02**	**0.03**
24 h XPC	48 h XPC	24	999	1.02	0.35	0.38	0.68	0.56	0.60
48 h CON	48 h XPC	**24**	**999**	**2.10**	**0.02**	**0.03**	2.40	0.04	0.06

Weighted

Group	**1**	**Sample**	**Permutations**
0exp3Sham	0exp3XPC	24	999
0exp3Sham	24exp3Sham	24	999
0exp3Sham	24exp3XPC	24	999
0exp3Sham	48exp3Sham	24	999
0exp3Sham	48exp3XPC	24	999
0exp3XPC	24exp3Sham	24	999
0exp3XPC	24exp3XPC	24	999
0exp3XPC	48exp3Sham	24	999
0exp3XPC	48exp3XPC	24	999
24exp3Sham	24exp3XPC	24	999
24exp3Sham	48exp3Sham	24	999
24exp3Sham	48exp3XPC	24	999
24exp3XPC	48exp3Sham	24	999
24exp3XPC	48exp3XPC	24	999
48exp3Sham	48exp3XPC	24	999

The relationships that are significantly different from one another as determined by ANISOM are bolded. A Q < 0.05 is considered significant.

compositional differences cannot be accomplished accurately by comparing individual OTU counts between group, nor can trends in diversity as the qualitative approach lacks the incorporation of an accurate false discovery rate (**Figure 6**). As beta diversity analysis demonstrated compositional divergence between groups, ANCOM was conducted at all taxonomical levels (data not shown) to evaluate which microorganisms are unique. ANCOM was chosen due to its ability to increase statistical power with small datasets while effectively incorporating a stringent false discovery rate (Mandal et al., 2015). The use of a false discovery rate of appropriate vigor is important for multi-dimensional, multi-response data characteristic of a single sample analyzed via next-generation sequencing.

Stark differences between XPC and CON groups at 24 and 48 h were observed at L7 (*species* epithet; **Figure 6**). Data was sorted to visualize the top nine species, though numerous species were present and exported into Excel. Importantly, the OTUs mapped to *Campylobacter* (light blue) paralleled the microbiological data. Therefore, the XPC mediated reduction in *C. jejuni* is supported by Petroff-Hausser counting, microscopy, and sequencing. The next highest abundant group is *Lactobacillus* spp. (gray), which was significantly enriched in the XPC groups as compared to the control groups. The OTUs that mapped to *Lactobacillus* spp. were over 3.8-fold more abundant at 24 h than XPC treated groups vs. CON. The next group with the greatest significant difference between groups was *Methanobrevibacter* spp. There were about 1.4-fold more *Methanobrevibacter* spp. in XPC treated groups, than the CON group. *Lachnospiraceae* spp. (orange) are differentially abundant between groups, however, unlike previously abundant species, *Lachnospiraceae* was reduced 7.9-fold at 24 h in XPC treated groups, then increased eightfold at 48 h. *Lachnospiraceae* at 48 h is slightly higher than CON

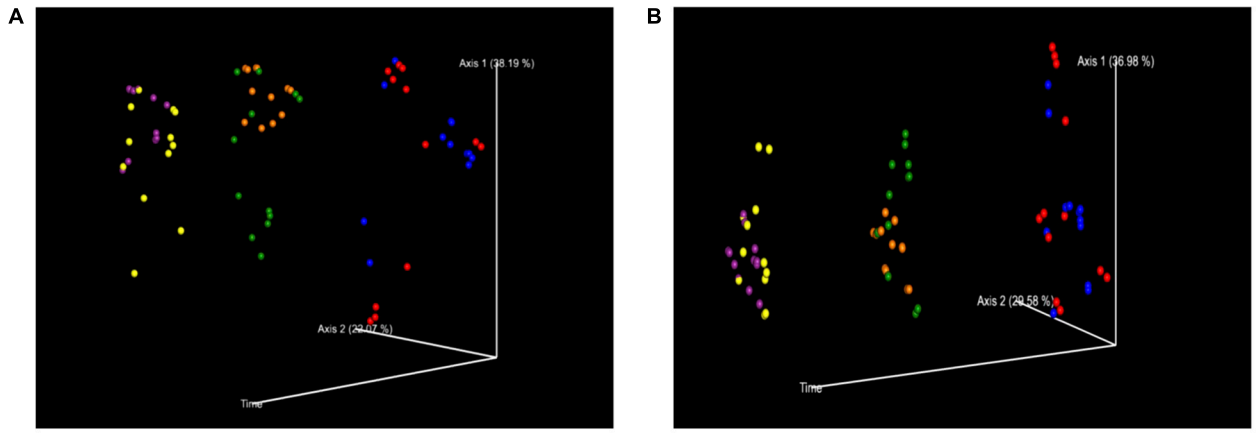

FIGURE 4 | (A) PCoA of Bray–Curtis Diversity Index separated out by time. Time was a significant factor in the change in diversification and therefore the treatment groups were plotted against the variable of time. Key: Red: 0 h; CON: Blue; 0 h XPC: Orange: 24 h CON; Green: 24 h XPC; Purple: 48 h CON; Yellow 48 h XPC. **(B)** PCoA of Weighted Unifrac Diversity Index Separated Out By Time. Time was a significant factor in the change in diversification and therefore the treatment groups were plotted against the variable of time. Key: Red: 0 h; CON: Blue; 0 h XPC: Orange: 24 h CON; Green: 24 h XPC; Purple: 48 h CON; Yellow 48 h XPC.

groups. Similarly, there is a contraction, followed by a bloom of an OTU identified as an uncultured Firmicutes termed *Phascolarctobacterium* spp. *Faecalbacterium* shares a similar trend, with a significant bloom in the population by 48 h. Two other groups that were enriched *Synergistes* and *Bacteroides* sp. Smarlab 3302398 shared similar trends. Finally, *Megamonas* spp., or formerly *Bacteriodes hypermegas*, showed a different trend, contracting significantly between CON and XPC groups at 24 h, then remaining contracted throughout the rest of the study. XPC groups had less *Megamonas* spp. present than other groups. Therefore, multiple species of bacteria are differentially expressed in XPC vs. the CON group, though their specific role remains to be elucidated.

DISCUSSION

Modulating the microbiome has the potential to control *Campylobacter* infections since the cecal microbiota and *Campylobacter* appear to be extensively interconnected (Indikova et al., 2015). This study was an *in vitro* approach for evaluating whether XPC could modulate the microbiome and if those effects could reduce *C. jejuni* independent of the immune system. Results suggest that XPC-mediated reductions of *Campylobacter* are cecal dependent, which is likely driven by changes in GIT microbial ecology, which is consistent with previously published reports for *Salmonella* (Rubinelli et al., 2016; Park et al., 2017). When the experiment was repeated and extended through 48 h, there was no increase in *C. jejuni* load and the effect of XPC was sustained as compared to the CON group. Incorporating the Petroff-Hausser counter was important in this study as it detected viable-but-not-culturable *C. jejuni*, which can result in foodborne disease and are not detected using traditional microbiological methods (Ziprin et al., 2003). However, direct evidence associated with the infectivity of viable-but-not-culturable bacteria as infectious agents is not strong (Ziprin et al., 2003).

Evidence that the microbiome can be harnessed to reduce *C. jejuni* fitness in the poultry GIT is twofold: first, *C. jejuni* colonization is influenced by total microbiome maturation and diversity; second, there are populations of bacteria that are directly antagonistic to *C. jejuni* (Awad et al., 2016; Connerton et al., 2018). In order for a feed additive to be successful, we theorize that it would likely have to exhibit a multi-dimensional influence on the microbial ecology of the gut to be sustainable enough to reduce *Campylobacter* colonization of poultry. The microbiome data independent of the immune system indicates that XPC seems to produce specific changes to the microbiome of the poultry ceca as demonstrated in this *in vitro* model. Importantly, there is not just one prokaryote population that seems to be driving these effects. In fact, ANCOM analysis indicates that the XPC-mediated changes to the microbiome are dynamic and potentially specific to XPC. For a pathogen such as *Campylobacter* that exhibits genomic and metabolic plasticity, total compositional changes to the microbiome may be essential in mitigating the threat of the pathogen. This could be why more one-dimensional approaches, such as probiotics that are transient, are not completely effective. For example, when Thibodeau et al. (2015) fed a selenium-yeast probiotic product traditionally tied to improved immunobiology and feed efficiency, the researchers did not detect microbiota, *Campylobacter* or general bird health responses in experimentally inoculated birds. *Lactobacillus* and other probiotics exhibit similar ranges in efficacy, which may be due, in part, to their ability to modulate the microbiome (Broom and Kogut, 2018).

However, additional evidence suggests that XPC is more beneficial to the bird, with microbiome-activating effects. Guyard-Nicodème et al. (2016) tested a number of commercial products, including XPC, as feed additives administered throughout the grow-out period to control experimentally infected *Campylobacter*. They found XPC significantly reduced a late *C. jejuni* challenge, but not early, in the grow-out period. Considering the *in vitro* data and other evidence indicate the

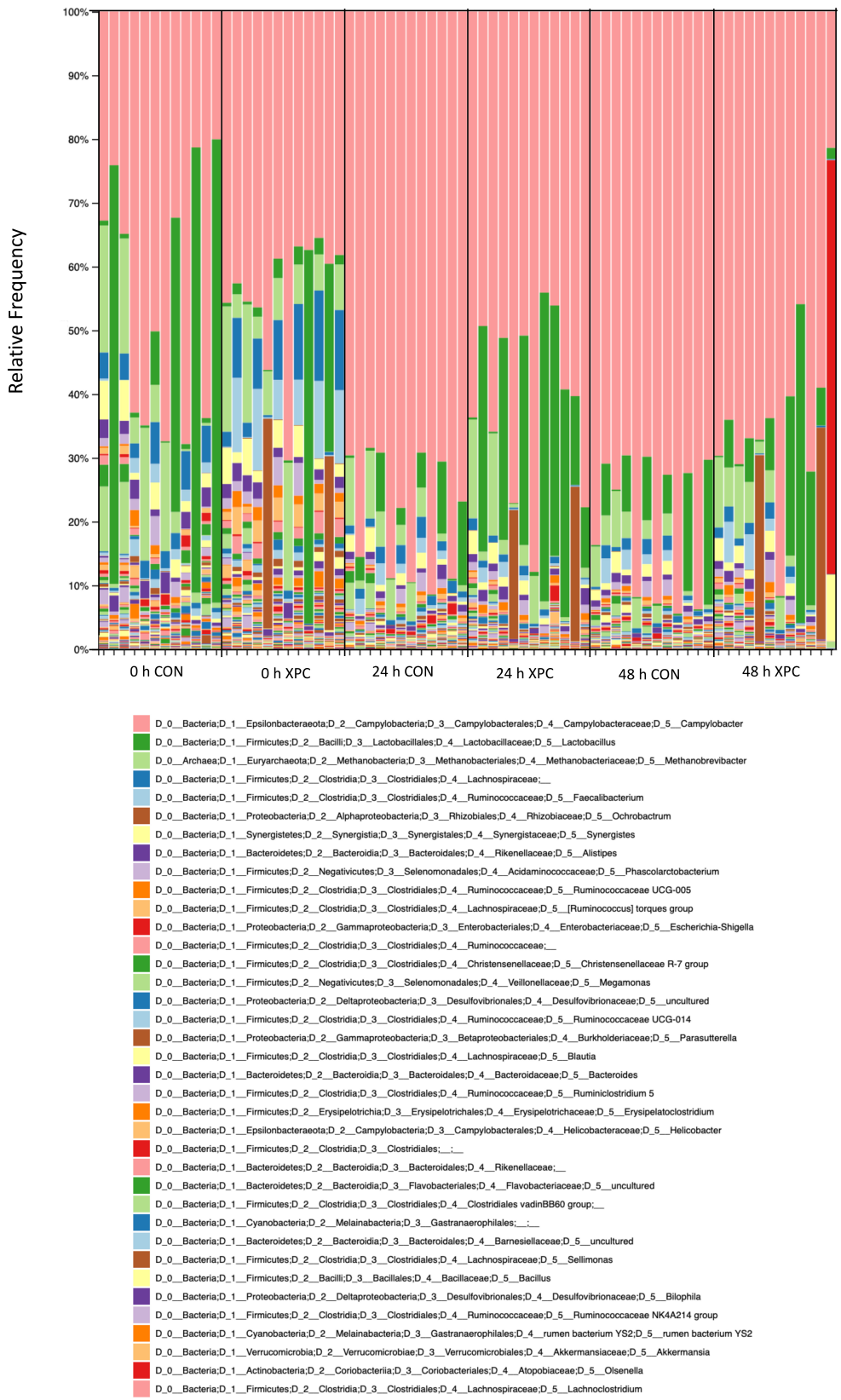

FIGURE 5 | Taxonomical bar graphs. Taxonomy bar plots are visualized through the species epithet and the colors are presented from the top of the graph (coral, *Campylobacter*) to the bottom of the graph (coral, *Lachnoclostridium*).

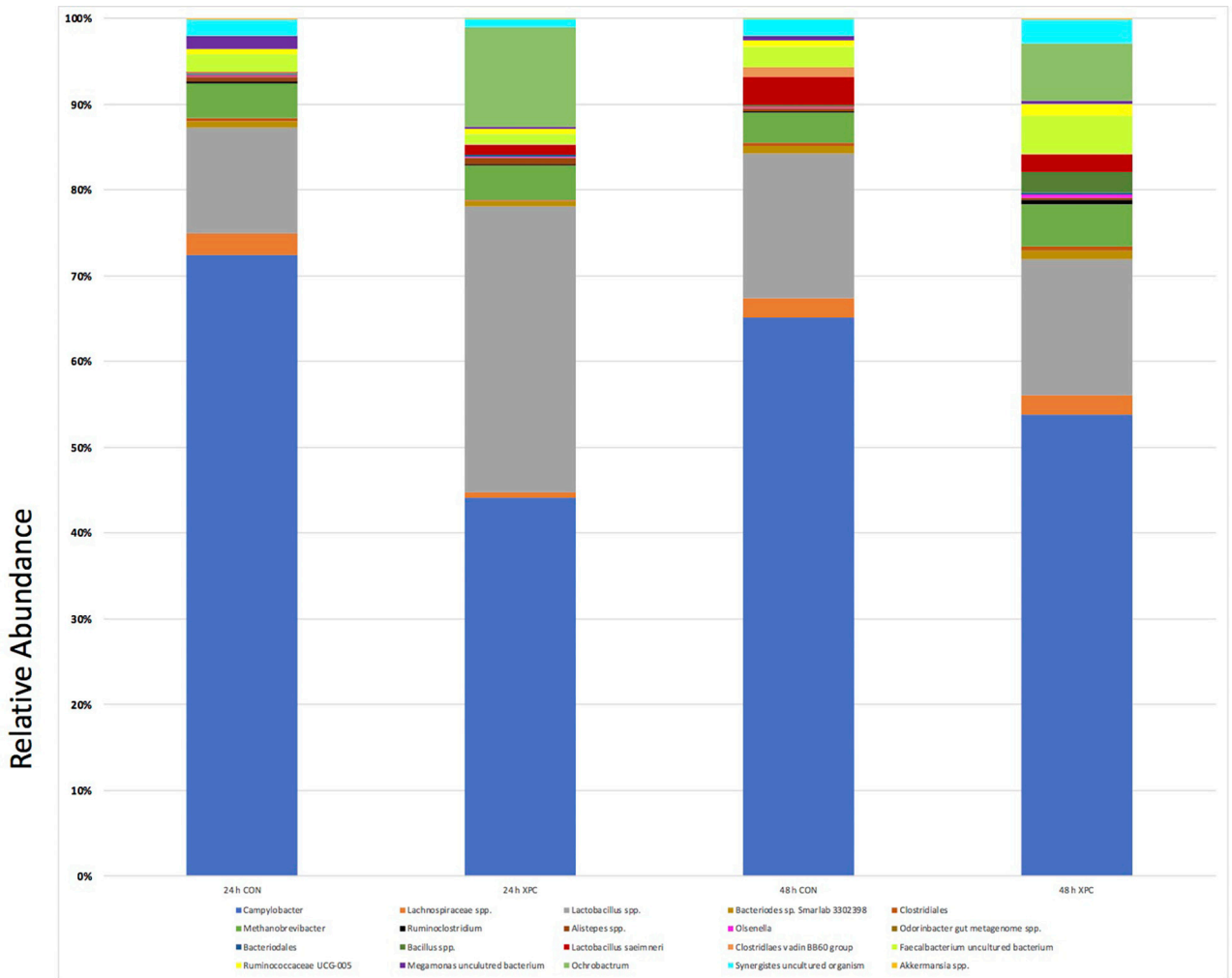

FIGURE 6 | ANCOM Analysis of Treatment Groups 24 and 48 h. The most prevalent groups that were identified by ANCOM as statistically significant are presented. Differences between the groups were identified as significant by ANCOM ($Q < 0.05$).

importance of a window of opportunity driven by the maturity index of the microbiome, it may be interesting to see if XPC changes the rate of diversification, maturity, and stability of the microbiome. Altogether, such data could drive the effects demonstrated by Guyard-Nicodème et al. (2016).

The identification of specific bacterial species, or cornucopia of bacterial species, as biomarkers associated with *Campylobacter* infection and resistance has been studied. Evidence suggests that microbiota natively and significantly enriched with *Bacteriodes* and *Escherichia* increased the likelihood of native infections of *Campylobacter* in chicken abattoir workers (Dicksved et al., 2014). However, a microbiota of human subjects prior to infection enriched with *Lachnospiraceae*, *Clostridiales*, and *Anaerovorax* conferred a general resistance to *Campylobacter* and resulted in less volatility over time (Dicksved et al., 2014). *Lachnospiraceae* and *Clostridiales* were the most differentially expressed species between treatments and across time throughout this study. More studies are needed to validate this phenotypically and if the microbiome identified in humans that confers robustness to *Campylobacter* assault is paralleled in the broiler chicken.

Poultry associated lactic acid bacteria have been previously demonstrated to inhibit the growth and repress virulence properties of *Campylobacter,* which is why *Lactobacillus* probiotics are popular in the poultry industry (Stern et al., 2005; Santini et al., 2010). Moreover, several studies have identified bacteriocins as the mechanism behind this effect. Bacteriocins like nisin and SMXD51 and NRRL B-30514, of *Lactobacillus salivarius*, kill *C. jejuni* and *Campylobacter coli* as well as several other bacterial species (Stern et al., 2005; Messaoudi et al., 2012). This same strain of *L. salivarius* also reduced a *C. jejuni* challenge in broilers when repeatedly administered orally (Stern et al., 2005; Saint-Cyr et al., 2017). Another potentially antagonistic species was *Megamonas* spp. Some evidence suggests that *Megamonas* may be antagonistic to *Campylobacter* persistence in the poultry GIT (Scumpham et al., 2010).

Faecalbacterium is a significant anaerobic butyrate metabolizer and signifies a healthy gut axis. *Faecalbacterium,* along with *Lactobacillus,* is positively associated with high performing broiler chickens as well as beneficially modulating the innate immune system (Yan et al., 2017). *Faecalbacterium* is also being investigated as a probiotic as increasing the populations of *Faecalbacterium* improve immune function, overall physiological functioning, and help fortify the microbiome against inflammatory assault (Sokol et al., 2008; Yan et al., 2017). *Faecalbacterium* is also associated with high microbial diversity (Sokol et al., 2008). It is theorized that *Faecalbacterium* increases the microbiome robustness to disbyosis by inducing IL-10 and other anti-inflammatory cytokines, as well as increasing butyrate production Sokol et al., 2018). As XPC in feed results in increased butyrate and other short chain fatty production, it is interesting that many of the differentially abundant bacteria are also high butyrate producers.

Data also suggests that volatility of that microbiota over time may predispose certain populations to Campylobacteriosis more so than the enrichment or loss of certain microbial populations (Dicksved et al., 2014). Those differences in alpha and beta diversity are likely consistent in susceptible populations. In poultry, *C. jejuni* thrives when the microbiome is its most volatile, which is prior to immunobiological and microbiome maturity (Connerton et al., 2018). Therefore, increasing the rate of maturation and the stability of the microbiome with in-feed additives may be a beneficial avenue for producers. The stabilization of the microbiome may be indicated as a potential mechanism for XPC efficacy via the differential abundance of *Methanobrevibacter* spp., which is a methanogen producing archaeon associated with the digestion of complex carbohydrates by being a major consumer of bacterial fermentation byproducts such as hydrogen and carbon dioxide (Armougom et al., 2009). Methanogens can be detected in the fecal material relatively early during a broiler life cycle and *Methanobrevibacter woesii* has been identified as the predominant methanogen in adult layer hen ceca (Saengkerdsub et al., 2007a,b). Additionally, *Methanobrevibacter* spp. and other methanogens are important in a gut rich in anaerobic fermentation as the accumulation of H_2 can be deleterious to the host (Gill et al., 2006). When microbial populations are healthy, anaerobic, and actively producing short chain fatty acids, *Methanobrevibacter* are usually enriched (Gill et al., 2006). This is consistent with detectable methane production occurring in poultry cecal *in vitro* anaerobic cultures grown on either a high fiber diet or a grain-based layer ration (Saengkerdsub et al., 2006).

Another important organism, *Phascolarctobacterium* spp., which produces acetate and propionate is also present and differentially abundant in this study, and are also considered indicators of an efficient metabolic state (Wu et al., 2017). *Synergistetes* are obligate anaerobic bacterial species that are amino acid reducers, although not much is known about the species beyond anecdotal knowledge (Jumas-Bilak et al., 2009). However, it is a known species associated with a "normal" microbiome. Therefore, an increase in anaerobic populations associated with a stable, energetically balanced microbiome, while also producing a microbiota that is competitively exclusive and antagonistic to *Campylobacter*, may be a mechanism for XPC anti-*Campylobacter* efficacy.

Significant caveats exist with the proposed *in vitro* model. First, short of oscillation, we do not simulate peristalsis or continuous culture environments. This likely reduces the mechanical stress on the microbiota as well as limits nutrient availability over time and greatly limits the potential applications of this model. We overcome the continuous culture limitation by only allowing the culture to go out to 48 h. Additionally, the effect of time was not likely driving the treatment effects as presented by our data. However, the authors agree that additional validations will be required, specifically the comparison of this model to a live bird study, to evaluate the specific caveats associated with time, nutrient availability, and antibiotic components of the media. Second, the difficulties in recovering *Campylobacter* were overcome by using the standard Bolton's Broth preparations. This absolutely can change the potential treatment-based fluctuations of the microbial ecology of the *in vitro* model and bias the data. Antibiotic resistance is favored with certain populations. Therefore, it is reasonable to believe that there may be important populations that may either benefit or antagonize *Campylobacter* that are not present in this study. Therefore, a full validation of this model will be required moving forward that tunes the trade-off between *Campylobacter* recovery and background microbiota. However, what is interesting about this study is that changes to the microbiota take place in the presence of XPC and ultimately XPC treatment groups carry fewer *Campylobacter*. This is mirrored in the cell counts as well as in the sequencing data. While caveated, with the controls in place, this study provides, preliminary understanding of the effects of XPC.

While single approaches, like a probiotic, have important effects, multiple antagonistic species were identified, potentially indicating that XPC effects may be multi-modal. This study provides preliminary evidence to suggest that XPC may ultimately change the ecology of the gut and be exclusive to not only *Salmonella*, but *Campylobacter* as well. Overall, it appears that there is an overall environmental shift from CON to XPC groups that are sustained over time that promote anaerobic production and oxidation of fatty acids and complex carbohydrates by Archeae and prokaryotes. Even if the population of bacteria or Archeae are reduced initially, they rebound by 48 h, which may indicate that XPC continues to beneficially modulate the microbiome over time. Additionally, bacteria that actively resist *Campylobacter*, such as *Lactobacillus* spp., were also identified in this study. Therefore, the total microbial shift in the environment may contribute to the reduction of *Campylobacter*. Further analyses evaluating metabolomics changes to the microbiome must also be conducted to determine if the phenotypic changes of the gut match the species identified through 16S microbiome sequencing.

CONCLUSION

A previous report estimated that reducing *Campylobacter* in the chicken cecum by as little as three logs could reduce

the risk of human disease by as much as 90–100% (Romero-Barrios et al., 2013). While the reductions in *Campylobacter* are not that biologically significant in this current study, it is promising. By understanding how *Campylobacter* persists, and the microbiota ecology associated with resistance and infection, new management strategies may be developed. Therefore, a necessary next step is an experimental infection of *Campylobacter jejuni* in broiler chickens to monitor birds for anti-*Campylobacter* effects. Necessarily, evidence presented in this paper highly suggests such data should include temporal studies in microbial diversity, maturation, and volatility. Overall, this study also suggests that XPC requires the microbiota for anti-pathogenic effects, which results in profound changes to the microbiome that both competitively exclude *Campylobacter* and may be directly antagonistic to the pathogen. By increasing butyrate producing bacteria, phenotypic data evaluating XPC-mediated metabolite changes in the microbiota from previous studies is further supported (Possemiers et al., 2013; Rubinelli et al., 2016).

Increases in butyrate producing bacteria are both anti-pathogenic and immune-balancing. Data presented herein supports other publications that evaluate the effects of XPC in poultry or the microbiome, where butyrate production was noted (Possemiers et al., 2013; Feye et al., 2016; Rubinelli et al., 2016). By fortifying the immunobiological-microbiome axis through the production of metabolites like butyrate, overall changes to host pathology are possible. Therefore, the underlying mechanism that drives the overall effects of XPC may rest at that nexus of XPC, the microbiome, and the immune system. Future studies need to elucidate that nexus, which could profoundly enhance the utility of XPC to producers.

DATA AVAILABILITY STATEMENT

The authors submitted the raw, unprocessed, and demultiplexed figures onto the laboratory GitHub site, along with the necessary metadata file. The repository is freely accessible and available at: https://github.com/RickeLab/FrontiersMicroManID493430.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because ceca were collected in a commercial plant, no need for IACUC approval as the animals were processed as per industry standards.

AUTHOR CONTRIBUTIONS

PR, SR, HP, and MK conceived the experiment. KF executed the microbiome analytics, developed the bioinformatics pipeline, conducted the microbiome analysis and interpretation, analyzed the data, prepared the figures, wrote the manuscript, and handled submissions and edits. PR conducted the *in vitro* work, executed the sequencing run, processed the samples, prepared the first figure, and edited the manuscript. KF, SR, HP, PR, and WC edited the final manuscript. WC provided significant insight to the analytics. All authors were given the opportunity to edit the manuscript prior to its final submission.

FUNDING

The authors declare that this study received funding from Diamond V. The funder was not involved in the study design, collection, analysis, and interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We would like to thank Diamond V (Cedar Rapids, IA, United States) for their support and funding for this project. Additionally, we would like to thank Dr. Young Min Kwon and his laboratory for the donation of the marker strain. Finally, we would like to extend our gratitude to the undergraduates and technicians who worked in the background to make sure that the laboratory ran smoothly during the generation of this, and so many other, publications.

REFERENCES

Armougom, F., Henry, M., Vialettes, B., Raccah, D., and Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals increase in *Lactobacillus* in obese patients and Methanogens in anorexic patients. *PLoS One* 4:e7125. doi: 10.1371/journal.pone.0007125

Awad, W. A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M., et al. (2016). Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with *Campylobacter jejuni* infection. *Front. Cell. Infect. Microbiol.* 6:154. doi: 10.3389/fcimb.2016.00154

Baffoni, L., Gaggìa, F., Gioia, D. D., Santini, C., Mogna, L., and Biavati, B. (2012). A *Bifidobacterium*-based synbiotic product to reduce the transmission of *C. jejuni* along the poultry food chain. *Int. J. Food Microbiol.* 157, 156–161. doi: 10.1016/j.ijfoodmicro.2012.04.024

Beery, J. T., Hugdahl, M. B., and Doyle, M. P. (1988). Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 54, 2365–2370. doi: 10.1128/aem.54.10.2365-2370.1988

Broom, L. J., and Kogut, M. H. (2018). Inflammation: friend or foe for animal production? *Poult. Sci.* 97, 510–514. doi: 10.3382/ps/pex314

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'Kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of timing of the exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory response of broiler chickens. *BMC Microbiome* 6:88. doi: 10.1186/s40168-018-0477-5

Dicksved, J., Ellstrom, P., Engstrand, L., and Rautelin, H. (2014). Suceptability to *Campylobacter* infection is associated with the species composition of the human fecal microbiota. *mBio* 5:e1212-14. doi: 10.1128/mBio.01212-14

Feye, K. M., Anderson, K. L., Scott, M. F., McIntyre, D. R., and Carlson, S. A. (2016). Inhibition of the virulence, antibiotic resistance, and fecal shedding of multiple antibiotic-resistant *Salmonella* Typhimurium in broilers fed Original XPCTM. *Poult. Sci.* 95, 2902–2910. doi: 10.3382/ps/pew254

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* 312, 1355–1359.

Guyard-Nicodème, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., et al. (2016). Efficacy of feed additives against *Campylobacter* in live broilers during the entire rearing period. *Poult. Sci.* 95, 298–305. doi: 10.3382/ps/pev303

Han, B., Sivaramakrishnan, P., Lin, C. C. J., Neve, I. A. A., He, J., Tay, L. W. R., et al. (2017). Microbial genetic composition tunes host longitivity. *Cell* 169, 1249–1262.

Indikova, I., Humphrey, T. J., and Hilbert, F. (2015). Survival with a helping hand: *Campylobacter* and microbiota. *Front. Microbiol.* 6:1266. doi: 10.3389/fmicb.2015.01266

Janssen, R., Krogfelt, K. A., Cawthraw, S. A., van Pelt, W., Wagenaar, J. A., and Owen, R. J. (2008). Host-pathogen interactions in *Campylobacter* infections: the host perspective. *Clin. Microbiol. Rev.* 21, 505–518. doi: 10.1128/CMR.00055-07

Jumas-Bilak, E., Roudiere, L., and Marchandin, H. (2009). Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'. *Int. J. Syst. Evol. Biol.* 59, 1028–1035. doi: 10.1099/ijs.0.006718-0

Kirk, M. D., Black, R. E., Caipo, M., Crump, J. A., and Devleesschauwer, B. (2015). World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Mandal, S., Van Truren, W., White, R. A., Eggsbo, M., Knight, R., and Peddada, S. D. (2015). Analysis of composition of microbimoes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26:27663. doi: 10.3402/mehd.v26.27663

Meade, K. G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B., and O'Farrelly, C. (2009). Comparative *in vivo* infection models yield insights on early host immune response to *Campylobacter* in chickens. *Immunogenetics* 61, 101–110. doi: 10.1007/s00251-008-0346-7

Messaoudi, S., Kergourlay, G., Dalgalarrondo, M., Choiset, Y., Ferchichi, M., Prévost, H., et al. (2012). Purification and characterization of a new bacteriocin active against *Campylobacter* produced by *Lactobacillus salivarius* SMXD51. *Food Microbiol.* 32, 129–134. doi: 10.1016/j.fm.2012.05.002

Park, S. H., Kim, S. A., Lee, S. I., Rubinelli, P. M., Roto, S. M., Pavlidis, H. O., et al. (2017). Original XPCTM effect on *Salmonella* Typhimurium and cecal microbiota from three different ages of broiler chickens when incubated in an anaerobic *in vitro* culture system. *Front. Microbiol.* 8:1070. doi: 10.3389/fmicb.2017.01070

Possemiers, S., Pinheiro, I., Verheist, A., Van den Abbeele, P., Maignien, L., Laukens, D., et al. (2013). A dried yeast fermentat selectively modulates both the luminal and mucosal gut microbiota and protects against inflammatino, as studied using an integrated in vitro approach. *J. Agric. Food Chem.* 61, 9380–9392. doi: 10.1021/jf402137r

Romero-Barrios, P., Hempen, M., Messens, W., Stella, P., and Hugas, M. (2013). Quantitative microbiological risk assessment (QMRA) of food-borne zoonoses at the European level. *Food Control* 29, 343–349. doi: 10.1016/j.foodcont.2012.05.043

Roto, S. M., Park, S. H., Lee, S. I., Kaldhone, P., Pavlidis, H. O., Frankenbach, S. B., et al. (2017). Effects of feeding Original XPCTM to broilers with a live coccidiosis-vaccine under industry conditions: part 1. Growth performance and *Salmonella* inhibition. *Poult. Sci.* 96, 1831–1837. doi: 10.3382/ps/pew445

Rubinelli, P., Roto, S., Kim, S. A., Park, S. H., Pavlidis, H. O., McIntyre, D., et al. (2016). Reduction of *Salmonella* Typhimurium by fermentation metabolites of Diamond V Original XPC in an *in vitro* anaerobic mixed chicken cecal culture. *Front. Vet. Sci.* 3:83. doi: 10.3389/fvets.2016.00083

Saengkerdsub, S., Anderson, R., Wilkinson, H., Kim, W., Nisbet, D., and Ricke, S. C. (2007a). Identification and quantification of methanogenic archaea in adult chicken ceca. *Appl. Environ. Microbiol.* 73, 353–356. doi: 10.1128/aem.01931-06

Saengkerdsub, S., Herrera, P., Woodward, C., Anderson, R., Nisbet, D., and Ricke, S. (2007b). Detection of methane and quantification of methanogenic archaea in feces from young broiler chickens using real-time PCR. *Lett. Appl. Microbiol.* 45, 629–634. doi: 10.1111/j.1472-765x.2007.02243.x

Saengkerdsub, S., Kim, W., Anderson, R. C., Nisbet, D. J., and Ricke, S. C. (2006). Effects of nitrocompounds and feedstuffs on the in vitro methane production in chicken cecal contents and rumen fluid. *Anaerobe* 12, 85–92. doi: 10.1016/j.anaerobe.2005.11.006

Saint-Cyr, M. J., Haddad, N., Taminiau, B., Poezevara, T., Quesne, S., Amelot, M., et al. (2017). Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control *Campylobacter jejuni* in broilers. *Int. J. Food Microbiol.* 247, 9–17. doi: 10.1016/j.ijfoodmicro.2016.07.003

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Gioia, D. D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Schneitz, C., and Hakkinen, M. (2016). The efficacy of a commercial competitive exclusion product on *Campylobacter* colonization in broiler chickens in a 5-week pilot-scale study. *Poult. Sci.* 95, 1125–1128. doi: 10.3382/ps/pew020

Scumpham, A. J., Jones, J. A., Rettedal, E. A., and Weber, T. E. (2010). Antibiotic manipulation of intestinal microbiota to identify microbes associated with *Campylobacter jejuni* exclusion in poultry. *Appl. Environ. Microbiol.* 76, 8026–8032. doi: 10.1128/AEM.00678-10

Sibanda, N., McKenna, A., Richmond, A., Ricke, S., Callaway, T., Stratakos, A., et al. (2018). A review of the effect of management practices on *Campylobacter* prevalence in poultry farms. *Front. Microb.* 9:2002. doi: 10.3389/fmicb.2018.02002

Sokol, C., Camire, R., Jones, M., and Luster, A. (2018). The chemokine receptor CCR8 promotes the migration of dendritic cells into the lymph node parenchyma to initiate the allergic response. *Immunity* 49, 449–463. doi: 10.1016/j.immuni.2018.07.012

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L., Gratadoux, J. J., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Chron disease patients. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16731–16736. doi: 10.1073/pnas.0804812105

Stern, N. J. (2008). *Salmonella* species and *Campylobacter jejuni* cecal colonization model in broilers. *Poult. Sci.* 87, 2399–2403. doi: 10.3382/ps.2008-00140

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Kovolev, Y. N., Volodina, L. I., Perelygin, V. V., et al. (2005). *Paenibacillus polymyxa* purified bacteriocin to control *Campylobacter jejuni* in chickens. *J. Food Prot.* 68, 1450–1453. doi: 10.4315/0362-028x-68.7.1450

Thibodeau, A., Fravalo, P., Yergeau, E., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by non-antibiotic feed additive. *PLoS One* 10:e0131978. doi: 10.1371/journal.pone.0131978

Wu, F., Guo, X., Zhang, J., Xhang, M., Ou, Z., and Peng, Y. (2017). *Phascolarctobacterium faecium* abundant colonization in human gastrointestinal tract. *Exp. Ther. Med.* 14, 3122–3126. doi: 10.3892/etm.2017.4878

Yan, W., Sun, C., Yuan, J., and Yang, N. (2017). Gut metagenomic analysis reveals prominent roles of *Lactobacillus* and cecal microbiota in chicken feed efficiency. *Sci. Rep.* 7:45308. doi: 10.1038/srep45308

Ziprin, R. L., Drolesky, R. E., Hume, M. E., and Harvey, R. B. (2003). Failure of viable non-culturable *Campylobacter jejuni* to colonize the cecum of newly hatched leghorn chicks. *Avian Dis.* 47, 753–758. doi: 10.1637/7015

Conflict of Interest: HP and WC are employees of Diamond V.

The remaining 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: 05 January 2022
doi: 10.3389/fmicb.2021.458721

Application of Bacteriophages to Limit *Campylobacter* in Poultry Production

*Elena G. Olson[1], Andrew C. Micciche[2], Michael J. Rothrock Jr.[3], Yichao Yang[4] and Steven C. Ricke[1]**

[1] Meat Science and Animal Biologics Discovery Program, Department of Animal and Dairy Sciences, University of Wisconsin–Madison, Madison, WI, United States, [2] Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, United States, [3] Agricultural Research Service, United States Department of Agriculture, Athens, GA, United States, [4] Department of Poultry Science, University of Arkansas, Fayetteville, AR, United States

Campylobacter is a major foodborne pathogen with over a million United States cases a year and is typically acquired through the consumption of poultry products. The common occurrence of *Campylobacter* as a member of the poultry gastrointestinal tract microbial community remains a challenge for optimizing intervention strategies. Simultaneously, increasing demand for antibiotic-free products has led to the development of several alternative control measures both at the farm and in processing operations. Bacteriophages administered to reduce foodborne pathogens are one of the alternatives that have received renewed interest. *Campylobacter* phages have been isolated from both conventionally and organically raised poultry. Isolated and cultivated *Campylobacter* bacteriophages have been used as an intervention in live birds to target colonized *Campylobacter* in the gastrointestinal tract. Application of *Campylobacter* phages to poultry carcasses has also been explored as a strategy to reduce *Campylobacter* levels during poultry processing. This review will focus on the biology and ecology of *Campylobacter* bacteriophages in poultry production followed by discussion on current and potential applications as an intervention strategy to reduce *Campylobacter* occurrence in poultry production.

Keywords: ***Campylobacter,*** **poultry, bacteriophage, post-harvest, pre-harvest**

OPEN ACCESS

Edited by:
Aldo Corsetti,
University of Teramo, Italy

Reviewed by:
Sílvio B. Santos,
University of Minho, Portugal
Giovanna Suzzi,
University of Teramo, Italy

***Correspondence:**
Steven C. Ricke
sricke@wisc.edu

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

Received: *07 March 2019*
Accepted: *29 November 2021*
Published: *05 January 2022*

Citation:
Olson EG, Micciche AC, Rothrock MJ Jr, Yang Y and Ricke SC (2022) Application of Bacteriophages to Limit Campylobacter in Poultry Production. Front. Microbiol. 12:458721. doi: 10.3389/fmicb.2021.458721

INTRODUCTION

According to the World Health Organization, *Campylobacter* is a leading cause of the diarrheal disease (World Health Organization [WHO], 2018). The genus *Campylobacter* is comprised of over 20 species. Strains of *Campylobacter jejuni* and *Campylobacter coli* are generally considered some of the more significant concerns among foodborne pathogens for human health (Korczak et al., 2006; Havelaar et al., 2012; World Health Organization [WHO], 2018; Centers for Disease Control and Prevention [CDC] (2019)). European Food Safety Authority (EFSA) has declared campylobacteriosis as being one of the most commonly reported foodborne diseases since 2005, with over 200,000 cases per year, representing 70% of the human zoonoses in the E.U. (European Food Safety Authority [EFSA], 2020). In one study in the United Kingdom (U.K.), it was reported that 50–80% of poultry harbored *Campylobacter* in their intestinal tract (Connerton et al., 2011;

European Food Safety Authority [EFSA], 2011, 2020). These numbers are similar to those found in poultry produced in the United States (U.S.) (Hanning et al., 2010; Chapman et al., 2016).

With populations in the bird cecum in some instances exceeding seven $\log_{10}$ colony-forming units (CFU) per gram of cecal content, this genus appears to be specifically well suited to reside within the poultry gastrointestinal tract (GIT) (Rudi et al., 2004; Connerton et al., 2011; Indikova et al., 2015). Consequently, *Campylobacter* can be released from the poultry GIT as birds are being processed, potentially contaminating poultry processing plant equipment and the finished product (Elvers et al., 2011; García-Sánchez et al., 2017). Furthermore, *Campylobacter* contamination in the poultry processing plant can remain a persistent problem. Given the poultry GIT establishment and subsequent likelihood of contamination in the poultry plant, poultry meat products are considered a significant source of potential infection for human campylobacteriosis (Umaraw et al., 2017). Pre-and postharvest interventions have been utilized and proposed over the years to reduce *Campylobacter* populations in poultry (Umaraw et al., 2017; Kim et al., 2019; Deng et al., 2020). One intervention that has received more interest as a potential intervention for *Campylobacter* is the administration of *Campylobacter* phages. This review aims to focus specifically on the ecology of *Campylobacter* phages, their mechanisms of bacterial host infection, host resistance, and their applications in both pre-and postharvest as intervention strategies toward reducing *Campylobacter* in poultry production. Due to the lytic characteristic that phages possess, multi-pronged interventions that include combination of phages with non-phage technologies, such as acids and endolysins, throughout poultry processing steps may result in efficient reduction of *Campylobacter* under commercial conditions.

CAMPYLOBACTER PHAGE – CLASSIFICATION AND MECHANISMS OF HOST INFECTION

The first *Campylobacter* phages were likely isolated in 1960 in cattle and pigs from then identified *Vibrio coli* and *Vibrio fetus*, now known as *C. coli* and *C. fetus* (Fletcher and Bertschinger, 1964; Fletcher, 1968). A few of the over 170 known phages infect *C. jejuni*, and most of them are specific to the bacterial host (Ushanov et al., 2020). Almost all currently isolated phages that infect *Campylobacter* are from the family *Myoviridae* and morphologically distinguished as Bradley's morphotype A1 with a contractile tail (Gencay et al., 2018; Jäckel et al., 2019; Ushanov et al., 2020; **Table 1**). Some *Campylobacter* bacteriophages are from the family of *Siphoviridae* and possess Bradley's morphotype B1 with a non-contractile tail (Ushanov et al., 2020). Furthermore, lytic phages that target *Campylobacter* are typically assembled into three groupings (I, II, III) based on genome size (Jäckel et al., 2019; Ushanov et al., 2020). Group I lytic phages are 320 kb in genome size and considered unstable, with only two isolates being identified (Frost et al., 1999; Connerton et al., 2018). Although both group II and III have been demonstrated to be useful for phage therapy, most isolated *Campylobacter* phages belong to group III (Sails et al., 1998; El-Shibiny et al., 2009; Carvalho et al., 2010). Group II phages with an average genome size of 162,601 base pairs, and Group III phages (133, 166 base pairs) comprise approximately half the group I phages' genomic size (Jäckel et al., 2019). Group II phages have a protein head diameter of 83 to 99 nm (Carvalho et al., 2010; Jäckel et al., 2019). Group III phages have a head diameter of 100–130 nm, whereas Group I phages possess much larger head proportions (Jäckel et al., 2019). Based on whole genome sequencing and protein analysis, groups II and III bacteriophages can be further combined into the Eucampyvirinae sub-family (Ushanov et al., 2020). Zampara et al. (2017) further grouped *C. jejuni* phages based on receptor dependency, namely, group III phages use CPS receptors, and group II phages contact the host *via* flagella.

Several phage receptors in Gram-negative bacteria have been recognized, which consist of bacterial surface components such as lipopolysaccharides (LPS), CPS, flagella, outer membrane proteins (Omps), and porins (Rakhuba et al., 2010). For instance, the T-even phages, such as *Escherichia coli* T4 phage, which is the model of the *Myoviridae* phages having contractile tail structure, are among the best-characterized phages (Sørensen et al., 2011). T-even phages recognize and bind to a variety of Omps or specific structures within LPS in *E. coli*. Bacterial receptors in other Gram-negative bacteria that are recognized by phages consist of structures within LPS such as O antigens and carbohydrate moieties (Kiljunen et al., 2005; Petty et al., 2007). The O antigens are central features of *E. coli* and *C. jejuni* cells surface and represent essential factors of infection and disease associated with humans (Mills et al., 1992; Stenutz et al., 2006). Application of phages that bind O antigens may help reduce the virulence of pathogens such as *Campylobacter* within human GIT and thus may be beneficial in postharvest applications for poultry meat intended for retail destinations such as ready to eat meats.

CAMPYLOBACTER PHAGE-ECOLOGY

Campylobacter phages have been isolated wherever their hosts exist, such as the feces of sheep, cows, pigs (Hansen et al., 2007; Rizzo et al., 2015; An et al., 2018); slaughterhouse run-offs, sewage, manure, excreta of chickens and their meat (Grajewski et al., 1985; Salama et al., 1989; Sails et al., 1998; Atterbury et al., 2005; Connerton et al., 2004, 2011; El-Shibiny et al., 2005; Loc Carrillo et al., 2007; Tsuei et al., 2007). Reports on the isolation of bacteriophages from poultry are considerably variable. For example, out of 205 broiler ceca, approximately 20% were positive for *Campylobacter* bacteriophages in the U.K., and a similar result was observed with broilers in South Korea (Atterbury et al., 2005; Hwang et al., 2009). However, in a Denmark study, the *Campylobacter* bacteriophages' isolation rate from conventionally raised broiler intestines was only 3% (Hansen et al., 2007). In contrast to these low rates of isolation, Owens et al. (2013) reported that 100% of the fecal samples from free-range broilers and egg layers tested positive for *C. jejuni* phages. El-Shibiny et al. (2005) isolated 51% *Campylobacter* phages from the *Campylobacter*-positive organic chickens from a U.K. flock. This increase makes sense because free-range birds

TABLE 1 | *Campylobacter* phages taxonomy and description.

Order (Harper et al., 2014)	**Family (Sharp, 2001; Gencay et al., 2018; Huang et al., 2020; Ushanov et al., 2020)**	**Nucleic acid type and phage example (Harada et al., 2018)**	**Morphotype (Sails et al., 1998)**	**Grouping not based on genome analysis (Jäckel et al., 2019)**	**Genome size (Huang et al., 2020)**	**Applicability (Carvalho et al., 2010; Connerton et al., 2018; Huang et al., 2020)**	**Genera based on genome sequencing (Javed et al., 2014)**	**Phages (Javed et al., 2014; Jäckel et al., 2019; Huang et al., 2020)**	**Phage resistance development (Janez and Loc-Carillo, 2013)**	**Host range (Jäckel et al., 2019; Huang et al., 2020)**
Caudovirales	*Myoviridae*	Linear dsDNA (T4)	Morphotype A1 with contractile tail	Group I	∼320 kb	Unstable			Motility defect	
				Group II	Average ∼162,601	Stable for phage therapy	CP220virus	CP21, CP220, Cpt10, vB-Ccom-IBB-35	Motility defect	*C. jejuni* and *C. coli*
				Group III	Average ∼133,166	Stable for phage therapy	CP8virus	CP81, CPX, NCTC12673, Cp30A, PC14, PC5, vB_CjeM_Los1, CP8	CPS structure	*C. jejuni*
	Siphoviridae	Linear dsDNA (Lambda)	Morphotype B1 with non-contractile tail		15–17 kb	Stable for phage therapy		CAM-P21		*C. coli*
	Podoviridae									

potentially encounter a wider variety of *Campylobacter* species and their phages because they are exposed to a broader range of environmental surroundings (Hald et al., 2001; Atterbury et al., 2003b). In general, the likelihood of phage recovery potentially increases with the presence of a susceptible host; therefore, birds with higher colonization rates of *Campylobacter* are more likely to be sources of phages (Atterbury et al., 2003b).

Campylobacter colonization in poultry can vary considerably and this can impact the contamination levels throughout the poultry production chain. In a study by Rudi et al. (2004), the concentration of *C. jejuni* in poultry ceca exhibited a 1,000-fold difference in range throughout a number of flocks. If the strain colonizing one community has an infectious dose that is 0.1% of the strain colonizing another flock, then these two strains would be considered relatively equivalent in their likelihood to cause illness through food contamination (from a human infectivity standpoint) (Rudi et al., 2004). Since up to 76% of chickens slaughtered can be *Campylobacter* positive, contamination management continues to be an important challenge to poultry production (Humphrey et al., 1993). In addition, with cross-contamination occurring in the slaughterhouse, *Campylobacter* and phages discovered on a single chicken carcass may have originated from more than one source (Atterbury et al., 2003b). Although, tracking of phages might be difficult throughout poultry processing due to cross-contamination effects, the capability of phages to persist throughout poultry processing demonstrated by Atterbury et al. (2003a) is an essential characteristic of their future use in the biocontrol of *Campylobacter* in poultry processing. The phages isolated in this study exhibited a broad range of recovery rates from chicken skin stored at 4°C (Atterbury et al., 2003a). Furthermore, the stated detection limit for phage recovery of 2×10^3 PFU/10 cm^2 of chicken skin suggests that there are at least that many phages persisting throughout the commercial poultry processing and packaging operation.

ISOLATION, PROPAGATION, CONCENTRATION, AND PURIFICATION OF *CAMPYLOBACTER* PHAGES

Isolation strategies may impact both the extent and the type of *Campylobacter* phages detected in different ecosystems. The outline and main concepts for the following procedures are briefly described in **Figure 1**. The first step for isolation is collecting the samples. Samples should not be frozen or vortexed as these treatments may significantly reduce the plaque-forming ability (Atterbury et al., 2003a; Jäckel et al., 2017). Although Atterbury et al. (2003a) were able to isolate *Campylobacter* phages from over 10% of chilled chicken thighs by plaque assay, Jäckel et al. (2017) reported reduced lytic activity of phages from samples that were previously frozen.

Even though phages have been detected with polymerase chain reaction (PCR) assays in frozen meats, they either did not exhibit lytic activity, were apparently unstable, or simply more difficult to propagate (Janež et al., 2014; Jäckel et al., 2017). Solid samples can be incubated in sodium chloride/magnesium sulfate (S.M.) buffer to resuspend the phages, and the use of a homogenizer, such as a Stomacher®, to remove phages from chicken skin provides the best results (Jäckel et al., 2019). Following the centrifugation of the resuspended samples and consequent filtration (0.45 and 0.22 μm) of the supernatant, samples can be scanned for lytic activity (Jäckel et al., 2019). The phages are then concentrated to a 10-fold concentration using centrifugal filter units before spotting on indicator strains (Jäckel et al., 2019). Pre-screening to discriminate group II and group III phages rapidly may be beneficial. Using PCR, Jäckel et al. (2017) distinguished over 45% of total phages as group II or III phages. PCR positive samples that do not show lytic activity with the indicator strain can be tested with other potential strains. Likewise, the PCR negative samples should be examined for lytic activity as they may contain rare group I phages (Jäckel et al., 2017).

The choice of a bacterial host is essential while harvesting the phages. *C. jejuni* NCTC12662 (PT14) is generally used as an indicator strain because it is vulnerable to a broad range of phages, although little is known about its response to phage infection (Hansen et al., 2007; Sørensen et al., 2015; Gencay et al., 2018). PT14 was isolated from chicken ceca, and its complete genome has been sequenced (Brathwaite et al., 2013; Sørensen et al., 2015). Nonetheless, a broad range of candidate host strains consisting of several *fla*-types and Penner serotypes should be considered since group II binds to receptors on the flagellum and group III phages binds to CPS receptors (Sails et al., 1998; Hammerl et al., 2011; Sørensen et al., 2011, 2015; Sorensen et al., 2017; Gencay et al., 2018). Sorensen et al. (2017) developed a protocol to determine receptor dependency of *Campylobacter* phages.

Jäckel et al. (2019) suggested that cultivation of indicator strains be *via Campylobacter* media in flasks rather than tubes, as amplification of bacteria is increased by an ample surface headspace for gas exchange. The selection of an overlay agar, such as NZCYM, is critical for the results of the activity tests and should include $CaCl_2$ and $MgSO_4$, which enable the attachment of phages to their host cell (Sails et al., 1998; Frost et al., 1999; Sambrook and Russell, 2001). Determination of lytic activity can be achieved *via* plaque assays and microplate tests (Fischer et al., 2013). In addition, to obtain single plaques, dilutions of phage preparations must be plated. The plaques formed by lytic *Campylobacter* phages are generally about one millimeter in diameter and somewhat turbid, so a zoom stereo microscope may be helpful to spot and count plaques (Jäckel et al., 2019). Specific phages may then be recovered by three sequential single plaque isolations (Hammerl et al., 2011; Jäckel et al., 2015).

Optimal propagation technique should be determined for each phage as propagation methods are not equally suited for all *Campylobacter* phages (Hammerl et al., 2011; Jäckel et al., 2015; Gencay et al., 2017; Sorensen et al., 2017). Propagation of lytic phages can be accomplished by either infection of bacterial cultures or creating agar plates and demonstrating confluent lysis (Green and Sambrook, 2012). Hammerl et al. (2011, 2014) and Jäckel et al. (2015, 2019) achieved over 10^8 PFU/mL by infecting 100 mL cultures of the indicator strain with OD588 of approximately 0.4 with phages at a multiplicity of infection (MOI) of 0.01 followed by incubation for 12–24 h at 42°C.

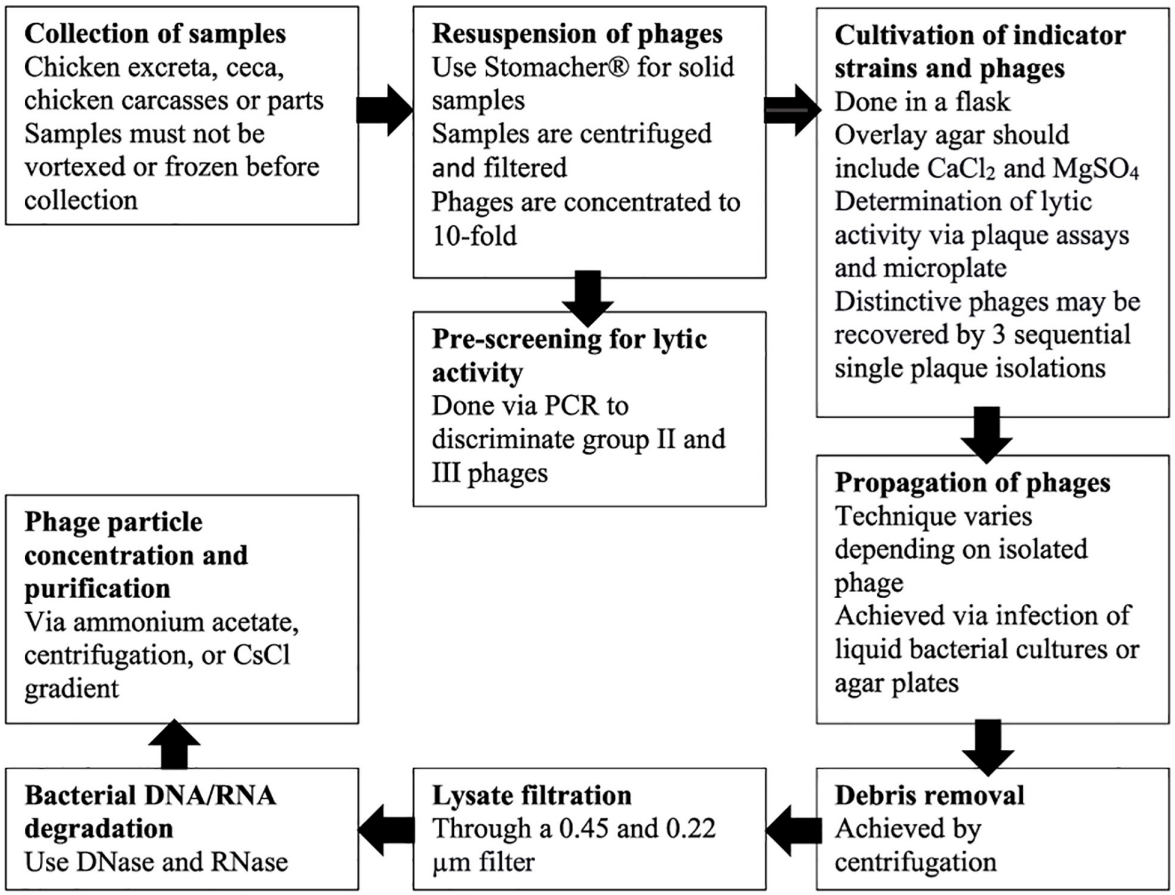

FIGURE 1 | Outline of main concepts of isolation, propagation, concentration, and purification of *Campylobacter* phages.

The cultures that appear to grow best in a flask with a filtered stopper can be placed in a box with a gas-producing sachet to mimic ideal growth conditions and incubated while mildly shaken (Jäckel et al., 2019). Since it is not recommended to use high centrifuge force, there is a possibility that remnants of agar can remain, which in turn may worsen filtration. This method has the additional benefit that the mass lysate does not include any agar, which may interfere with filtration (Jäckel et al., 2019). However, in another technique utilized by Loc Carrillo et al. (2007), Owens et al. (2013), and Gencay et al. (2017), the overlay agar is not harvested; instead, the visible plaques are extracted and suspended in buffer using the typical steps described by Frost et al. (1999).

Before the lysate is filtrated through a 0.45 and 0.22 μm filter, the residual agar, cells, and debris are removed by centrifugation (Jäckel et al., 2019). Bacterial DNA and RNA are then degraded using 20 mg/mL of DNase, and RNase added to the lysate and incubated at 37°C for 30–60 min (Jäckel et al., 2019). Phage particles can be subsequently concentrated using a variety of techniques depending on the amount of the lysate, such as ammonium acetate purification done by Ackermann (2009), centrifugal force utilized in Loc Carrillo et al. (2007), or CsCl density gradient done by Hammerl et al. (2011) and Jäckel et al. (2015). CsCl density gradient is also used to purify *Campylobacter* phages, where the application of 10^9 phage particles is suggested to obtain a prominent band (Jäckel et al., 2019). The extracted phages can consequently be applied to various studies such as morphological typing, selection of host range, and genomic or protein analysis.

ISOLATION AND ANALYSIS OF *CAMPYLOBACTER* PHAGE DNA

All sequenced *Campylobacter* phages have double-stranded DNA (Hammerl et al., 2011; Jäckel et al., 2015, 2019; Harada et al., 2018). Furthermore, genomic differences between group II and group III phages affect the choice of DNA extraction method (Hammerl et al., 2011; Jäckel et al., 2015, 2019). For instance, although the standard protocol described by Green and Sambrook (2012) is suitable for the extraction of group II DNA, the use of phenol-chloroform fails in the extraction of group III DNA (Hammerl et al., 2011; Jäckel et al., 2015, 2019). Commercially available kits for phage DNA extraction are available for both phage groups.

Campylobacter is resistant against digestion by numerous restriction endonucleases, such as *Ava*II, *Bam*HI, CIaI, *Eco*RV, *Eco*RI, *Hae*III, HinfI, *Hin*dIII, HpaIII, PstL, PvuII, RsaI, and ScaI (Sails et al., 1998). However, PFGE analysis using restriction endonucleases that cut pure A/T sequences, such as DraI, SmiI, or VspI, can be performed to establish the genome size of the phages and to assign them to their respective groups (Loc Carrillo et al., 2007; Hammerl et al., 2011; Sorensen et al., 2017). Restriction patterns can subsequently be analyzed on standard agarose gel yielding a more rapid and cost-effective evaluation. Jäckel et al. (2019) suggest applying DreamTaq DNA polymerase amplification constituents or whole genome amplification kits for phage DNA amplification such as those used by Hammerl et al. (2011). To date, all *Campylobacter* phage genomes have been sequenced by short-read sequencing, which is predisposed to homopolymer errors (Jäckel et al., 2019). Long DNA repeats that occur in group II phages hinder the assembly of reads using short-read sequencing. Long-read sequencing platforms such as PacBio or MinION can resolve the problem but necessitate a high amount of DNA (2–10 g) of phages, which can be challenging to obtain (Jäckel et al., 2019).

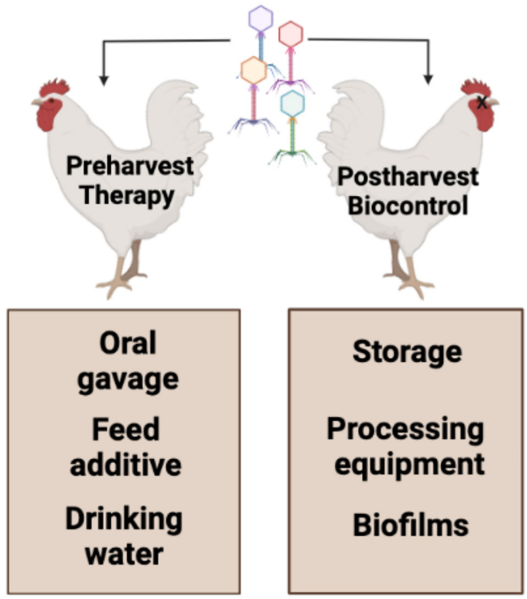

FIGURE 2 | Poultry pre- and post-harvest phage applicability. Figure was created with Biorender.com.

POULTRY PREHARVEST *CAMPYLOBACTER* PHAGE THERAPY

Research is ongoing to reduce pathogen occurrence on poultry farms for *Campylobacter* (Deng et al., 2020). Decreasing pathogen concentrations on poultry farms can affect the reduction of pathogen populations entering the food chain. Preharvest strategies include successful oral application of phages to reduce *C. jejuni* colonization in birds (**Figure 2**; Carvalho et al., 2010) and phages against *C. jejuni* as an alternative feed additive (Kittler et al., 2013). The phage-bacterial interaction is a typical association in the chicken GIT. Lower levels of *Campylobacter* have been observed to innately occur in the ceca of chickens (5.1 $\log_{10}$ CFU/g) in the presence of indigenous *Campylobacter* phages compared to those chickens' lacking phages in the ceca (6.9 $\log_{10}$ CFU/g) (Atterbury et al., 2005). Thus, the majority of preharvest intervention strategies of *Campylobacter* are focused on the reduction or removal of the microorganism from the ceca (Callaway et al., 2004; Hermans et al., 2011; Wheeler et al., 2014; Kim et al., 2019; Deng et al., 2020).

Bacteriophage treatment of *Campylobacter* in chickens was first reported by Loc Carrillo et al. (2005) and Wagenaar et al. (2005). Wagenaar et al. (2005) studied the effects of preventative versus therapeutic phage applications. The study consisted of a 10-day phage treatment trial with the preventive group infected with *C. jejuni* on day four of phage treatment, whereas in the therapeutic group treatment with phage was administered on sixth day of *C. jejuni* infection. Both groups had at least a two $\log_{10}$ CFU/g *C. jejuni* reduction, which tapered off within a week to one $\log_{10}$ lower than the untreated group. Wagenaar et al. (2005) compared single phage application versus a cocktail of group III phages *via* birds' oral administration (**Table 2**). In their study, while the initial reduction was only maintained for 48 h, a one $\log_{10}$ CFU/g decrease was sustained after 30 days, independent of when the therapy was administered. In addition, the birds utilized in this study were at the age for slaughter (day 42), indicating that, despite the phage effectiveness peaking at 48 h, *Campylobacter* concentrations could still be reduced in time for processing. More importantly, when Wagenaar et al. (2005) added another group III phage 69 along with phage 71, they observed a 1.5 $\log_{10}$ CFU/g reduction that eventually leveled off at one log lower than the untreated birds, which suggested a synergistic effect when phages were applied simultaneously (Wagenaar et al., 2005). Ultimately, Wagenaar et al. (2005) conducted the first *in vivo* study that indicated no signs of pathology to the chickens despite a dose phage administration. Although bacterial and phage strains were not obtained from the poultry meat or excreta, the model is not considered appropriate for broader remedial purposes in poultry manufacturing.

In the same year, Loc Carrillo et al. (2005) demonstrated that phage treatment of birds infected with *C. jejuni* reduced the cecal concentration of the marker strains by 0.5–5 $\log_{10}$ CFU/g of cecal contents over 5 days compared to the control birds. The decrease in bacterial load was determined by the phage load, phage-*Campylobacter* grouping, and post-administration time (Loc Carrillo et al., 2005). Loc Carillo and associates used two group III *Campylobacter* phages (CP8 and CP34) in 25-day old broilers administered *via* oral gavage with an antacid (**Table 2**). Interestingly, phage CP8 was active against one tested strain (*C. jejuni* GIIC8) and not the other (*C. jejuni* HPC5). In contrast, phage CP34 reduced both marker strains and sustained the reduction for 5 days (Loc Carrillo et al., 2005). Host strain specificity has been demonstrated as a potential therapeutic drawback of using *Campylobacter* phages. Few phages infect distinctive bacterial species, and the host range for most of them

contains various strains of one bacterial species (Loc Carrillo et al., 2007). Similar to the Loc Carrillo findings, when phage CP8 tested against *C. jejuni* strain HPC5 *in vitro*, no statistically significant reduction was detected by Rabinovitch et al. (2003).

Multiplicity of infection is the ratio of adsorbed or infecting phages to predisposed hosts. Assuming the unlimited time for adsorption, the MOI ratio denotes the threshold of the infecting ratio (Abedon, 2011). Knowing the MOI value is essential for optimizing efficacy. For instance, when the MOI is too high, virions may inactivate through clumping and aggregation (Brown and Bidle, 2014; Roach and Debarbieux, 2017). In addition, MOI depends on the host-phage interaction and varies with medium, phage, and host (Brown and Bidle, 2014; Roach and Debarbieux, 2017). As such, an MOI above the point of saturation of infection, which is host-phage specific, may explain why a higher concentration of phages would not further reduce bacterial concentrations (Kasman et al., 2002; Rabinovitch et al., 2003; Brown and Bidle, 2014). Interestingly, Loc Carrillo et al. (2005) observed that the administration of 9 $\log_{10}$ PFU was less effective than 7 $\log_{10}$ PFU, which may be explained by potential phage aggregation and non-specific association that can occur once the MOI is achieved. One notable drawback with the Loc Carrillo et al. (2005) and Wagenaar et al. (2005) studies is their phage therapy testing employed only one or two *C. jejuni* strains. While these studies demonstrated reductions in tested strains by phage therapy, they did not evaluate how the phage treatment would interact with a more diverse array of background *Campylobacter* strains.

El-Shibiny et al. (2009) utilized *Campylobacter* group II phage CP220 against *C. jejuni* and *C. coli* colonized 20-day old broilers and observed a 2-$\log_{10}$ CFU/g reduction in cecal *C. jejuni* HPC5 populations in 48 h with a single 7-$\log_{10}$ PFU dose (**Table 2**). However, to accomplish a comparable decrease in *C. coli* OR12-colonized chickens, a 9-log PFU dose of CP220 was necessary (El-Shibiny et al., 2009). The critical distinction observed between CP220 and group III phages by El-Shibiny et al. (2009) demonstrated the ability of group II phages to lyse *C. coli* OR12 and 30% of other broiler strains, including the ones that were not lysed by group III phages. A cocktail mixture of two groups could contribute to a broader host range to give the most coverage of *Campylobacter* species. Physiologically, *C. coli* and *C. jejuni* are similar with comparable cell wall structure and energy metabolism. The primary difference is the ability of *C. jejuni* to hydrolyze hippurate, a compound commonly detected in urine (Totten et al., 1987; Jauk et al., 2003). However, the genomes of *C. jejuni* and *C. coli* are approximately 12% divergent (Sheppard and Maiden, 2015). Thus, it may be possible for phages to infect and lyse *C. coli* but unable to replicate due to variations within the bacterial host cellular machinery. While the phenomenon has not been studied within *Campylobacter* phages, it has been observed that bacterial lysis can occur without phage propagation in the presence of a high MOI environment (Cairns et al., 2009; Abedon, 2011). Therefore, as El-Shibiny et al. (2009) suggested, this may offer a possible explanation for the need of a higher concentration of phages to reduce *C. coli* populations compared to *C. jejuni* populations.

The route of phage administration was examined by Carvalho et al. (2010). In their study the authors evaluated the success of the two different paths for phage application *via* oral gavage or feed intake (**Table 2**). In the first study, an orally gavaged cocktail of three broad-spectrum phages (phiCcoIBB35, phiCcoIBB37, phiCcoIBB12) was administered to 1-week-old birds infected with *C. jejuni* 2140CD1. In the second study, Carvalho et al. (2010) compared the oral gavage route and application of phages in feed on birds colonized with *C. coli* A11. Administration of phages *via* the feed route resulted in a higher reduction of *Campylobacter* fecal counts versus the oral route 4 days post administration (**Table 2**; Carvalho et al., 2010). The results of the Carvalho et al. (2010) study indicated that a successful reduction of the two most prevalent *Campylobacter* strains in poultry is possible through the administration of a phage cocktail. In addition, better reduction of *Campylobacter* counts *via* feed intake is advantageous. It is a simple and more practical method of applying phage therapy on farms than oral gavage.

Another route of phage therapy where the cocktail was administered through the drinking water was assessed by Kittler et al. (2013). Despite utilizing the same feed, vaccinations, and living conditions, phages were only significantly effective in reducing *Campylobacter* in one of the three trials, where *Campylobacter* populations were reduced below the detection limit in fecal samples (less than 50 CFU/g) (Kittler et al., 2013). Within 1 day of phage administration *via* drinking water *C. jejuni* cecal counts decreased more than three $\log_{10}$ CFU/g in the cecal contents. Three days post administration, at slaughter, *Campylobacter* counts were still reduced by 1.66–2.14 $\log_{10}$ CFU/g (Kittler et al., 2013; **Table 2**). Similar to previously described studies, at least one $\log_{10}$ *Campylobacter* reduction could be detected 6 days post-administration, indicating successful self-replication and pathogen biocontrol (**Table 2**).

Fischer et al. (2013) compared the combinatory effect of a four-phage cocktail to a single phage administration versus *C. jejuni* counts over time. Using group III one phage or a four-phage cocktail administered at eight time points over 4 weeks directly into the crop of broilers, *Campylobacter* concentrations within the ceca were monitored over days 1, 3, 7, 14, 21, 28, 35, and 42 after phage application (Fischer et al., 2013). Over time, significant reductions were observed, with the highest decrease being $\log_{10}$ 2.8 CFU *Campylobacter*/g of cecal contents on day 21 in both groups (Fischer et al., 2013). When the phage was utilized in a cocktail containing an additional three phages, no significant differences between the cocktail treatment and the single phage treatment were observed (Fischer et al., 2013). The drawback of the study is that Fischer et al. (2013) only used group III phages in a cocktail, which may explain no significant difference when compared to the one phage application. Because the group III phages bind to the same receptors on *Campylobacter* and the phages replicate based on bacterial density, this may explain why the use of group III phages as cocktail and a single phage could produce similar results.

Hammerl et al. (2014) compared combinations of group II (CP68) and III phages with a cocktail of only group III phages (CP14 and CP81) administered *via* oral gavage against *Campylobacter* (**Table 2**). While a combination of group III

TABLE 2 | *Campylobacter* phage treatments during *in vivo* preharvest studies.

Phage	Group	Source	Administration	Flock age	*Campylobacter* spp.	Outcome	Developed resistance	Time of sustained reduction	Study
Phage 71 (NCTC 12671)	Group III	NCTC	$\sim 10^{10}$ PFU by oral gavage	32 days	*C. jejuni* C356	3 $\log_{10}$ CFU/g reduction in 24 h in cecal contents	n/a	1 log sustained reduction over 30 days compared to control in both trials	Wagenaar et al., 2005
Phage 71, Phage 69	Group III		$\sim 10^{10}$ PFU by oral gavage		*C. jejuni* C356	1.5 $\log_{10}$ CFU/g initial reduction			
CP8	Group III	Broiler chicken excreta, free-range layer chicken excreta, processed chicken meat	7 $\log_{10}$ PFU by oral gavage	25 days	*C. jejuni* GIIC8	5.6 $\log_{10}$ CFU/g cecal content	<4%	2.1–1.8 $\log_{10}$ sustained reduction for 5 days	Loc Carrillo et al., 2005
CP34	Group III		7 $\log_{10}$ PFU by oral gavage		*C. jejuni* HPC5	3.9 $\log_{10}$ CFU/g cecal counts			
CP220	Group II	Poultry sources	7 $\log_{10}$ PFU by oral gavage	20 days	*C. jejuni* HPC5	2.1 $\log_{10}$ CFU/g reduction 24 h post phage	2%	2 $\log_{10}$ sustained reduction for 2 days	El-Shibiny et al., 2009
CP220	Group II		9 $\log_{10}$ PFU by oral gavage		*C. coli* OR12	2 $\log_{10}$ reduction			
PhiCcoIBB35, PhiCcoIBB37, PhiCcoIBB12	Group II	Poultry intestinal contents	1×10^6 PFU phage cocktail by oral gavage or 1.5 × 107 PFU through feed	7 days	*C. jejuni* 2140 CD1 and *C. coli* A11	1.25 $\log_{10}$ CFU/g reduction in feces by oral gavage 2 days post administration; 2 log10 CFU/g reduction in feces by feed route 2 days post administration	13%	1.7 $\log_{10}$ CFU/g sustained reduction in feces by oral gavage 7 days post administration; 2 log10 CFU/g sustained reduction in feces by feed route 7 days post administration	Carvalho et al., 2010
NCTC 12672, NCTC 12673, NCTC 12674, NCTC 12678	Group III	NCTC	Phage cocktail of 7.4 $\log_{10}$ PFU/bird *via* drinking water	36 d	*C. jejuni* NCTC 12661, *C. jejuni* NCTC 12664, *C. jejuni* NCTC 12660	3.2 log CFU/g reduction in cecal content compared to control 1 day post administration	n/a	1.66–2.14 PFU/g sustained reduction in cecal content 6 days post administration	Kittler et al., 2013
NCTC 12673	Group III	NCTC	10^7 PFU into crop of broiler	9 d	*C. jejuni* 1474-06	1.3 CFU/g $\log_{10}$ reduction of cecal contents compared to control up to 3 days post administration	Initially 43%	2.8 $\log_{10}$ sustained reduction 21 days post administration in trial II	Fischer et al., 2013
NCTC 12673, NCTC 12674, NCTC 12678, NCTC 12672	Group III	NCTC	Phage cocktail of 10^7 PFU directly into crop of broiler		*C. jejuni* 1474-06	1.3 CFU/g $\log_{10}$ reduction of cecal content compared to control	Initially 24%		
CP14	Group III	Chicken fecal samples of organic origin	5×10^8 PFU by oral gavage	27 days	*C. jejuni* 3871	1 $\log_{10}$ reduction of cecal contents	5%	*C. jejuni* counts increased 4 days post administration	Hammerl et al., 2014

(Continued)

TABLE 2 | (Continued)

Phage	Group	Source	Administration	Flock age	*Campylobacter* spp.	Outcome	Developed resistance	Time of sustained reduction	Study
CP14, CP81	Group III	Retail chicken	5×10^8 PFU by oral gavage			No reduction	7%(CP14); 8% (CP81)		
CP14, 24 h later CP68	Group II, III	Chicken fecal samples of organic origin	5×10^8 PFU of CP14; 5×10^{10} PFU of CP68 by oral gavage			3 $\log_{10}$ reduction of cecal contents 2 days post administration of CP68	5% (CP14) 2% (CP68)	*C. jejuni* counts increased 4 days post administration but were still lower than in control group and group with CP14 alone	
CP20, CP30A	Group II, III	Commercial broiler chicken excreta	7 $\log_{10}$ PFU *via* oral gavage	24 d	*C. jejuni* HPC5	2.4 log CFU g^{-1} reduction of cecal contents 2 days post treatment.	0.1	1.3 $\log_{10}$ CFU g^{-1} sustained reduction after 5 days	Richards et al., 2019

phages did not elicit a detectable *C. jejuni* reduction, application of CP14 phage alone achieved one $\log_{10}$ CFU/g reduction. These results were similar to the reduction of *Campylobacter* counts achieved by Wagenaar et al. (2005) using only one phage cocktail. However, Hammerl et al. (2014) observed that a sequential combination of CP14 phage followed by CP68 phage led to a three $\log_{10}$ CFU/g *Campylobacter* reduction in the cecal contents. Similar to the observations of El-Shibiny et al. (2009), Hammerl et al. (2014) concluded that phage cocktails should be composed of group II and group III phages to successfully combat *Campylobacter*.

Furthermore, Richards et al. (2019) showed that the utilization of phage therapy offers a minimal targeted intervention that is not harmful to the intestinal microbiota of broilers. Richards et al. (2019) demonstrated that a cocktail of CP30A and CP20 phages against *C. jejuni* colonized birds produced significant reductions in intestinal *C. jejuni* populations compared to control birds over 5 days and did not affect the alpha-diversity and richness of microbiota in ceca and ileum of birds compared to control (**Table 2**). The phage cocktail produced the most *Campylobacter* reduction in the ceca where the bacterial counts were decreased by 2.4 $\log_{10}$ CFU/g. The bacterial reductions were also significant in ileum (1.36 $\log_{10}$ CFU/g on day 2) and colon (1.74 $\log_{10}$ CFU/g on day 3). Richards et al. (2019) were able to recover both phages of the cocktail for 5 days throughout the experiment in all three compartments of the chicken's GIT, indicating successful self-replication *in vivo* and no competition between phages allowing them to co-exist. Overall, the time of phage application to limit *Campylobacter* colonization in chickens have been shown to be most effective over a 2- or 3-day period post phage administration (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Richards et al., 2019). In addition, when the time to slaughter after phage application was prolonged, numerous studies showed that cecal *Campylobacter* counts did not reach the counts detected in non-treated controls (**Table 2**). These findings suggest that phages' success of self-replication is possible *in vivo* and can provide an antimicrobial safety net in cases when a slaughter day may be postponed. In addition, the diminished competitive advantage of the resistant types, as reported by Loc Carrillo et al. (2005), reinforces the hypothesis of Wagenaar et al. (2005) that the release of potent *Campylobacter* phages into the environment would not comprise any more significant risk.

Achieving complete elimination of *Campylobacter* in the bird GIT may be unrealistic with phage therapy for various reasons. However, the partial reduction could still be a productive outcome for reducing potential exposure to *Campylobacter*. This possible reduction can be estimated using quantitative microbial risk assessment (QMRA), which is the probability of infection and illness when a population (usually of humans) is exposed to pathogens in the environment (Chapman et al., 2016). QMRA is based on hazard identification, exposure assessment, dose-response, and risk characterization (Vose, 2008). Based on this approach, quantitative risk assessment models suggest that reducing two $\log_{10}$ CFU/g of *Campylobacter* in the ceca at the time of slaughter would significantly impact campylobacteriosis's human incidence by approximately 30-fold (Rosenquist et al., 2003). Understanding how reductions

in *Campylobacter* populations impact disease occurrence is essential, as, in nature, phages seldom eradicate their host bacterium populations (Connerton et al., 2008). The inability of complete microbial elimination may partly be due to natural bacterial resistance and the failure of phage particles to find host cells when occurring in low concentrations (Chibani-Chennoufi et al., 2004). Low concentrations of host cells should not be a concern when considering phage remediation in the ceca of broilers, as *Campylobacter* concentrations in the ceca often range from 4 to 8 $\log_{10}$ CFU/g (Rudi et al., 2004).

POULTRY POSTHARVEST *CAMPYLOBACTER* PHAGE BIOCONTROL

Postharvest application of lytic phages could selectively target *Campylobacter* populations without interfering with the remaining microbiota. Phage treatment can be used to inactivate *Campylobacter* attached to food contact surfaces or grown as biofilms. *Campylobacter* bacteriophages isolated from retail poultry have been used in some post slaughter experiments (Umaraw et al., 2017). While *Campylobacter* phages have been isolated from poultry carcasses, they occur in relatively low concentrations compared to the *Campylobacter* bacterial loads that have been reported on poultry skin in retail environments up to 10^4 CFU per carcass (Dufrenne et al., 2001; Atterbury et al., 2003a; Scherer et al., 2006).

Campylobacter species can often be isolated from chicken skin and feathers, because the chicken skin has a protective effect on *Campylobacter* and other pathogens (Humphrey and Lanning, 1987; Berrang et al., 2000; Whyte et al., 2001; Atterbury et al., 2003a). This phenomenon is most likely due to the presence of feather follicles and skin folds that contain oils and fats that may protect bacterial cells from crystalizing during the freezing process. Since the infective dose of *Campylobacter* for humans is less than 500 cells, the research on the persistence of *Campylobacter* on chicken skin under freezing conditions is a significant food safety concern (Black et al., 1988). One of the approaches in the industry to decrease broiler carcass contamination includes the use of hyperchlorite in scald water and chillers (Atterbury et al., 2003a). However, this approach has been demonstrated to not significantly reduce pathogen loads, such as *Campylobacter* (Whyte et al., 2001). In addition, increasing the dilution of hyperchlorite increases its efficacy but reduces the quality of the product, which is intolerable by consumers (Atterbury et al., 2003a).

Host-specific phages have been successfully used in preharvest operations to control enteric *Campylobacter* counts in poultry (**Table 2**). Using a single phage therapy, Atterbury et al. (2003a) demonstrated over a one $\log_{10}$ CFU/cm^2 reduction of *C. jejuni* on chicken skin inoculated with 10^6 CFU of *C. jejuni* PT14 with the administration of 10^7 PFU of group III phage φ2 when the skin was stored at 4°C for 10 days (**Table 3**). Atterbury et al. (2003a) reported an improved reduction to 2.5 $\log_{10}$ CFU/cm^2 during additional cold storage (–20°C) of poultry skin, which was greater than the effect of cold storage without phage application. However, environmental conditions may have enhanced phage efficacy. It is commonly accepted that *Campylobacter* species cannot replicate at 4°C, and Atterbury et al. (2003a) findings verified that premise since the number of *C. jejuni* populations on chicken skin without phage stored at 4°C for 10 days were reduced by one $\log_{10}$ CFU. There was no increase in phage counts on any of the chicken skins inoculated with *Campylobacter*, indicating that phages may not reduce *Campylobacter* counts *in situ* without bacterial replication. The subsequent rationalization for the reduction in *Campylobacter* cells is that during inoculation, a fraction of phages effectively adsorbed to the surface of the host but did not replicate until the bacterium became more metabolically active. Their data supported this concept since no reduction was observed in either *Campylobacter* concentration or phage number when the phage was mixed with a non-susceptible bacterial host. The authors concluded that a combinatory phage cocktail of a broad host range must be used for the therapy to be practical. More so, combining phage application with freezing may cause further reduction of *Campylobacter* on broiler carcasses. In another study, Goode et al. (2003) demonstrated a more significant reduction of *C. jejuni* counts with a phage application than *C. jejuni* counts without phage due to the low temperature alone. Ninety-five percent reduction of *C. jejuni* occurred on chicken skin *via* group III phage 12673 at 10^6 PFU/cm^2 inoculated with 10^4 CFU/cm^2 *C. jejuni* C222 and incubated for 24 h compared to a non-phage treated group that resulted in a 90% reduction of *C. jejuni,* which was statistically different. Similar to the results of Atterbury et al. (2003a), the phages were able to persist on chicken skin over 48 h at 4°C. These results indicate that phage administration may lessen cross-contamination with pathogens from other carcasses and processing environments.

Improvement of efficacy with a decrease in temperature was also noted in other meat matrices. For example, the use of phage Cj6 on raw beef at 5°C with high MOI and high host density reduced *C. jejuni* by 2.4 $\log_{10}$ CFU/cm^2 in cooked meat and a 1.5 $\log_{10}$ CFU/cm^2 in raw beef (Bigwood et al., 2008; **Table 3**). Conversely, at a high host density and high MOI, 2.8 CFU/cm^2 at 6 h post phage administration and 2.2 $\log_{10}$ CFU/cm^2 reductions at 24 h were observed on cooked and raw meat, respectively (Bigwood et al., 2008). However, at 24°C and a low host density, no significant reductions were observed even with a high MOI (Bigwood et al., 2008). The results were different from the *Salmonella* outcome in the same study where the introduction of *Salmonella* phage P7 produced a 4.7 $\log_{10}$ CFU *Salmonella* reduction when incubated at 24°C with a high MOI and low host density in cooked meat and two $\log_{10}$ reduction in raw beef. The decrease of *Salmonella* populations in the presence of the phage demonstrated that at low host cell load and high phage MOI, the number of bacterial cells eliminated does not rely upon the host cell load. These findings were consistent with Bigwood et al. (2009). This study also noted that inactivation of *Campylobacter* by phages continued and increased with time for 8 days incubated at 5°C for both cooked and raw types of meat. This finding is essential, as ready-to-eat meats are usually consumed within 7 days of purchase (Gilbert et al., 2007). In further work, Bigwood et al. (2009) concentrated on applying

TABLE 3 | *Campylobacter* phage treatments during the postharvest studies.

Phage	Group	Source	Administration	*Campylobacter* inoculation	Results	Resistance	Study
φ2	Group III	NCTC 12674, ACTC 35922-B2	10^7 PFU/cm^2	10^6 CFU/cm^2 *C. jejuni* PT14 on chicken skin	4°C >1 $\log_{10}$ CFU reduction 30 min, 3 days, 5 days –20°C 2.3 $\log_{10}$ CFU reduction 5 days post administration	None	Atterbury et al., 2003a
NCTC 12673	Group III	NCTC	10^6 PFU/cm^2 on chicken skin	10^4 CFU/cm^2 *C. jejuni* C222 on chicken skin	4°C non-phage 90% reduction 24 h post administration 4°C with phage 95% reduction 24 h post administration	n/a	Goode et al., 2003
Cj6	Group not specified	Chicken feces	Low MOI (10) or high MOI (10^4)	<100 cm^{-2} or 10^4 cm^{-2} of *C. jejuni* FGCSCFT onto cooked or raw beef	5°C(high MOI, high host density) >2 log cm^{-2} reduction 24 h post administration for cooked meat and 1.5 log cm^{-2} for raw beef	n/a	Bigwood et al., 2008
Cj6	Group not specified	Chicken feces	10^2-10^8 PFU mL^{-1}	10-10^4 CFU mL^{-1} of *C. jejuni* into tube with inoculum	24°C and 2 h post administration: 1.8×10^5 PFUmL^{-1} 3–8% reduction 1.2×10^6 PFUmL^{-1} 33–52.3% reduction 1.1×10^7 PFUmL^{-1} >96% reduction Without phages 8.9% reduction	n/a	Bigwood et al., 2009
CP8 or CP 30	Group III	Poultry excreta	10^6 or 10^9 PFU/ml	*C. jejuni* NCTC 11168 or PT14 at 10^5 CFU/ml incubated at 37°C for 5 days grown in biofilms on glass	In biofilms: CP30 or CP8 versus 11168 or PT14 3 $\log_{10}$ CFU/cm^2 reduction 2 h post administration CP8 versus 11168 barely detectable limits 24 h post administration CP8 versus PT14 1 $\log_{10}$ CFU/cm^2 reduction 24 h post administration CP30 versus PT14 2.5 $\log_{10}$ CFU/cm^2 reduction 4 h post administration Planktonic cells: CP30 versus PT14 < 1 log CFU/cm^2 reduction 4 h post administration	NCTC 11168 à CP8 (84%) à CP30 (90%) None in PT14	Siringan et al., 2011
F356 + F357	Group not specified	Free range poultry farms	10^7 PFU/cm^2	10^4 CFU/cm^2 of *C. jejuni* NCTC 12662 on chicken skin	0.73 $\log_{10}$ reduction at 5°C 24 h post administration	n/a	Zampara et al., 2017

phage in the liquid food stored at room temperature for 2 h, as it is considered the maximum time for food storage at room temperature (**Table 3**). At the lowest phage concentration, the number of surviving host bacteria was close to 100%, but with the increasing concentration of phages, the inactivation of *C. jejuni* and *Salmonella* cells increased (**Table 3**). Decreased survival over time was observed for *C. jejuni* populations when the host concentration was low, indicating that with a given concentration of phages, the reduction of *Campylobacter* cells was more significant for lower concentrations of host bacterial cells.

In a more recent study, Zampara et al. (2017) demonstrated the application of lytic phages that targeted both CPS (group III) and flagella (group II) of *C. jejuni* on chicken skin under conditions that imitated a storage environment (**Table 3**). A combination of group III phages (F356 and F357) produced a 0.73 $\log_{10}$ reduction of *C. jejuni* counts on chicken skin at 5°C in 24 h. These results are in contrast to the results from CPS targeting phages used alone that produced a 0.55 $\log_{10}$ reduction (F356) and 0.49 $\log_{10}$ reduction (F357), and phage targeting the flagella (F379) that failed to reduce *Campylobacter* at a low temperature significantly. Previous studies have shown that motility may affect the infection proficiency of flagellotropic phages (Sørensen et al., 2015). Thus, the group II phage could be unsuccessful due to the potentially compromised motility of *C. jejuni* at lower temperatures. However, Zampara et al. (2017) observed that motility of *C. jejuni* was not a factor and further indicated that the temperature did not affect phage binding as no differences of phage binding between 37 and 5°C were observed within an hour of incubation. However, group III phages exhibited greater binding capacity with an average of 96% of phages adsorbing to the bacterial cells at 5°C after 24 h of incubation, compared to 55% of attached group II phages. The authors also observed an increase in free group II phage concentration after 24 h of incubation compared to 1 h incubation. They postulated that group II phages bound to the bacterial cell within the first hour but not permanently, explaining the increased concentration of free phages after 24 h.

Most phage studies involve the *Myoviridae* family of *Campylobacter* phages. However, Huang et al. (2020) characterized and described a rare member of the *Siphoviridae* family, CAM-P21, isolated from the beef grind. CAM-P21 was described to possess a broad host range, a better titer, and enhanced performance under diverse stress conditions compared to the *Myoviridae* family of phages. CAM-P21 reduced viable *C. coli* counts by more than two logs after a 12–24 h incubation period at both 42 and 37°C *in vitro*, respectively (Huang et al., 2020). The findings reported by Huang et al. (2020) suggest that a prospective combination of families of phages in a cocktail can potentially control for multiple *Campylobacter* species.

A significant concern for processing facilities is the buildup of biofilms on processing surfaces and equipment (Arnold and Silvers, 2000). *Campylobacter*, along with other pathogens, can form biofilms by producing a polysaccharide matrix (Gunther and Chen, 2009). In comparison to chemical sanitizers such as chlorine and peracetic acid, which appeared to be inefficient in removing biofilms, several bacteriophages have successfully reduced pathogen populations within biofilms (Deborde and Von Gunten, 2008; Siringan et al., 2011; Van der Veen and Abee, 2011). For example, bacteriophages CP8 and CP30 effectively reduced *C. jejuni* by one to three $\log_{10}$ in biofilms formed on glass surfaces (Siringan et al., 2011). While glass may not represent the surfaces typically found in poultry processing facilities, Siringan et al. (2011) detected a three $\log_{10}$ CFU/cm^2 reduction within 2 h post phage administration in *Campylobacter* counts in biofilm incubated at 37°C using group II phages CP8 or CP30 (**Table 3**). However, bacterial cells were recovered at 4–8 h post phage administration that may be correlated with the reattachment of *C. jejuni* biofilm that was previously separated by phage treatment. Regardless of the final level of recovered *Campylobacter* cells after 4 h, instantaneous effects of phage application could still be used to initiate the dispersal of biofilms in poultry processing that high-pressure water treatments could follow. Remarkably, application of CP8 on *C. jejuni* NCTC 11168 resulted in nearly undetectable counts 24-h post phage application compared to *C. jejuni* PT14 strain, which produced a greater quantity of biofilm and exhibited less than one $\log_{10}$ CFU/cm^2 reduction over 24 h period in 11168 counts when CP8 phage was used. However, CP30 reduced *C. jejuni* PT14 counts by 2.5 $\log_{10}$ CFU/cm^2 in 4 h regardless of the excessive amount of biofilm matrix.

Interestingly, although CP30 showed such success on *C. jejuni* PT14 cells in biofilm, the phage produced less than one $\log_{10}$ CFU/cm^2 reduction in the planktonic cells under the same conditions. This finding contradicts previous observations by Sharma et al. (2005) that concluded similar effects of phages on attached and planktonic cells. Furthermore, similarly to several preharvest studies, there appeared to be stasis in phage concentration in biofilm and planktonic cultures throughout the experiment regardless of the reduced *Campylobacter* numbers. This phenomenon indicates a threshold above which phage counts do not increase despite being constantly supplemented into the matrix through replication. This could illustrate a passive biocontrol in which the quantity of phages is adequate to decrease cell counts without the necessity for excessive levels of phage replication.

Biofilms represent an accumulation of various cells enclosed by a matrix of extracellular polymeric substance (EPS) produced by bacterial members of the biofilm (Sillankorva et al., 2011). The main constituents of the EPS are long-chain sugars, DNA, and other various biological macromolecules that can be very diverse (Flemming, 2008). Bacteria within a biofilm have demonstrated high resistance to antibiotics and other antimicrobial agents (Harper et al., 2014). In addition, the concentration of the respective agent required to generate antimicrobial effects can be more than a thousand times higher than the amount necessary for free-living microorganisms (Ceri et al., 1999). Yet, bacteriophage application has shown high success in biofilm dispersal within bacterial species (Siringan et al., 2011).

Since biofilms' extracellular contents depend on the microbial populations present, phage cocktails should be evaluated against biofilms comprised of various bacterial genera. The success of specific phages in removing biofilms can be potentially due to polysaccharide depolymerase production, which breaks up the polysaccharide matrix (Hughes et al., 1998). For instance,

many caudovirales, such as T4 and HK620 of *E. coli*, possess a polysaccharide depolymerase protein at the end of their tail that can degrade microbial capsules and allow cellular attachment (Harper et al., 2014). Lu and Collins (2007) engineered such a phage that stimulated host polysaccharide depolymerase expression resulting in the breakup of the polysaccharide matrix. They also reported that its application reduced *E. coli* biofilms by nearly 100% and produced a reduction two times better than non-enzymatic phage (Lu and Collins, 2007). The polysaccharide depolymerase expressing phages may improve efficacy in preharvest strategies where the sustained phage replication is less expected, such as the poultry GIT. However, host specificity insinuates that a thorough library of phages must be preserved so that a proper administration can be designated for each bacterial community within a biofilm. The possibility of combinations within biofilms suggests that it may be challenging for any created phage to be successful on a wide range of biofilms; although, the concept could work with other biofilm-destroying enzymes (Lu and Collins, 2007). Future developments for the strategy may incorporate several phage promoters amplifying enzyme production that would target multiple EPS constituents and target multiple bacterial species (Lu and Collins, 2007). Unlike T7 *E. coli* phage, *Campylobacter* phages do not possess RNA polymerase and there are no strong promoters that have been identified within the *Campylobacter* phages, which would make genome cloning within the phage difficult.

POTENTIAL *CAMPYLOBACTER* PHAGE HOST RESISTANCE MECHANISMS

A potential concern of phage resistance arises from the increased or prolonged phage application for medical applications or in the food industry (Goodridge and Bisha, 2011). In the environment, bacteria and bacteriophages exist in a co-evolution cycle, in which phage-insensitive hosts survive or prevent phage predation by passing on the corresponding resistance mechanisms (Weinbauer, 2004; Labrie et al., 2010). Phage resistance usually arises due to the loss or modification of cell surface molecules, such as capsules, LPS, pili, or flagella (Labrie et al., 2010).

Labrie et al. (2010), Bradde et al. (2017), and Jiang and Doudna (2017) briefly described general phage resistance mechanisms. The mechanisms can be classified into two categories: (1) prevent initial phage interaction with the host and/or (2) survive phage infection. The former is commonly accomplished *via* modification of phage receptor sites, preventing DNA entry by changing the injection site conformation, and producing physical barriers through the extracellular matrix (Labrie et al., 2010). For instance, the MeO*P*N moiety of the CPS has been identified as a receptor site for lytic phages, such as F336 (Sørensen et al., 2011, 2012). Sørensen et al. (2012) observed phage resistance when the phase variable phosphoramidate (MeO*P*N) moiety of CPS of *C. jejuni* was modified. In addition, over 70% of *C. jejuni* were found to possess modifications in their MeO*P*N moiety (McNally et al., 2007; Alphen et al., 2014). Although these surface structures often function as virulence factors and can contribute to bacterial survival, studies have shown that phage resistant *C. jejuni* have a competitive disadvantage in terms of fitness compared to phage sensitive isolates in the same environment without the phage's presence (Levin and Bull, 2004; Scott et al., 2007a,b; Carvalho et al., 2012; Hooton et al., 2020).

Scott et al. (2007b) showed that phage therapy affects the growth of resident *Campylobacter* in the avian GIT. Scott et al. (2007b) showed that amongst *C. jejuni* that survive phage infection in broiler chickens are phage-resistant types that exhibit genomic rearrangements. Scott et al. (2007b) investigated group III phage CP34 predation on *C. jejuni* HPC5 and R14-CampMu and R20-CampMu on R14 and R20 *C. jejuni* strains within the avian GIT and isolated phage-resistant mutants. These mutants were most likely not the dominant *Campylobacter* strain; since the mutations primarily altered the flagella, which caused a significant negative impact on colonization. The authors concluded that phage resistance is rare in poultry because the mutants that avoid phages are not capable of chicken GIT colonization and quickly mutate back to colonization-capable sensitive forms.

Prevention of phage infection is not the only bacterial response utilized. Other phage resistance mechanisms focus not on preventing phage entry but instead on bacterial host survival once infected by phage. Clustered regularly interspaced short palindromic repeats (CRISPR) have been studied extensively and provide a mechanism for bacteria to survive multiple phage infections (Barrangou et al., 2007). CRISPR loci are present in 45% of the bacteria, according to Grissa et al. (2007). Initially observed in *E. coli* and described by Ishino et al. (1987), CRISPR is an arrangement of short repeated sequences split by spacers with unique sequences and are located in plasmid and chromosomal DNA. These spacers are frequently the nucleic acids of plasmids and viruses (Rath et al., 2015). CRISPR activity involves the CRISPR-associated (*cas*) genes located adjacent to the CRISPR that code for proteins fundamental for the proper immune response (Barrangou et al., 2007). There are distinct types of CRISPR systems. *C. jejuni* NCTC 11168 and PT14 have been reported to contain subtype II-C CRISPR systems that lack Cas4 proteins (Dugar et al., 2013). While the absence of Cas4 has been noted in subtype II-C CRISPR systems, the protein has been shown to possess an exonuclease activity which is required for CRISPR adaptation (Shah et al., 2013). Thus, the deficiency of Cas4 may inhibit spacer integration, such as phage defense.

By studying carrier state life cycle (CSLC) of *C. jejuni* PT14 for CP8 and CP30A phages, Hooton and Connerton (2015) had the opportunity to assess the process of the CRISPR-Cas system in the company of Class III phage carrying Cas4. The CSLC represented a mixture of bacteria and phages in a state of equilibrium (Lwoff, 1953). Under CSLC conditions, phages could continue to associate with a well-suited host and generate free virions in a search for new hosts (Siringan et al., 2014). While a fraction of the bacteria attain resistance, some sensitive cells happen to maintain the phage population so that both survive (Siringan et al., 2014). Although appearing as lysogens, strains exhibiting CSLC do not integrate phage nucleic acid into the host genome (Siringan et al., 2014). In addition, CSLC has been strictly observed with the lytic phages, and there are several experimental

examples of such relationships (Li et al., 1961; Jones et al., 1962; Jarling et al., 2004; Bastías et al., 2010). During their experiment, Hooton and Connerton (2015) observed that the carrier state populations was comprised of bacteria that had extended the CRISPR array by acquiring naive spacers. Markedly, all the new spacer sequences could be located in the host genome sequence and were not noted in either co-propagating phage genomes. This mechanism prevents phage DNA inclusion, which allows the phage to replicate undetected (Hooton and Connerton, 2015). The phenomenon indicates that even internal host resistance mechanisms can be complicated and mechanistically elusive to understand. The authors could not conclude how long bacteria carrying these spacers survived (Hooton and Connerton, 2015). Potentially, the CRISPR facilitated immunity is sustained in this setting as a self-sacrificing response to the constant exposure to phage infection but this still does not explain why the phage resides as a practical component of the system. Hooton and Connerton (2015) suggested that *Campylobacter* phages can use Cas4-like protein as an anti-CRISPR technique to initiate a spacer integration to use host DNA as an operational distraction to phage DNA. Therefore, *Campylobacter* that obtain self-spacers and avoid phage infection must overcome CRISPR-facilitated immunity against itself by either withstanding alterations in gene regulation or losing the interference functions exposing them to the foreign DNA invasion (Hooton and Connerton, 2015).

Other factors may be associated with phage resistance. It has been insinuated that the persistence of phage infection in microbial cultures could be facilitated by gradual adsorption rates of the phages, permitting the bacterial host time to replicate before the cellular machinery is overcome (Torsvik and Dundas, 1980). Remarkably, bacteriophages generated by *Campylobacter* CSLC strains retained phage adsorption constants comparable to those propagated by traditional lysis (Siringan et al., 2014). Siringan et al. (2014) showed that 70–90% of the phage population in *Campylobacter* CSLC strains were closely related with their host, either bound to CPS of the cell or preserved within the host cell with the potential that the phage genomes are carried as episomes (Siringan et al., 2014). Siringan et al. (2014) proposed that disbanded phage elements are associated with the bacteria, and the outcome of the phages is not subject to the fate of the host. Nevertheless, the authors also suggested that the presence of the phages is not totally passive, and that the host's replication contributed to the detected phage concentration. Phenotypic analysis of the CSLC *Campylobacter* strains showed that the cells were non-motile, and their flagella were shortened (Siringan et al., 2014). These findings are consistent with impaired motility of most phage-resistant types retrieved from post-infection cultures, where mutants with non-functional flagella have been shown not to establish phage infection (Coward et al., 2006; Scott et al., 2007a,b). Consequently, CSLC strains were incapable of colonizing chickens in the Siringan et al. (2014) study. These features make CSLC strains an essential ecological reservoir for phage propagation and conceivable commercial interest as a constant source of phages for remedial and biological sanitation purposes in the food and farming productions directed at reducing human exposure *to Campylobacter*.

An interesting observation was noted by Burmeister et al. (2020), indicating an evolutionary compromise between phage and antibiotic resistance in bacteria. When studying the interactions between phage and antibiotic resistance genes, Burmeister et al. (2020) observed increased antibiotic sensitivity in bacteria where the phage resistance emerged. The relationships between phages, antibiotic-resistant bacteria, and antibiotic-sensitive bacteria are complicated. Occasionally when phage resistance develops, the bacteria increase antibiotic sensitivity (Burmeister et al., 2020). To achieve a better understanding of these associations, Burmeister et al. (2020) screened 33 commercial and environmental *E. coli* phages for their dependence on the antibiotic efflux pump gene *tolC*. They identified phage U136, which depends on the core of the LPS and the antibiotic resistance gene *tolC*. Burmeister et al. (2020) also noted that U136B selects host mutants with genes encoding its essential host entry elements, *tolC* and LPS. These phage-resistant mutants exhibited phenotypic modifications to their tetracycline sensitivity, which was facilitated by *tolC*, as well as colistin, stimulated by LPS associated elements, or both (Burmeister et al., 2020). These results demonstrate the potential to reverse the existing antibiotic resistance and potentially alleviate some of the public health problems associated with treatment by antibiotics.

Lysogenic bacteriophages of *Campylobacter* species have been the emphasis of numerous studies directed at comprehending the complicated relations that have become established between bacteria and viruses throughout the millions of years of co-existence (Hooton et al., 2020). Studying lysogenic phages of *Campylobacter* species have been used to assess different protein expression responses in host cells, genomic reshuffling, and methods of resistance to phage infection in *Campylobacter* species such as CRISPR-facilitated immunity and phase variation (Hooton et al., 2020). *C. jejuni* integrative elements (CJIE) and Mu-like phage sequences have been previously detected in *Campylobacter* species (Parker et al., 2006; Scott et al., 2007b; Clark et al., 2012). Genomic rearrangements triggered by Mu-like prophages are considered as main features for administering host resistance to phage infection (Hooton et al., 2020). It is commonly recognized that integrated phages can potentially change the virulence phenotype of the host (Brussow et al., 2004). For example, a study implicating *C. jejuni* encoding homologs of Mu-like phages (CJIE1) indicated increased adherence and invasion of cells compared to *C. jejuni* cells lacking integrated phage components (Clark et al., 2012). However, in an analogous study, no statistical differences were detected between the adhesion and presence of CJIE1-like elements (Skarp et al., 2017). In addition, genomic rearrangements by CJIE1-like prophages contributed to the host resistance to phage infection in *Campylobacter* and have been observed to hinder the host's ability to attain extracellular DNA through the natural transformation process (Scott et al., 2007b; Gaasbeek et al., 2009, 2010; Brown et al., 2015).

Campylobacter use various strategies to avoid phages, such as genetic rearrangements, utilization of alternate flagellin, phase variation, and attainment of CRISPR spacers to abolish phage predation (Scott et al., 2007b; Hooton and Connerton, 2015; Lis and Connerton, 2016; Gencay et al., 2018). More

recently, researchers from Singapore-MIT Alliance for Research and Technology (SMART) discovered another new and remarkable type of bacterial defense system. The SspABCD-SspE phosphorothioate (PT) system is different from previously studied mechanisms. For instance, SspE protein inhibits phage replication by nicking the phage DNA rather than degrading dsDNA as seen in previous mechanisms and the protection against bacteriophages is stimulated by sequence-specific PTs (Xiong et al., 2020). Xiong et al. (2020) described this unique defense system for *Vibrio cyclitrophicus*, *E. coli*, and *Streptomyces yokosukanensis*, which have different genetic structures, metabolism, and phenotypes. These findings have expanded the understanding of the diversity of a bacterial defense system. In summary, when designing a phage therapeutic cocktail, the discovered bacterial defense mechanisms must be considered.

CAMPYLOBACTER PHAGE RESISTANCE IN POULTRY PRODUCTION AND STRATEGIES FOR CIRCUMVENTION

The use of phages to control *Campylobacter* in poultry deviates from the more clinical applications of phage therapy because the bacteria are not explicitly pathogenic in birds they reside as a part of the GIT. *Campylobacter* colonize the chicken intestine to a high density and are certainly an optimal phage therapy target (Connerton et al., 2011). However, concerns have been raised that *Campylobacter* will merely develop resistance to phages, making this strategy ultimately ineffective in the long term (Barrow, 2001). Although a multiplicity of spontaneous phage-resistant bacterial mutants develops in *in vitro* populations, resistance to phages has been associated with reduced virulence *in vivo* and reduced survival (Adams, 1959; Connerton et al., 2004; Loc Carrillo et al., 2005; Capparelli et al., 2010). Unlike bacterial resistance to bacteriostatic chemical agents, phages continually evolve to evade host barriers, leading to an evolutionary balance that allows both host and phage to multiply. To achieve success in phage therapy, modifying the balance in favor of phage serves as a momentary opportunity to reduce bacterial numbers, at least in the short term. For the application in preharvest procedures, the return to equilibrium can potentially be avoided by the slaughter of the birds while the *Campylobacter* populations are still reduced and have not yet recovered.

In practice, the required replication for successful phage treatment has its advantages and disadvantages. The benefits are that phage application is economical as there is no need to match the administration dose to the concentration of *Campylobacter* colonization in the bird because the phages will propagate according to the number of hosts present (El-Shibiny et al., 2009). A potential disadvantage is the possible development of bacterial host resistance due to the increased number of replications (El-Shibiny et al., 2009). However, El-Shibiny et al. (2009) stated that this specific disadvantage did not appear to be a concern even 5 days post phage administration during their experiment. Furthermore, the authors hypothesized that the slaughter of birds 2 days following phage therapy would be the most optimal management approach by allowing phage replication and decrease the risk of developing bacterial resistance. Other *in vivo* preharvest studies support this approach (**Table 2**). Thus, phage application closer to the end of poultry production can mitigate selective pressure on *Campylobacter* and prevent the transmission of infection to other birds.

Another strategy to circumvent the potential problem of developing phage resistance is administering phages from different phage groups. Thus, group II and group III phages are capable of binding multiple host cell receptors (Coward et al., 2006; Sørensen et al., 2011). Hammerl et al. (2014) showed that the application of a phage cocktail consisting of the same group of phages contributed to a higher resistance frequency versus the application of one type of phage and a combination of different groups of phages (**Table 2**). However, the administration of group III phage alone or in combination with group II phage yielded a lower resistance rate than when applied in combination with another group III phage (Hammerl et al., 2014). In addition, Hammerl et al. (2014) observed that the resistance to group II phages was much more constant than resistance to phages of group III, which was similar to reports from other studies (Loc Carrillo et al., 2005; Fischer et al., 2013), where host cells rapidly reverted to susceptible types. Therefore, it would be beneficial to examine the resistance potential of all phages of a cocktail to construct the final cocktail of phages from different groups, which vary in their host ranges, lytic spectra, and resistance mechanisms. Furthermore, postharvest phage application to poultry meat may restrict the emergence of phage-resistant strains since *C. jejuni* cannot grow at lower temperatures (Atterbury et al., 2003a). Thus, phage-resistant variants of *C. jejuni* cannot arise under these conditions (Atterbury et al., 2003a).

Several pathogens, such as *C. jejuni* have a phase variation (PV) mechanism that allows the bacterium to rapidly adapt to external environment, specifically for a host-associated bacteria, through modifications of surface structures (Sandhu et al., 2021; Sorensen et al., 2021; Yamamoto et al., 2021). PV occurs *via* hypermutation of simple sequence repeats (SSR) through slipped-strand mispairing during DNA replication (Sandhu et al., 2021). The PV mechanism involves differences of protein expression in an on-off fashion and is located within protein-coding regions within genes that regulate the expression of surface structures, such as CPS and flagella (Parkhill et al., 2000). PV can affect phage infection, as surface structures function as receptors for phages. Due to PV, treatment of birds with phages have shown to produce high degree of resistance by modifications in the formation or expression of receptors (Sandhu et al., 2021).

Through computational analysis considering different mutational structures of the PV within *C. jejuni* and phage F336 interaction in a nutrient-controlled continuous culture system, Sandhu et al. (2021) studied how phage infections effect the evolution of PV in *C. jejuni*. The authors observed that extremely low and extremely high mutation rates are evolutionarily unfavorable and rather evolutionary stable mutation rates are affected by fluctuating density of the phage and the reduction of bacterial numbers. The equilibrium between counter-selection and phage infection can result in the progression of PV phage receptor and maintenance of the PV

receptor-dependent phage (Sandhu et al., 2021). From a practical standpoint, Sandhu et al. (2021) predicted that introducing phage into the system with no continuing bacterial growth, such as in post-harvest applications, may result in nearly 10-fold reduction in bacterial counts. Whereas pre-harvest application of F336 phage should be considered when counter-selection acts only on the phage resistant variants of *C. jejuni* strains or in combination with another phage that binds F336-resistant variants (Sandhu et al., 2021).

Interestingly, Sorensen et al. (2021) indicated that PV *Campylobacter*-dependent phages can imitate their hosts and avoid bacterial resistance. Sorensen et al. (2021) observed that the *Fletchervirus* genus from *Myovirideae* family of *Campylobacter* phages similar to their host contain hypermutable traits, which influence phase variable expression of some of the receptor-binding proteins. In addition, the resulting phenotypically varied phage populations contain sub-populations that can infect the host when PV-receptor is not present, supporting co-existence of phage and host in the shared environment (Sorensen et al., 2021). Phenotypic variability that can be generated by PV can limit duplicability of results with *C. jejuni*. Currently, there does not appear to be routine commercial applications for *Campylobacter* phages, due to *Campylobacter* phase variation and genomic instability. However, this may be resolved with the latest research from Yamamoto et al. (2021) demonstrating a potential to stabilize the *Campylobacter* genome in one phase variation state that could be used to devise practical phage applications.

FUTURE STRATEGIES FOR OPTIMIZING PHAGE APPLICATION IN POULTRY PRODUCTION

Campylobacter phage biocontrol for poultry production appears to have promise. However, several strategies involving current and future developments need to be explored to optimize their efficacy from a practical standpoint. These current and future strategies represent some possible development strategies to improve phage effectiveness and potentially achieve routine commercial application in poultry. One of the considerations for practical phage therapy is developing the appropriate delivery system to apply the bacteriophages. Since phages are self-replicating, single dosing of *Campylobacter* phages in birds may be sufficient, though it depends on high bacterial concentrations (Loc Carrillo and Abedon, 2011). When and where to apply *Campylobacter* phages in poultry processing requires strategic approaches that consider the biology of the bacteriophage and its interaction with target host cells. Capparelli et al. (2010) found an inverse relationship between the incidence of lytic phages and their hosts. Different from what happens with antibiotics, administration of phages when the bacterial count is low may be ineffective. Thus, one phage dose may be appropriate only when the target bacterial population divides rapidly while multiple phage administration doses are employed when bacteria divide slowly (Capparelli et al., 2010). In contrast, Bigwood et al. (2009) observed that a certain threshold of phages was sufficient to decrease the *Campylobacter* counts without the need of excessive replication. In addition, the reduction of *Campylobacter* cells was more significant for lower concentrations of host bacterial cells in the liquid culture. Conveniently, because of their low toxicity and resistance to degradation, phages can be supplied within the drinking water or feed to allow for continuous dosing throughout the rearing period (Kittler et al., 2013).

More radical approaches for applying *Campylobacter* phages in poultry may offer promise to overcome some of the issues encountered with general phage therapy, such as the development of host cell resistance. For example, phage endolysins have a broader host specificity than lytic phages (Siringan et al., 2011). Along these lines, Zampara et al. (2021) suggested the application of phage-derived enzymes instead of lytic phages to overcome some of the problems inherent with intact phages. Endolysins are phage-programmed enzymes that destroy the peptidoglycan layer when externally added leading to osmotic imbalance and cell death (Zampara et al., 2021). Although endolysins have successfully been used in an antibacterial application and can exhibit low development of resistance, their application is currently more suitable toward Gram-positive bacteria, as Gram-negative bacteria contain an outer membrane that inhibits entry of endolysins to the peptidoglycan layer (Gutiérrez and Briers, 2021). Previous studies have successfully utilized fusion of endolysins with binding domains of bacteriocins or polycationic and amphipathic peptides to surmount the outer membrane barrier of Gram-negative bacteria (Lukacik et al., 2012; Yan et al., 2017; Heselpoth et al., 2019). Recently, Zampara et al. (2020) demonstrated successful application of the fusion of phage T5 receptor-binding protein (RBP) and endolysin (Innolysins) against *E. coli*. In their study, the bactericidal activity proved to be dependent on the phage T5 RBP cognate receptor, FhuA.

Based on Zampara et al. (2020) reporting of a successful bactericidal effect of Innolysins against *E. coli*, they (Zampara et al., 2021) applied the same concept against *C. jejuni*. Zampara et al. (2021) demonstrated that the H-fiber derived from a *C. jejuni* integrated elements (CJIEs)-1 like prophage CAMSA2147 functions as a unique RBP. Zampara et al. (2021) designed Innolysins that target *C. jejuni* by fusing the H-fiber and T5 endolysin. Zampara et al. (2021) noted that the application of Innolysins on chicken skin at 5°C contaminated with *C. jejuni* CAMSA2147 led to an average of 1.4 $\log_{10}$ reduction of bacteria, indicating that Innolysins can eradicate *C. jejuni in situ*. Zampara et al. (2021) concluded that the H-fiber potentially recognizes a different receptor on the host cells than the lytic *C. jejuni* phages that bind to the host cell CPS or flagella.

Phage intervention strategy may also be advantageous during the somewhat static steps of poultry processing, where slow *Campylobacter* growth occurs, such as poultry plant surfaces that may contain biofilms, at the endpoints of processing, and poultry plants that utilize reuse water systems. Phage application can be exceptionally favorable in water reuse systems, which use filters to remove large chemical particles but would allow phages to pass through, offering the potential for a single cocktail to be utilized multiple times as an ongoing component of the recycled water (Mannapperuma and Santos, 2004; Meneses et al., 2017; Micciche et al., 2018). Theoretically, phages can persist in water indefinitely, although inactivation does occur

(Pinon and Vialette, 2018). Thus, the investigation of phage biocontrol's reuse possibilities within poultry processing water, such as carcass wash waters and chilled water, is warranted. Optimizing *Campylobacter* phage administration in poultry processing water and reuse water will require determining whether inactivation problems occur and can be overcome. It also would be worth determining whether *Campylobacter* phage can remain active in the presence of antimicrobials such as acids typically used in poultry processing plants. If phages remain viable and sustain their lytic properties, the potential of introducing phages as a component of multiple hurdle interventions that include non-phage antimicrobials such as acids would be highly attractive.

Bacteriophages may hold other advantages for application in the poultry processing plant. Due to their very low toxicity and specific host range (Loc Carrillo and Abedon, 2011), large quantities of phages could be added to processing waters with little to no risk to workers, which can concern when employing traditional chemical sanitizers. Most lytic phages, including *Campylobacter* phages, do not produce toxic by-products and have little to no harmful effect on humans or birds (Skurnik et al., 2007; Abedon et al., 2011; Loc Carrillo and Abedon, 2011). However, it has been noted that phages can interact with host immune systems, resulting in a harmful but reversible immune response (Alisky et al., 1998; Kutateladze and Adamia, 2010). In addition, since phages are grown and isolated from cultures containing pathogenic host cell bacteria, improper purification can lead to pathogenic bacterial components triggering severe immune responses (Skurnik and Strauch, 2006; Skurnik et al., 2007). To overcome this, ion-exchange chromatography or high-speed centrifugation can ensure separation between the phage and residual bacterial components (Bogovazova et al., 1992; Sulakvelidze et al., 2001). Regardless, in preharvest environments, appropriate controls will need to be implemented to optimize the application of phages and the immune response. In addition, unlike traditional sanitizers and regardless of concentration, phages have no deleterious impacts regarding food quality (Greer, 1986, 2005; Barrow and Soothill, 1997).

CONCLUSION

Quantitative risk assessments have concluded that reductions in *Campylobacter* both pre-and postharvest poultry production would potentially mitigate the health risk posed by campylobacteriosis (Havelaar et al., 2007; Lake et al., 2007; Nauta and Havelaar, 2008; Nauta et al., 2009). While bacteriophages may not eliminate *Campylobacter* from chicken ceca or carcass, their ability to reduce bacterial counts represents a promising avenue for eliminating the risk of contamination from a finished product. The precise mechanisms of *Campylobacter* phage lytic activity and host cell resistance must be elucidated to utilize phages for widespread control of *Campylobacter*. Some of these may be overcome by a targeted selection of *Campylobacter* phages and phage cocktails that contain multiple *Campylobacter* families or groups of phages with a wide range of specificities to accommodate host cell variability. Development of non-phage technologies such as isolation of endolysins and fusion with various host cell binding proteins may offer a novel strategy that overcomes *Campylobacter* host cell resistance in a more general fashion.

Evaluation of phage cocktails should also be investigated to remediate biofilms on processing surfaces. However, biofilm extracellular composition depends on the microbial populations present. Therefore, phages that code for polysaccharide depolymerase may effectively be applied with other phages against biofilms comprised of diverse microbial populations. As such, *Campylobacter* phage cocktails should be evaluated against biofilms that contain multiple bacterial genera to assess if *Campylobacter* population reductions are still observed in the presence of these mixed bacterial populations. Ultimately, mixtures of *Campylobacter* phages and non-*Campylobacter* phages may need to be employed to overcome biofilms that consist of fairly complex microbial ecosystems. It would be interesting to determine whether synergism for disruption of these more complex biofilms would occur when a multiple mixed phage cocktail is applied.

Campylobacter phage treatment can be implemented in poultry live bird production and processing operations with appropriate safety and quality practices. Nevertheless, understanding how phages acquire resistance and specifically how *Campylobacter* phages infect their host is essential for optimizing the efficacy under commercial conditions. As *Campylobacter* phage biology is better understood, optimizing their routine application in poultry production should be achievable. Indeed, better search tools for isolating *Campylobacter* phage with more broad-spectrum host specificity and optimizing delivery systems for maximum efficacy should offer incremental improvements. However, equally important will be the strategic application of *Campylobacter* phages within the various phases of poultry production. For example, postharvest employment during poultry processing may result in reducing risk more effectively than elsewhere. However, even within poultry processing, certain stages such as those that involve carcass rinses or reuse water may be more optimal delivery systems. With potential developments in phage technologies and a more strategic application management approach in both pre-and postharvest poultry environments, *Campylobacter* phages offer a viable potential hurdle for administration at multiple places throughout poultry production.

AUTHOR CONTRIBUTIONS

EO wrote the latest version of the manuscript. SR helped with writing and editing of the manuscript. AM, YY, and MR contributed to the first draft of the manuscript. All authors listed approved the manuscript for publication.

FUNDING

AM was supported by a Distinguished Doctoral Fellowship and support from the Department of Food Science at the University of Arkansas.

REFERENCES

Abedon, S. (2011). Chapter 1 – Phage therapy pharmacology: calculating phage dosing. *Adv. Appl. Microbiol.* 77, 1–40. doi: 10.1016/B978-0-12-387044-5.00001-7

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., and Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage* 1, 66–85. doi: 10.4161/bact.1.2.15845

Ackermann, H. W. (2009). Basic phage electron microscopy. *Methods Mol. Biol.* 501, 113–126. doi: 10.1007/978-1-60327-164-6_12

Adams, M. (1959). *Bacteriophages*. New York, NY: Wiley Interscience.

Alisky, J., Iczkowski, K., Rapoport, A., and Troitsky, N. (1998). Bacteriophages show promise as antimicrobial agents. *J. Infect.* 36, 5–15. doi: 10.1016/s0163-4453(98)92874-2

Alphen, L. B., Wenzel, C. Q., Richards, M. R., Fodor, C., Ashmus, R. A., Stahl, M., et al. (2014). Biological roles of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*. *PLoS One* 9:e87051. doi: 10.1371/journal.pone.0087051

An, J. U., Ho, H., Kim, J., Kim, W. H., Kim, J., Lee, S., et al. (2018). Dairy cattle, a potential reservoir of human campylobacteriosis: epidemiological and molecular characterization of *Campylobacter jejuni* from cattle farms. *Front. Microbiol.* 9:3136. doi: 10.3389/fmicb.2018.03136

Arnold, J. W., and Silvers, S. (2000). Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. *Poult. Sci.* 79, 1215–1221. doi: 10.1093/ps/79.8.1215

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E., and Connerton, I. F. (2003b). Isolation and characterization of *Campylobacter bacteriophages* from retail poultry. *Appl. Environ. Microbiol.* 69, 4511–4518. doi: 10.1128/aem.69.8.4511-4518.2003

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E., and Connerton, I. F. (2003a). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 69, 6302–6306. doi: 10.1128/AEM.69.10.6302-6306.2003

Atterbury, R. J., Dillon, E., Swift, C., Connerton, P. L., Frost, J. A., Dodd, C. E., et al. (2005). Correlation of *Campylobacter bacteriophage* with reduced presence of hosts in broiler chicken ceca. *Appl. Environ. Microbiol.* 71, 4885–4887. doi: 10.1128/AEM.71.8.4885-4887.2005

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Barrow, P. A. (2001). The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. *J. Chem. Technol. Biotechnol.* 76, 677–682. doi: 10.1002/jctb.436

Barrow, P. A., and Soothill, S. (1997). Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends Genet.* 5, 268–271. doi: 10.1016/S0966-842X(97)01054-8

Bastías, R., Higuera, G., Sierralta, W., and Espejo, R. T. (2010). A new group of cosmopolitan bacteriophages induce a carrier state in the pandemic strain of *Vibrio parahaemolyticus*. *Environt. Microbiol.* 12, 990–1000. doi: 10.1111/j.1462-2920.2010.02143.x

Berrang, M. E., Buhr, R. J., and Cason, J. A. (2000). *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poult. Sci.* 79, 286–290. doi: 10.1093/ps/79.2.286

Bigwood, T., Hudson, J. A., and Billington, C. (2009). Influence of host and bacteriophage concentrations on the inactivation of food-borne pathogenic bacteria by two phages. *FEMS Microbiol. Lett.* 291, 59–64. doi: 10.1111/j.1574-6968.2008.01435.x

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., and Heinemann, J. A. (2008). Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiol.* 25, 400–406. doi: 10.1016/j.fm.2007.11.003

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., and Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157, 472–479. doi: 10.1093/infdis/157.3.472

Bogovazova, G. G., Voroshilova, N. N., Bondarenko, V. M., Gorbatkova, G. A., Afanas'eva, E. V., Kazakova, T. B., et al. (1992). Immunobiological properties and therapeutic effectiveness of preparations from *Klebsiella bacteriophages*. *Zh. Mikrobiol. Epidemiol. Immunobiol.* 3, 30–33.

Bradde, S., Vucelja, M., Teşileanu, T., and Balasubramanian, V. (2017). Dynamics of adaptive immunity against phage in bacterial populations. *PLoS Comput. Biol.* 13:e1005486. doi: 10.1371/journal.pcbi.1005486

Brathwaite, K. J., Siringan, P., Moreton, J., Wilson, R., and Connerton, I. F. (2013). Complete genome sequence of universal bacteriophage host strain *Campylobacter jejuni* subsp. jejuni PT14. *Genome Announc.* 1:e00969-13. doi: 10.1128/genomeA.00969-13

Brown, C. M., and Bidle, K. D. (2014). Attenuation of virus production at high multiplicities of infection in *Aureococcus anophagefferens*. *Virol. J.* 466, 71–81. doi: 10.1016/j.virol.2014.07.023

Brown, H. L., Hanman, K., Reuter, M., Betts, R. P., and van Vliet, A. H. (2015). *Campylobacter jejuni* biofilms contain extracellular DNA and are sensitive to DNase I treatment. *Front. Microbiol.* 6:699. doi: 10.3389/fmicb.2015.00699

Brussow, H., Canchaya, C., and Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.* 68, 560–602. doi: 10.1128/MMBR.68.3.560-602.2004

Burmeister, A. R., Fortier, A., Roush, C., Lessing, A. J., Bender, R. G., Barahman, R., et al. (2020). Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proc. Natl. Acad. Sci. U.S.A.* 117, 11207–11216. doi: 10.1073/pnas.1919888117

Cairns, B. J., Timms, A. R., Jansen, V. A., Connerton, I. F., and Payne, R. J. (2009). Quantitative models of *in vitro* bacteriophage–host dynamics and their application to phage therapy. *PLoS Pathog.* 5:e1000253. doi: 10.1371/journal.ppat.1000253

Callaway, T. R., Anderson, R. C., Edrington, T. S., Genovese, K. J., Harvey, R. B., Poole, T. L., et al. (2004). Recent pre-harvest supplementation strategies to reduce carriage and shedding of zoonotic enteric bacterial pathogens in food animals. *Anim. Health Res. Rev.* 5, 35–47. doi: 10.1079/ahr200462

Capparelli, R., Nocerino, N., Iannaccone, M., Ercolini, D., Parlato, M., Chiara, M., et al. (2010). Bacteriophage therapy of *Salmonella enterica*: a fresh appraisal of bacteriophage therapy. *J. Infect. Dis.* 201, 52–61. doi: 10.1086/648478

Carvalho, C. M., Gannon, B. W., Halfhide, D. E., Santos, S. B., Hayes, C. M., Roe, J. M., et al. (2010). The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiol.* 10:232. doi: 10.1186/1471-2180-10-232

Carvalho, C. M., Santos, S. B., Kropinski, A. M., Ferreira, E. C., and Azeredo, J. (2012). *Phages as Therapeutic Tools to Control Major Foodborne Pathogens: Campylobacter and Salmonella, Bacteriophages, Ipek Kurtboke*. London: IntechOpen.

Centers for Disease Control and Prevention [CDC] (2019). *Campylobacteriosis. Information for Health Professionals*. Atlanta: CDC.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., and Buret, A. (1999). The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* 37, 1771–1776. doi: 10.1128/JCM.37.6.1771-1776.1999

Chapman, B., Otten, A., Fazil, A., Ernst, N., and Smith, B. A. (2016). A review of quantitative microbial risk assessment and consumer process models for *Campylobacter* in broiler chickens. *Microb. Risk Anal.* 2, 3–15. doi: 10.1016/j.mran.2016.07.001

Chibani-Chennoufi, S., Bruttin, A., Dillmann, M. L., and Brüssow, H. (2004). Phage-host interaction: an ecological perspective. *J. Bacteriol. Res.* 186, 3677–3686. doi: 10.1128/JB.186.12.3677-3686.2004

Clark, C. G., Grant, C. C., Pollari, F., Marshall, B., Moses, J., Tracz, D. M., et al. (2012). Effects of the *Campylobacter jejuni* CJIE1 prophage homologs on adherence and invasion in culture, patient symptoms, and source of infection. *BMC Microbiol.* 12:269. doi: 10.1186/1471-2180-12-269

Connerton, I. F., Connerton, P. L., Barrow, P., Seal, B. S., and Atterbury, R. J. (2008). "Bacteriophage therapy and *Campylobacter*," in *Campylobacter*, 3rd Edn, eds I. Nachamkin, C. M. Szymanski, and M. J. Blaser (Washington, DC: ASM Press), 679–693. doi: 10.1128/9781555815554.ch38

Connerton, P. L., Loc Carrillo, C. M., Swift, C., Dillon, E., Scott, A., Rees, C. E., et al. (2004). Longitudinal study of *Campylobacter jejuni* bacteriophages and their hosts from broiler chickens. *Appl. Environ. Microbiol.* 70, 3877–3883. doi: 10.1128/AEM.70.7.3877-3883.2004

Connerton, P. L., Richards, P. J., Lafontaine, G. M., O'Kane, P. M., Ghaffar, N., Cummings, N. J., et al. (2018). The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome* 6:88. doi: 10.1186/s40168-018-0477-475

Connerton, P. L., Timms, A. R., and Connerton, I. F. (2011). *Campylobacter* bacteriophages and bacteriophage therapy. *J. Appl. Microbiol.* 111, 255–265. doi: 10.1111/j.1365-2672.2011.05012.x

Coward, C., Grant, A. J., Swift, C., Philp, J., Towler, R., Heydarian, M., et al. (2006). Phase-variable surface structures are required for infection of *Campylobacter jejuni* by bacteriophages. *Appl. Environ. Microbiol.* 72, 4638–4647. doi: 10.1128/AEM.00184-06

Deborde, M., and Von Gunten, U. R. S. (2008). Reactions of chlorine with inorganic and organic compounds during water treatment—kinetics and mechanisms: a critical review. *Water Res.* 42, 13–51. doi: 10.1016/j.watres.2007.07.025

Deng, W., Dittoe, D. K., Pavilidis, H. O., Chaney, W. E., Yang, Y., and Ricke, S. C. (2020). Current perspectives and potential of probiotics to limit foodborne *Campylobacter* in poultry. *Front. Microbiol.* 11:583429. doi: 10.3389/fmicb.2020.583429

Dufrenne, J., Ritmeester, W., Asch, E. D. V., Van Leusden Frans, A., and De Jonge, R. (2001). Quantification of the contamination of chicken and chicken products in the Netherlands with *Salmonella* and *Campylobacter*. *J. Food Prot.* 64, 538–541. doi: 10.4315/0362-028x-64.4.538

Dugar, G., Herbig, A., Förstner, K. U., Heidrich, N., Reinhardt, R., Nieselt, K., et al. (2013). High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet.* 9:e1003495. doi: 10.1371/journal.pgen.1003495

El-Shibiny, A., Connerton, P. L., and Connerton, I. F. (2005). Enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens. *Appl. Environ. Microbiol.* 71, 1259–1266. doi: 10.1128/aem.71.3.1259-1266.2005

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P., and Connerton, I. (2009). Application of a group II *Campylobacter bacteriophage* to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *J. Food Prot.* 72, 733–740. doi: 10.4315/0362-028x-72.4.733

Elvers, K. T., Morris, V. K., Newell, D. G., and Allen, V. M. (2011). Molecular tracking, through processing, of *Campylobacter* strains colonizing broiler flocks. *Appl. Environ. Microbiol.* 77, 5722–5729. doi: 10.1128/AEM.02419-2410

European Food Safety Authority [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.

European Food Safety Authority [EFSA] (2020). Update and review of control options for *Campylobacter* in broilers at primary production. *EFSA J.* 18:6090. doi: 10.2903/j.efsa.2020.6090

Fischer, S., Kittler, S., Klein, G., and Glünder, G. (2013). Impact of a single phage and a phage cocktail application in broilers on reduction of *Campylobacter jejuni* and development of resistance. *PLoS One* 8:e78543. doi: 10.1371/journal.pone.0078543

Flemming, H. C. (2008). *Biofilms. The Encyclopedia of Life Sciences*. Chichester: John Wiley and Sons.

Fletcher, R. D. (1968). Activity and morphology of *Vibrio coli* phage. *Am. J. Vet. Res.* 26, 361–364.

Fletcher, R. D., and Bertschinger, H. U. (1964). A method of isolation of *Vibrio coli* from swine fecal material by selective filtration. *Zentralbl. Veterinärmed. Reihe B* 11, 469–474. doi: 10.1111/j.1439-0450.1964.tb01075.x

Frost, J. A., Kramer, J. M., and Gillanders, S. A. (1999). Phage typing of *Campylobacter jejuni* and *Campylobacter coli* and its use as an adjunct to serotyping. *Epidemiol. Infect.* 123, 47–55. doi: 10.1017/s095026889900254x

Gaasbeek, E. J., Wagenaar, J. A., Guilhabert, M. R., van Putten, J. P., Parker, C. T., and van der Wal, F. J. (2010). Nucleases encoded by the integrated elements CJIE2 and CJIE4 inhibit natural transformation of *Campylobacter jejuni*. *J. Bacteriol.* 192, 936–941. doi: 10.1128/JB.00867-09

Gaasbeek, E. J., Wagenaar, J. A., Guilhabert, M. R., Wosten, M. M., van Putten, J. P., van der Graaf-van Bloois, L., et al. (2009). A DNase encoded by integrated element CJIE1 inhibits natural transformation of *Campylobacter jejuni*. *J. Bacteriol.* 191, 2296–2306. doi: 10.1128/JB.01430-08

García-Sánchez, L., Melero, B., Jaime, I., Hänninen, M.-L., Rossi, M., and Rovira, J. (2017). Campylobacter jejuni survival in a poultry processing plant environment. *Food Microbiol.* 65, 185–192. doi: 10.1016/j.fm.2017.02.009

Gencay, Y. E., Birk, T., Sørensen, M. C. H., and Brøndsted, L. (2017). "Methods for isolation, purification, and propagation of bacteriophages of *Campylobacter jejuni*," in *Campylobacter jejuni Methods in Molecular Biology*, Vol. 1512, eds J. Butcher and A. Stintzi (New York, NY: Humana Press). doi: 10.1007/978-1-4939-6536-6_3

Gencay, Y. E., Sørensen, M. C., Wenzel, C. Q., Szymanski, C. M., and Brøndsted, L. (2018). Phase variable expression of a single phage receptor in *Campylobacter jejuni* NCTC12662 influences sensitivity toward several diverse CPS-dependent phages. *Front. Microbiol.* 9:82. doi: 10.3389/fmicb.2018.00082

Gilbert, S. E., Whyte, R., Bayne, G., Paulin, S. M., Lake, R. J., and van der Logt, P. (2007). Survey of domestic food handling practices in New Zealand. *Int. J. Food Microbiol.* 117, 306–311. doi: 10.1016/j.ijfoodmicro.2007.05.004

Goode, D., Allen, V. M., and Barrow, P. A. (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Appl. Environ. Microbiol.* 69, 5032–5036. doi: 10.1128/AEM.69.8.5032-5036.2003

Goodridge, L. D., and Bisha, B. (2011). Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage* 1, 130–137. doi: 10.4161/bact.1.3.17629

Grajewski, B. A., Kusek, J. W., and Gelfand, H. M. (1985). Development of a bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli*. *J. Clin. Microbiol.* 22, 13–18. doi: 10.1128/jcm.22.1.13-18.1985

Green, M. R., and Sambrook, J. (2012). *Molecular Cloning: A Laboratory Manual*, Fourth Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Greer, G. G. (1986). Homologous bacteriophage control of *Pseudomonas* growth and beef spoilage. *J. Food Prot.* 49, 104–109. doi: 10.4315/0362-028x-49.2.104

Greer, G. G. (2005). Bacteriophage control of foodborne bacteria. *J. Food Prot.* 68, 1102–1111. doi: 10.4315/0362-028x-68.5.1102

Grissa, I., Vergnaud, G., and Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res.* 35, W52–W57. doi: 10.1093/nar/gkm360

Gunther, N. W., and Chen, C. Y. (2009). The biofilm forming potential of bacterial species in the genus *Campylobacter*. *Food Microbiol.* 26, 44–51. doi: 10.1016/j.fm.2008.07.012

Gutiérrez, D., and Briers, Y. (2021). Lysins breaking down the walls of Gram negative bacteria, no longer a no-go. *Curr. Opin. Biotechnol.* 68, 15–22. doi: 10.1016/j.copbio.2020.08.014

Hald, B., Rattenborg, E., and Madsen, M. (2001). Role of batch depletion of broiler houses on the occurrence of *Campylobacter* spp. in chicken flocks. *Lett. Appl. Microbiol.* 32, 253–256. doi: 10.1046/j.1472-765x.2001.00896.x

Hammerl, J. A., Jäckel, C., Alter, T., Janzcyk, P., Stingl, K., Knüver, M. T., et al. (2014). Reduction of *Campylobacter jejuni* in broiler chicken by successive application of group II and group III phages. *PLoS One* 9:e114785. doi: 10.1371/journal.pone.0114785

Hammerl, J. A., Jäckel, C., Reetz, J., Beck, S., Alter, T., Lurz, R., et al. (2011). *Campylobacter jejuni* group III phage CP81 contains many T4-like genes without belonging to the T4-type phage group: implications for the evolution of T4 phages. *J. Virol.* 85, 8597–8605. doi: 10.1128/JVI.00395-11

Hanning, I., Biswas, D., Herrera, P., Roesler, M., and Ricke, S. C. (2010). Prevalence and characterization of *Campylobacter jejuni* isolated from pasture flock poultry. *J. Food Sci.* 75, M496–M502. doi: 10.1111/j.1750-3841.2010.01747.x

Hansen, V. M., Rosenquist, H., Baggesen, D. L., Brown, S., and Christensen, B. B. (2007). Characterization of *Campylobacter* phages including analysis of host range by selected *Campylobacter* Penner serotypes. *BMC Microbiol.* 7:90. doi: 10.1186/1471-2180-7-90

Harada, L. K., Silva, E. C., Campos, W. F., Del Fiol, F. S., Vila, M., Dabrowska, K., et al. (2018). Biotechnological applications of bacteriophages: state of the art. *Microbiol. Res.* 212-213, 38–58. doi: 10.1016/j.micres.2018.04.007

Harper, D. R., Parracho, H. M. R. T., Walker, J., Sharp, R., Hughes, G., Werthen, M., et al. (2014). Bacteriophages and biofilms. *Antibiotics* 3, 270–284. doi: 10.3390/antibiotics3030270

Havelaar, A. H., Haagsma, J. A., Mangen, M. J., Kemmeren, J. M., Verhoef, L. P., and Vijgen, S. M. (2012). Disease burden of foodborne pathogens in the Netherlands. *Int. J. Food Microbiol.* 156, 231–238. doi: 10.1016/j.ijfoodmicro.2012.03.029

Havelaar, A. H., Mangen, M. J. J., De Koeijer, A. A., Bogaardt, M.-J., Evers, E. G., Jacobs- Reitsma, W. F., et al. (2007). Effectiveness and efficiency of controlling *Campylobacter* on broiler chicken meat. *Risk Anal.* 27, 831–844. doi: 10.1111/j.1539-6924.2007.00926.x

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., et al. (2011). *Campylobacter* control in poultry by current

intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol.* 152, 219–228. doi: 10.1016/j.vetmic.2011.03.010

Heselpoth, R. D., Euler, C. W., and Schuch, R. (2019). Lysocins: bioengineered antimicrobials that deliver lysins across the outer membrane of Gram-negative bacteria. *Antimicrob. Agents Chemother.* 63:e00342-19. doi: 10.1128/aac.00342-19

Hooton, S., and Connerton, I. F. (2015). *Campylobacter jejuni* acquire new host-derived CRISPR spacers when in association with bacteriophages harboring a CRISPR-like Cas4 protein. *Front. Microbiol.* 5:744. doi: 10.3389/fmicb.2014.00744

Hooton, S., D'Angelantonio, D., Hu, Y., Connerton, P. L., Aprea, G., and Connerton, I. F. (2020). *Campylobacter bacteriophage* DA10: an excised temperate bacteriophage targeted by CRISPR-cas. *BMC Genomics* 21:400. doi: 10.1186/s12864-020-06808-3

Huang, H. H., Hoang, M. D., Masuda, Y., Honjoh, K., and Miyamoto, T. (2020). Characterization and utilization of phages specific to *Campylobacter coli*. *Access Microbiol.* 2:18. doi: 10.1099/acmi.fis2019.po0112

Hughes, K. A., Sutherland, I. W., and Jones, M. V. (1998). Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* 144, 3039–3047. doi: 10.1099/00221287-144-11-3039

Humphrey, T. J., Henley, A., and Lanning, D. G. (1993). The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. *Epidemiol. Infect.* 110, 601–607. doi: 10.1017/s0950268800051025

Humphrey, T. J., and Lanning, D. G. (1987). *Salmonella* and *Campylobacter* contamination of broiler chicken carcasses and scald tank water: the influence of water pH. *J. Appl. Bacteriol.* 63, 21–25. doi: 10.1111/j.1365-2672.1987.tb02413.x

Hwang, S., Yun, J., Kim, K. P., Heu, S., Lee, S., and Ryu, S. (2009). Isolation and characterization of bacteriophages specific for *Campylobacter jejuni*. *Microbiol. Immun.* 53, 559–566. doi: 10.1111/j.1348-0421.2009.00163.x

Indikova, I., Humphrey, T. J., and Hilbert, F. (2015). Survival with a helping hand: *Campylobacter* and microbiota. *Front. Microbiol.* 6:1266. doi: 10.3389/fmicb.2015.01266

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J. Bacteriol.* 169, 5429–5433. doi: 10.1128/jb.169.12.5429-5433.1987

Jäckel, C., Hammerl, J. A., and Hertwig, S. (2019). *Campylobacter* phage isolation and characterization: what we have learned so far. *Methods Protoc.* 2:18. doi: 10.3390/mps2010018

Jäckel, C., Hammerl, J. A., Rau, J., and Hertwig, S. (2017). A multiplex real-time PCR for the detection and differentiation of *Campylobacter* phages. *PLoS One* 12:e0190240. doi: 10.1371/journal.pone.0190240

Jäckel, C., Hammerl, J. A., Reetz, J., Kropinski, A. M., and Hertwig, S. (2015). *Campylobacter* group II phage CP21 is the prototype of a new subgroup revealing a distinct modular genome organization and host specificity. *BMC Genom.* 16:629. doi: 10.1186/s12864-015-1837-1

Janež, N., Kokošin, A., Zaletel, E., Vranac, T., Kovač, J., Vučković, D., et al. (2014). Identification and characterisation of new *Campylobacter* group III phages of animal origin. *FEMS Microbiol. Lett.* 359, 64–71. doi: 10.1111/1574-6968.12556

Janez, N., and Loc-Carillo, C. (2013). Use of phages to control *Campylobacter* spp. *J. Microbiol. Method* 95, 68–75. doi: 10.1016/j.mimet.2013.06.024

Jarling, M., Bartkowiak, K., Robenek, H., Pape, H., and Meinhardt, F. (2004). Isolation of phages infecting *Actinoplanes* SN223 and characterization of two of these viruses. *Appl. Microbiol. Biotechnol.* 64, 250–254. doi: 10.1007/s00253-003-1473-6

Jauk, V., Neubauer, C., Szölgyényi, W., and Vasicek, L. (2003). Phenotypic and genotypic differentiation of *Campylobacter* spp. isolated from Austrian broiler farms: a comparison. *Avian Pathol.* 32, 33–37. doi: 10.1080/0307945021000070697

Javed, M. A., Ackermann, H. W., Azeredo, J., Carvalho, C. M., Connerton, I., Evoy, S., et al. (2014). A suggested classification for two groups of *Campylobacter myoviruses*. *Arch. Virol.* 159, 181–190. doi: 10.1007/s00705-013-1788-2

Jiang, F., and Doudna, J. A. (2017). CRISPR–Cas9 structures and mechanisms. *Annu. Rev. Biophys.* 46, 505–529. doi: 10.1146/annurev-biophys-062215-010822

Jones, L. M., Mcduff, C. R., and Wilson, J. B. (1962). Phenotypic alterations in the colonial morphology of Brucella abortus due to a bacteriophage carrier state. *J. Bacteriol.* 83, 860–866. doi: 10.1128/jb.83.4.860-866.1962

Kasman, L. M., Kasman, A., Westwater, C., Dolan, J., Schmidt, M. G., and Norris, J. S. (2002). Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. *J. Virol.* 76, 5557–5564. doi: 10.1128/jvi.76.11.5557-5564.2002

Kiljunen, S., Hakala, K., Pinta, E., Huttunen, S., Pluta, P., Gador, A., et al. (2005). *Yersiniophage* phiR1-37 is a tailed bacteriophage having a 270 kb DNA genome with thymidine replaced by deoxyuridine. *Microbiology* 151, 4093–4102. doi: 10.1099/mic.0.28265-0

Kim, S. A., Jang, M. J., Kim, S. Y., Yang, Y., Pavlidis, H. O., and Ricke, S. C. (2019). Potential for prebiotics as feed additives to limit foodborne *Campylobacter* establishment in the poultry gastrointestinal tract. *Front. Microbiol.* 10:91. doi: 10.3389/fmicb.2019.00091

Kittler, S., Fischer, S., Abdulmawjood, A., Glünder, G., and Klein, G. (2013). Effect of bacteriophage application on *Campylobacter jejuni* loads in commercial broiler flocks. *Appl. Environ. Microbiol.* 79, 7525–7533. doi: 10.1128/AEM.02703-13

Korczak, B. M., Stieber, R., Emler, S., Burnens, A. P., Frey, J., and Kuhnert, P. (2006). Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. *Int. J. Syst. Evol. Microbiol.* 56, 937–945. doi: 10.1099/ijs.0.64109-64100

Kutateladze, M., and Adamia, R. (2010). Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol.* 28, 591–595. doi: 10.1016/j.tibtech.2010.08.001

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8:317. doi: 10.1038/nrmicro2315

Lake, R., Hudson, A., Cressey, P., and Bayne, G. (2007). *Quantitative Risk Model: Campylobacter spp. in the Poultry Food Chain*. Christchurch: Institute of Environmental Science and Research Limited, 1–91.

Levin, B. R., and Bull, J. J. (2004). Population and evolutionary dynamics of phage therapy. *Nat. Rev. Microbiol.* 2, 166–173. doi: 10.1038/nrmicro822

Li, K., Barksdale, L., and Garmise, L. (1961). Phenotypic alterations associated with the bacteriophage carrier state of *Shigella* dysenteriae. *J. Gen. Microbiol.* 24, 355–367. doi: 10.1099/00221287-24-3-355

Lis, L., and Connerton, I. F. (2016). The minor flagellin of *Campylobacter jejuni* (FlaB) confers defensive properties against bacteriophage infection. *Front. Microbiol.* 7:1908. doi: 10.3389/fmicb.2016.01908

Loc Carrillo, C., and Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage* 1, 111–114. doi: 10.4161/bact.1.2.14590

Loc Carrillo, C. L., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl. Environ. Microbiol.* 71, 6554–6563. doi: 10.1128/AEM.71.11.6554-6563.2005

Loc Carrillo, C. M., Connerton, P. L., Pearson, T., and Connerton, I. F. (2007). Free-range layer chickens as a source of *Campylobacter bacteriophage*. *Antonie Van Leeuwenhoek* 92, 275–284. doi: 10.1007/s10482-007-9156-9154

Lu, T. K., and Collins, J. J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. NatL. Acad. Sci. U.S.A.* 104, 11197–11202. doi: 10.1073/pnas.0704624104

Lukacik, P., Barnard, T. J., Keller, P. W., Chaturvedi, K. S., Seddiki, N., Fairman, J. W., et al. (2012). Structural engineering of a phage lysin that targets Gram negative pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9857–9862. doi: 10.1073/pnas.1203472109

Lwoff, A. (1953). Lysogeny. *Bacteriol. Rev.* 17, 269–337.

Mannapperuma, J. D., and Santos, M. R. (2004). Reconditioning of poultry chiller overflow by ultrafiltration. *J. Food Process Eng.* 27, 497–516. doi: 10.1111/j.1745-4530.2004.00474.x

McNally, D. J., Lamoureux, M. P., Karlyshev, A. V., Fiori, L. M., Li, J., Thacker, G., et al. (2007). Commonality and biosynthesis of the O-methyl phosphoramidate capsule modification in *Campylobacter jejuni*. *J. Biol. Chem.* 282, 28566–28576. doi: 10.1074/jbc.M704413200

Meneses, Y. E., Stratton, J., and Flores, R. A. (2017). Water reconditioning and reuse in the food processing industry: current situation and challenges. *Trends Food Sci. Technol.* 61, 72–79. doi: 10.1016/j.tifs.2016.12.008

Micciche, A. C., Rubinelli, P. M., and Ricke, S. C. (2018). Source of water and potential sanitizers and biological antimicrobials for alternative poultry

processing food safety applications. *Front. Sustain. Food Syst.* 2:82. doi: 10.3389/fsufs.2018.00082

Mills, S. D., Kuzniar, B., Shames, B., Kurjanczyk, L. A., and Penner, J. L. (1992). Variation of the O antigen of *Campylobacetr jejuni in vivo*. *J. Med. Microbiol.* 36, 215–219. doi: 10.1099/00222615-36-3-215

Nauta, M., Hill, A., Rosenquist, H., Brynestad, S., Fetsch, A., van der Logt, P., et al. (2009). A comparison of risk assessments on *Campylobacter* in broiler meat. *Int. J. Food Microbiol.* 129, 107–123. doi: 10.1016/j.ijfoodmicro.2008.12.001

Nauta, M. J., and Havelaar, A. H. (2008). Risk-based standards for *Campylobacter* in the broiler meat chain. *Food Control.* 19, 372–381. doi: 10.1016/j.foodcont.2007.04.016

Owens, J., Barton, M. D., and Heuzenroeder, M. W. (2013). The isolation and characterization of *Campylobacter jejuni* bacteriophages from free range and indoor poultry. *Vet. Microbiol.* 162, 144–150. doi: 10.1016/j.vetmic.2012.08.017

Parker, C. T., Quinones, B., Miller, W. G., Horn, S. T., and Mandrell, R. E. (2006). Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. *J. Clin. Microbiol.* 44, 4125–4135. doi: 10.1128/JCM.01231-06

Parkhill, J., Wren, B., Mungall, K., Ketley, J., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403:665. doi: 10.1038/35001088

Petty, N. K., Foulds, I. J., Pradel, E., Ewbank, J. J., and Salmobnd, G. P. C. (2007). A generalized transducing phage for the murine pathogen *Citrobacter rodentium*. *Microbiology* 153, 2984–2988. doi: 10.1099/mic.0.2007/008888-0

Pinon, A., and Vialette, M. (2018). Survival of viruses in water. *Intervirology* 61, 214–222. doi: 10.1159/000484899

Rabinovitch, A., Aviram, I., and Zaritsky, A. (2003). Bacterial debris – an ecological mechanism for coexistence of bacteria and their viruses. *J. Theor. Biol.* 224, 377–383. doi: 10.1016/s0022-5193(03)00174-177

Rakhuba, D. V., Kolomiets, E. I., Szwajcer Dey, E., and Novik, G. I. (2010). Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol. J. Microbiol.* 59, 145–155. doi: 10.33073/pjm-2010-023

Rath, D., Amlinger, L., Rath, A., and Lundgren, M. (2015). The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie* 117, 119–128. doi: 10.1016/j.biochi.2015.03.025

Richards, P. J., Connerton, P. L., and Connerton, I. F. (2019). Phage biocontrol of *Campylobacter jejuni* in chickens does not produce collateral effects on the gut microbiota. *Front. Microbiol.* 10:476. doi: 10.3389/fmicb.2019.00476

Rizzo, H., Gregory, L., Beraldi, F., Carvalho, A. F., and Pinheiro, E. S. (2015). *Campylobacter* isolation from the feces of sheep with a history of reproductive disorders bred in the state of Sao Paulo, Brazil. *Semina Ciências Agrárias* 36, 4207–4214. doi: 10.5433/1679-0359.2015v36n6Supl2p4207

Roach, D. R., and Debarbieux, L. (2017). Phage therapy: awakening a sleeping giant. *Emerg. Top. Life Sci.* 1, 93–103. doi: 10.1042/ETLS20170002

Rosenquist, H., Nielsen, N. L., Sommer, H. M., Norrung, B., and Christensen, B. B. (2003). Quantitative risk assessment of human *Campylobacteriosis* associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol.* 83, 87–103. doi: 10.1016/s0168-1605(02)00317-313

Rudi, K., Hoidal, H. K., Katla, T., Johansen, B. K., Nordal, J., and Jakobsen, K. S. (2004). Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. *Appl. Environ. Microbiol.* 70, 790–797. doi: 10.1128/aem.70.2.790-797.2004

Sails, A. D., Wareing, D. R. A., Bolton, F. J., Fox, A. J., and Curry, A. (1998). Characterisation of 16 *Campylobacter jejuni* and *C. coli* typing bacteriophages. *J. Med. Microbiol.* 47, 123–128. doi: 10.1099/00222615-47-2-123

Salama, S., Bolton, F. J., and Hutchinson, D. N. (1989). Improved method for the isolation of *Campylobacter jejuni* and *Campylobacter coli* bacteriophages. *Lett. Appl. Microbiol.* 8, 5–7. doi: 10.1111/j.1472-765x.1989.tb00211.x

Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edn, Vol. 1. New York, NY: Cold Spring Harbor Laboratory Press.

Sandhu, S. K., Bayliss, C. D., and Morozov, A. Y. (2021). How does feedback from phage infections influence evolution of phase variation in *Campylobacter*? *PLoS Comput. Biol.* 17:e1009067. doi: 10.1371/journal.pcbi.1009067

Scherer, K., Bartelt, E., Sommerfeld, C., and Hildebrandt, G. (2006). Comparison of different sampling techniques and enumeration methods for the isolation and quantification of *Campylobacter* spp. in raw retail chicken legs. *Int. J. Food Microbiol.* 108, 115–119. doi: 10.1016/j.ijfoodmicro.2005.08.031

Scott, A. E., Timms, A. R., Connerton, P. L., El-Shibiny, A., and Connerton, I. F. (2007a). Bacteriophage influence *Campylobacter jejuni* types populating broiler chickens. *Environ. Microbiol.* 9, 2341–2353. doi: 10.1111/j.1462-2920.2007.01351.x

Scott, A. E., Timms, A. R., Connerton, P. L., Loc Carrillo, C., Radzum, K. A., and Connerton, I. F. (2007b). Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. *PLoS Pathog.* 3:119. doi: 10.1371/journal.ppat.0030119

Shah, S. A., Erdmann, S., Mojica, F. J., and Garrett, R. A. (2013). Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biol.* 10, 891–899. doi: 10.4161/rna.23764

Sharma, M., Ryu, J. H., and Beuchat, L. R. (2005). Inactivation of *Escherichia coli* O157:H7 in biofilm on stainless steel by treatment with an alkaline cleaner and a bacteriophage. *J. Appl. Microbiol.* 99, 449–459. doi: 10.1111/j.1365-2672.2005.02659.x

Sharp, R. (2001). Bacteriophages: biology, history. *J. Chem. Technol. Biotechnol.* 76, 667–672.

Sheppard, S. K., and Maiden, M. C. (2015). The evolution of *Campylobacter jejuni* and *Campylobacter coli*. *Cold Spring Harb. Perspect. Biol.* 7:a018119. doi: 10.1101/cshperspect.a018119

Sillankorva, S., Neubauer, P., and Azaredo, J. (2011). *Use of Bacteriophages to Control Biofilms*. Saarbrücken: LAP Lambert Academic Publishing.

Siringan, P., Connerton, P. L., Cummings, N. J., and Connerton, I. F. (2014). Alternative bacteriophage life cycles: the carrier state of *Campylobacter jejuni*. *Open Biol.* 4:130200. doi: 10.1098/rsob.130200

Siringan, P., Connerton, P. L., Payne, R. J., and Connerton, I. F. (2011). Bacteriophage mediated dispersal of *Campylobacter jejuni* biofilms. *Appl. Environ. Microbiol.* 77, 3320–3326. doi: 10.1128/AEM.02704-10

Skarp, C. P. A., Akinrinade, O., Kaden, R., Johansson, C., and Rautelin, H. (2017). Accessory genetic content in *Campylobacter jejuni* ST21CC isolates from feces and blood. *Int. J. Med. Microbiol.* 307, 233–240. doi: 10.1016/j.ijmm.2017.04.001

Skurnik, M., Pajunen, M., and Kiljunen, S. (2007). Biotechnological challenges of phage therapy. *Biotechnol. Lett.* 29, 995–1003. doi: 10.1007/s10529-007-9346-1

Skurnik, M., and Strauch, E. (2006). Phage therapy: facts and fiction. *Int. J. Med. Microbiol.* 296, 5–14. doi: 10.1016/j.ijmm.2005.09.002

Sorensen, M. C., Gencay, Y. E., and Brondsted, L. (2017). Methods for initial characterization of *Campylobacter jejuni* bacteriophages. *Methods Mol. Biol.* 1512, 91–105. doi: 10.1007/978-1-4939-6536-6_9

Sørensen, M. C. H., Gencay, Y. E., Birk, T., Baldvinsson, S. B., Jäckel, C., Hammerl, J. A., et al. (2015). Primary isolation strain determines both phage type and receptors recognised by *Campylobacter jejuni* bacteriophages. *PLoS One* 10:e0116287. doi: 10.1371/journal.pone.0116287

Sørensen, M. C. H., Van Alphen, L. B., Frodor, C., Crowley, S., Christensen, B. B., Szymanski, C. M., et al. (2012). Phase variable expression of capsular polysaccharide modifications allows *Campylobacter jejuni* to avoid bacteriophage infection in chickens. *Front. Cell. Infect. Microbiol.* 2:11. doi: 10.3389/fcimb.2012.00011

Sørensen, M. C. H., van Alphen, L. B., Harboe, A., Li, J., Christensen, B. B., Szymanski, C. M., et al. (2011). The F336 bacteriophage recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *J. Bacteriol.* 193, 6742–6749. doi: 10.1128/JB.05276-11

Sorensen, M. C. H., Vitt, A., Neve, H., Soverini, M., Ahern, S. J., Klumpp, J., et al. (2021). *Campylobacter* phages use hypermutabkle polyG tracts to create phenotypic diversity and evade bacterial resistance. *Cell Rep.* 35:109214. doi: 10.1016/j.celrep.2021.109214

Stenutz, R., Weintraub, A., and Widmalm, G. (2006). The structures of *Escherichia coli* O-polysaccharide antigens. *FEMS Microbiol. Rev.* 30, 382–403.

Sulakvelidze, A., Alavidze, Z., and Morris, J. G. (2001). Bacteriophage therapy. *Antimicrob. Agents and Chemother.* 45, 649–659. doi: 10.1128/AAC.45.3.649-659.2001

Torsvik, T., and Dundas, I. D. (1980). Persisting phage infection in *Halobacterium salinarium* str. 1. *J. Gen. Virol.* 47, 29–36. doi: 10.1099/0022-1317-47-1-29

Totten, P. A., Patton, C. M., Tenover, F. C., Barrett, T. J., Stamm, W. E., Steigerwalt, A. G., et al. (1987). Prevalence and characterization of hippurate-negative *Campylobacter jejuni* in King County, Washington. *J. Clin. Microbiol.* 25, 1747–1752.

Tsuei, A. C., Carey-Smith, G. V., Hudson, J. A., Billington, C., and Heinemann, J. A. (2007). Prevalence and numbers of coliphages and *Campylobacter jejuni* bacteriophages in New Zealand foods. *Int. J. Food Microbiol.* 116, 121–125. doi: 10.1016/j.ijfoodmicro.2006.12.028

Umaraw, P., Prajapati, A., Verma, A. K., Pathak, V., and Singh, V. P. (2017). Control of *Campylobacter* in poultry industry from farm to poultry processing unit: a review. *Crit. Rev. Food Sci. Nutr.* 57, 659–665. doi: 10.1080/10408398.2014.935847

Ushanov, L., Lasareishvili, B., Janashia, I., and Zautner, A. E. (2020). Application of *Campylobacter jejuni* phages: challenges and perspectives. *Animals* 10:279. doi: 10.3390/ani10020279

Van der Veen, S., and Abee, T. (2011). Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. *Int. J. Food Microbiol.* 144, 421–431. doi: 10.1016/j.ijfoodmicro.2010.10.029

Vose, D. (2008). *Risk Analysis: A Quantitative Guide, Third ed*. West Sussex: John Wiley & Sons Ltd.

Wagenaar, J. A., Van Bergen, M. A., Mueller, M. A., Wassenaar, T. M., and Carlton, R. M. (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Vet. Microbiol.* 109, 275–283. doi: 10.1016/j.vetmic.2005.06.002

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* 28, 127–181. doi: 10.1016/j.femsre.2003.08.001

Wheeler, T. L., Kalchayanand, N., and Bosilevac, J. M. (2014). Pre-and post-harvest interventions to reduce pathogen contamination in the US beef industry. *Meat Sci.* 98, 372–382. doi: 10.1016/j.meatsci.2014.06.026

Whyte, P., Collins, J. D., McGill, K., Monahan, C., and O'Mahony, H. (2001). Quantitative investigation of the effects of chemical decontamination procedures on the microbiological status of broiler carcasses during processing. *J. Food Protect.* 64, 179–183. doi: 10.4315/0362-028x-64.2.179

World Health Organization [WHO] (2018). *Campylobacter*. Geneva: WHO.

Xiong, X., Wu, G., Wei, Y., Liu, L., Zhang, Y., Su, R., et al. (2020). SspABCD–SspE is a phosphorothioation-sensing bacterial defence system with broad anti-phage activities. *Nat. Microbiol.* 5, 917–928. doi: 10.1038/s41564-020-0700-6

Yamamoto, S., Iyoda, S., and Ohnishi, M. (2021). Stabilizing genetically unstable simple sequence repeats in the *Campylobacter jejuni* genome by multiplex genome editing: a reliable approach for delineating multiple phase-variable genes. *bioRxiv* [Preprint]. doi: 10.1101/2021.05.14.444138

Yan, G., Liu, J., Ma, Q., Zhu, R., Guo, Z., Gao, C., et al. (2017). The N-terminal and central domain of colicin A enables phage lysin to lyse *Escherichia coli* extracellularly. *Antonie Van Leeuwenhoek* 110, 1627–1635. doi: 10.1007/s10482-017-0912-9

Zampara, A., Sorensen, M. C. H., Elsser-Gravesn, A., and Brondsted, L. (2017). Significance of phage-host interactions for biocontrol of *Campylobacter jejuni* in food. *Food Control* 73(Part B), 1169–1175. doi: 10.1016/j.foodcont.2016.10.033

Zampara, A., Sorensen, M. C. H., Gencay, Y. E., Grimon, D., Kristiansen, S. H., Jorgenses, L. S., et al. (2021). Developing innolysins against *Campylobacter jejuni* using a novel prophage receptor-binding protein. *Front. Microbiol.* 12:619028. doi: 10.3389/fmicb.2021.619028

Zampara, A., Sørensen, M. C. H., Grimon, D., Antenucci, F., Vitt, A. R., Bortolaia, V., et al. (2020). Exploiting phage receptor binding proteins to enable endolysins to kill Gram-negative bacteria. *Sci. Rep.* 10:12087. doi: 10.1038/s41598-020-68983-3

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership