ABOUT THE FOODBORNE PATHOGEN CAMPYLOBACTER

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ABOUT THE FOODBORNE PATHOGEN CAMPYLOBACTER

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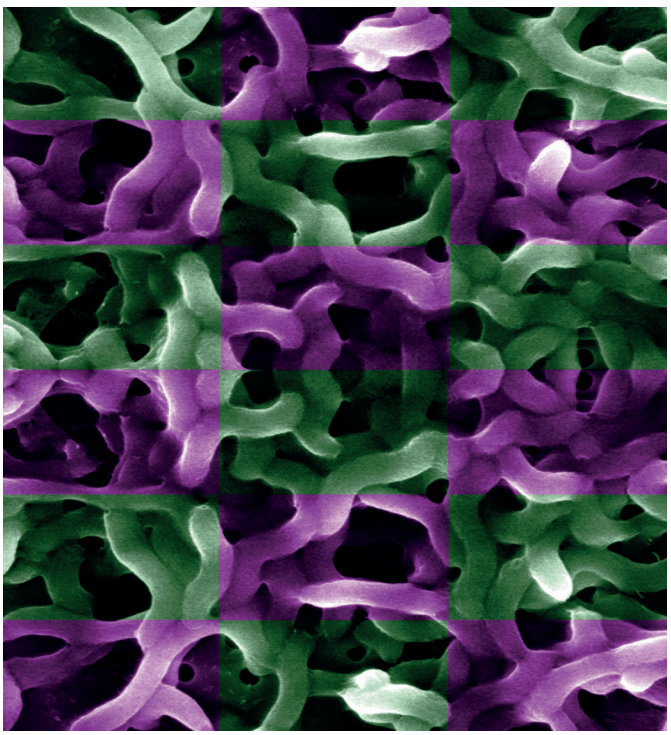

Campylobacter jejuni Bf. Image: Odile Tresse.

A significant increase in the prevalence of campylobacteriosis cases has been observed over the past years. Campylobacter has emerged as the leading cause of bacterial foodborne disease worldwide with a significant impact on human health and an associated economic burdens. Campylobacteriosis human cases have been generally correlated with the handling, preparation and consumption of poultry. In 2017, the European Commission regulation has amended Regulation (EC) No 2073/2005 on the hygiene of foodstuffs as regards Campylobacter on broiler carcasses stating a limit of 1000 cfu/g. Campylobacter is also present in other farm animals and

is frequently found on a range of foodstuffs due to cross contamination. Among the pathogenic species, C. jejuni is the most prevalent species followed by C. coli. Current guidelines highlight the importance of biosecurity but these measures are failing to mitigate the risk of pathogenic Campylobacter. As an obligate microaerophile, Campylobacter does not multiply under atmospheric oxygen concentration at ambient temperatures. It therefore constitutes a puzzle as to how it can survive from farm to retail outlets. The underlying molecular mechanisms of persistence, survival and pathogenesis appear to be unique to this pathogen. Recent research has indicated how genomic polymorphism, restricted catabolic capacity, self regulation or deregulation of genes, bacterial cooperation and unknown contamination routes may be connected to this specificity.

This book includes original studies on both C. jejuni and C. coli species dealing with epidemiology and animal carriage, host interaction, control strategies, metabolism and regulation specificities of these two pathogenic species, methodology to improve cultural techniques and chicken gut microbiota challenged with Campylobacter.

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EDITORIAL
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Editorial: About the Foodborne Pathogen *Campylobacter*

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Editorial on the Research Topic

About the Foodborne Pathogen *Campylobacter*

INTRODUCTION

The name "Campylobacter" comes from ancient Greek meaning "curved rod" which describes the shape of this microorganism. *Campylobacter* was firstly isolated as a *Vibrio* species from epizootic ovine abortion in 1906 by McFadyean and Stockman (1913), and renamed in 1973 as the neotype strain *Campylobacter* after showing significant biological differences with *Vibrio* species (Véron and Chatelain, 1973). Rather than a curved rod, the shape looks more like to a spiral and can develop in to filamentous or coccoid forms under stressful conditions (Tangwatcharin et al., 2006; Ghaffar et al., 2015; Rodrigues et al., 2016). Nowadays, *Campylobacter* spp. are classified among the ε-proteobacteria in the family of Campylobacteriaceae (Vandamme et al., 1991). *Campylobacter* has emerged as the leading cause of bacterial foodborne infections in developed countries, having surpassed *Salmonella* several years ago, and represents a significant economic burden (EFSA and ECDC, 2016). Although new species of *Campylobacter* have been recently discovered, human cases of campylobacterosis are dominated by two main species, *Campylobacter jejuni* and, to a lesser extent, *Campylobacter coli*.

Quantitative epidemiology reports reveal high rates of contamination for broiler chickens and carcasses by *Campylobacter* (Hue et al., 2010; Lawes et al., 2012; Powell et al., 2012). The presence of *Campylobacter* was also detected in other farm animals or foodstuffs due to cross contamination (EFSA and ECDC, 2016). *Campylobacter* in poultry remains a problem with no effective control measures available that can be recommended for microbial food/farm safety guidelines to mitigate the risk of flock colonization. *Campylobacter* also remains a puzzle as to how an obligate microaerobic bacterium can survive from farm to retail outlets. The underlying molecular mechanisms of persistence, survival and pathogenesis appear to represent a combination peculiar to this pathogen, which are not shared with other foodborne bacterial pathogens such as *Listeria monocytogenes*, *Salmonella enterica, Escherichia coli,* and *Staphylococcus aureus*.

This topic includes 18 published articles describing original studies of *C. jejuni* and *C. coli* that deal with (1) epidemiology and animal carriage, (2) host interaction, (3) control strategies, (4) metabolism and regulation specificities of these two pathogen species, (5) methodology to improve cultural technique and (6) chicken gut microbiota challenged with *Campylobacter*.

Epidemiology and Animal Carriage

Organic animal production schemes differ in many ways (antibiotic use, herd structure, feeding regimes, access to outdoor areas, space allowance) from conventional rearing systems, and therefore can have an impact in the occurrence, transmission and pathogenicity of foodborne

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pathogens, including *Campylobacter* spp. In this research topic, is however shown that organic pig production schemes have a minor impact on the epidemiology of *C. coli*. Kempf et al. monitored the prevalence and antimicrobial resistance of *C. coli* isolated from conventional and organic pigs in farms from France and Sweden and observed no significant difference in prevalence between pigs in organic and conventional productions. They however observed in France a higher occurrence of antimicrobial resistant *C. coli* isolates (particularly against the antibiotics tetracycline and erythromycin) from conventional rearing systems. Denis et al. characterized *C. coli* isolates obtained from 19 organic pig farms and 24 conventional pig farms through pulsed field gel electrophoresis, multilocus sequence typing, detection of nine virulence-associated genes and evaluation of the adhesion and invasion capacity on Caco-2 cells. They concluded that pig farm management strategies did not influence the diversity and virulence of *C. coli*.

Host Interactions

Ayllón et al. used a proteomic approach to examine relative differences in protein expression levels between *C. jejuni* interacting with human (INT-407) and porcine (IPEC-1) cell lines. The study revealed 366 differentially expressed proteins after 3 h infection and 485 after 24 h. The identities of the protein enabled analysis of the response pathways that indicate differences in the timing of inflammatory responses between the cell lines and comparative down regulation of the signaling pathways that control cell migration, endocytosis and cell cycle progression in the porcine cell line. The authors attribute the differences in the cellular pathway responses to *C. jejuni* exposure as indicative of the processes that establish either infective or commensal behavior respectively in human or porcine hosts.

Upadhyay et al. examined the potential of three phytochemicals generally recognized as safe (GRAS) and applied at sub-inhibitory concentrations to bacterial growth, to prevent or reduce the severity of human infection. Phytochemical treatments of *C. jejuni* resulted in reduced motility and a reduction in the expression of cytolethal distending toxin that could result in favorable effects on human infection. Using human intestinal epithelial (Caco-2) cell-based assays the authors demonstrated the abilities of trans-cinnamaldehyde (0.01%), carvacrol (0.002%), and eugenol (0.01%) to reduce the processes of attachment, invasion and translocation of *C. jejuni*.

Control Strategies

Effective control of *Campylobacter* on commercial broiler chicken farms is proving more than challenging. Microbiological risk assessments suggest that if reductions in the intestinal loads carried by chickens could be translated on to poultry meat then these measures could make a significant impact on the cases of human campylobacterosis (Boysen et al., 2013). Two studies within the topic report the results of feed supplement additions to broiler chicken diets directed to reduce *Campylobacter* colonization levels. Guyard-Nicodème et al. reported the effects of five treatments applied on a French free-range broiler farm that became naturally positive for *C. jejuni* when the birds went on to range after 35 days. A combination treatment of a cation exchange clay-based product and an organic acid mixture containing formic acid, sodium formate, lactic acid and propionic acid resulted in a modest but significant reduction of $\log_{10}$ 0.82 CFU per g of cecal contents at day 78 when the birds were scheduled for processing. This treatment was also found to be associated with a significant reduction of $\log_{10}$ 0.68 CFU per g on the neck skins of the carcasses of the chickens. Wagle et al. examined the impact of β-resorcylic acid as in-feed supplement and the impact of the phytophenolic on the *in vitro* infection of Caco-2 intestinal epithelial cells. Broiler chickens were challenged with *C. jejuni* at day 7 and fed β-resorcylic acid supplemented feed (0.25 to 1%) until day 14, which resulted in significant reductions up to $\log_{10}$ 2.5 CFU per g of cecal contents. Sub-inhibitory concentration also reduced *C. jejuni* motility and their ability to attach and invade Caco-2 cells.

Bacteriophage therapy is a sustainable biological control that has the potential to reduce *Campylobacter* colonization of broiler chickens (Connerton et al., 2011). Lis and Connerton investigated factors that impact on virulent bacteriophage infection of *C. jejuni*. As discussed above motility is a critical trait for successful intestinal colonization and infection by *C. jejuni*. These studies confirmed that motility is also critical for one class of bacteriophage and required for efficient infection by a second. For *C. jejuni* any loss in motility to escape phage infection would result in an inability to colonize animal hosts. As a response to this Achilles heel, campylobacters utilize a second minor flagellin protein, FlaB, to enable regrowth post phage infection. These populations arise still largely at the expense of motility but have the potential to revert to full motility and survive once separated from the bacteriophage. Although, once motility has been recovered, they will become susceptible once more to bacteriophage infection.

Metabolism and Regulation

Glucose Metabolism

C. jejuni is an asaccharolytic micro-organism characterized by the absence of a functional Embden-Meyerhof-Parnas glycolysis pathway due to the absence of glucokinase (Glk) and phosphofructokinase (Pfk). Gluconeogenesis fueled by amino acids is the main pathway for *C. jejuni* to synthesize anabolic hexose phosphate. The Entner-Doudoroff (ED) pathway is an alternative in bacteria to synthesize pyruvate from extracellular glucose via phosphogluconate in order to bypass the absence of Pfk. In this research topic, the key genes of the ED pathway have been found in the genomes of rare isolates of both *C. jejuni* and *C. coli* (Vegge et al.). A complete gene set encoding a functional ED pathway in these *C. jejuni* and *C. coli* isolates are located on a transferable genomic island, similar to that for the genes involved in the utilization of L-fucose in human gut by few *C. jejuni* (Stahl et al., 2011). Interestingly, the presence of a functional ED pathway gives advantages to these bacterial isolates for survival and biofilm formation (Vegge et al.).

Oxidative Stress Response

Campylobacter species are obligate microaerobic microorganisms characterized by the inability to multiply in ambient levels of oxygen (Macé et al., 2015). However, some

strains are able to better counteract aerobic conditions for their survival. Recently, the atypical *C. jejuni* Bf was described to be able to grow under aerobic conditions in contrast to other *C. jejuni* (Rodrigues et al., 2015, 2016). As for *C. jejuni* Bf, *C. coli* OR12 is also able to develop an habituation to aerobic conditions. In this research topic, the hyperaerotolerance of *C. coli* was found to be associated with an increased resistance to peroxide stress (O'Kane and Connerton). On the other hand, *C. jejuni* is not able to grow anaerobically, even though it can produce functional alternative electron acceptors to O_2, such as fumarate, nitrate, nitrite, trimethylamine-N-oxide or dimethylsulfoxide (Weingarten et al., 2009). This was attributed to the absence of an alternative to the O_2-dependent Class I type of ribonucleotide reductase, an enzyme essential for DNA synthesis (Sellars et al., 2002). In this research topic, the regulation and function of various C4-dicarboxylate components as alternative electron acceptors or transporters in *C. jejuni* were explored. It was suggested that the DctA transporter participates in the uptake of succinate at high oxygen levels while *dcuA* and *dcuB* genes, controlled by RacRS system, are up-regulated in oxygen-limited conditions (Wösten et al.). The main enzymes constituting the sub-system of oxygen detoxification in *C. jejuni* has been described. Nonetheless, the regulation of these enzymes remain elusive as the main regulators previously described to play this role in other Gram-negative bacteria (SoxRS and OxyR regulons) are absent in *C. jejuni* genome. These enzymes could be potentially controlled by the iron homeostasis and transcriptional regulating mechanisms including the essential pleiotropic regulator CosR and the inessential RrpA and RrpB regulators. In this research topic, Gundogdu et al. have brought new insights concerning the roles of RrpA and RrpB in the response to oxidative and aerobic stress conditions. The gene *rrpA* is present in over 99% of *C. jejuni* strains while *rrpB* seems to be restricted to livestock clonal complexes. This latter gene is located to a transferable hypervariable region in association with the type I R-M (*hsd*) system. Consequently, the presence of RrpB suggests a specific adaptation of *C. jejuni* to host.

Phase Transition

Phase transition in bacteria is important for survival and adaptation to harmful conditions. As mentioned earlier, *C. jejuni* is able to modulate its shape in response to environmental conditions. The life cycle of *Campylobacter* is an alternation of states from dormancy to multiplication. Bacterial growth is also punctuated by different phases. The lag phase represents the time for the bacteria to adapt to new conditions, the log phase is characterized by the cell doubling and the stationary phase is a way of survival in growth limiting conditions. The timing of the latter phase is conditioned by the adaptation capability of the cells. It is usually driven by growth factors in response to general stress. In contrast to most of Gram-negative bacteria, the main transcriptional factor, RpoS, which controls the switch between the log and the stationary phases, is absent in *Campylobacter*. Reported in this research topic is the identification of proteins showing significantly different abundances between the two growth phases (Turonova et al.). These proteins belong to biological pathways including metabolism, general and specific oxidative stress response, translation and motility. In addition, the regulator CosR was identified among these differently abundant proteins. The dynamics of the transcript levels of CosR throughout the growth of *C. jejuni* reveal transient differences between the log and stationary phases, suggesting the transcriptional regulator is under negative control. As aforementioned, CosR was originally ascribed a role in the control of enzymes involved in oxygen detoxification. Further analyses indicated that CosR is able to bind to its own promoter region indicating its potential for auto-regulation. The DNA binding consensus sequence of CosR was refined by bioinformatic analysis of the promotor region of CosR and other genes previously described to be able to bind this protein. Although the complete regulatory framework associated with CosR remains to be discovered, these data suggest a major contribution of CosR during the switch between exponential and stationary phases in *C. jejuni*.

Characterization of New Genes

Whole genome sequencing provides comprehensive set of features by which *Campylobacter* demonstrates genetic variation and plasticity. Many well described molecular mechanisms in bacteria are not transferable to *Campylobacter* due to the absence of homologous genes. Consequently, the mutational analysis of specific genes with putative functions continues to turn up new aspects of the biology of *Campylobacter*. For instance, the putative transcriptional regulator Cj0440c, belonging to the TENA/THI-4 family of proteins, could play a role in compensating the fitness cost of erythromycin resistance through a positive relationship with flagellar proteins (Hao et al.). The protein Cj1199 seems to be involved in the leucine biosynthesis and transport but could also indirectly affect the development of erythromycin resistance in *C. jejuni* (Hao et al.). In another study also published in this research topic (Taylor et al.), the analysis of two putative chaperone genes (Cj1289 and Cj0694) was investigated using a mutational approach. The protein Cj0694 is predicted as an inner membrane anchored protein but possesses a peptidyl-prolyl cis/trans isomerase (PPIase) activity, which could be involved in the initial folding and outer membrane translocation of Cj1289, a SurA-like chaperone (SalC). These two proteins likely participate to the outer membrane protein biogenesis and integrity.

Methodology to Improve Cultural Technique

Culture-based isolation methods targeting *Campylobacter* spp. are usually challenging for some type of highly contaminated samples, due to its outgrowth by major competing bacteria in the enrichment conditions. In this research topic, an improved culture-dependent methodology for the selective isolation of *C. jejuni* from wastewater samples is described. Kim et al. assessed a few different enrichment conditions using five different antibiotics (i.e., cefoperazone, vancomycin, trimethoprim, polymyxin B, and rifampicin), to which *C. jejuni* is intrinsically resistant. They showed that *Enterococcus* spp. and *Pseudomonas aeruginosa* are major competing bacteria in the

enrichment conditions and that the addition of polymyxin B, rifampicin or both to the selective media enhanced the selective isolation of *C. jejuni*.

Chicken Gut Microbiota Challenged with *Campylobacter*

The gut microbiota plays an essential role in nutrition, feed conversion, growth performance and protection against pathogenic bacteria such as *Campylobacter* spp. However, and despite the increasing number of articles focused on the gut microbiome of humans and animals, there is little information yet about the diversity and function of the gut microbiota in chickens and its impact on the establishment of certain pathogens, including *Campylobacter* spp. This research topic includes a couple of articles dealing with this issue. Thibodeau et al. assessed the impact of feed supplementation with selenium on the gut microbiota of chickens in a *C. jejuni* colonization model. Results obtained by these authors evidenced that, for healthy chickens raised in good hygienic conditions, selenium-yeast did not influence neither the body weight nor the caecal microbiota or the colonization status by *C. jejuni*. Awad et al. monitored in a longitudinal study from day 1 to day 28 of age the composition and structure of the microbiota of the gut content and the mucosa, as well as the consequences of a *C. jejuni* infection on the gut microbiome. These authors show in their article that the chicken gut microbiota significantly changes during the first 28 days of age, that numerous significant differences in microbial profiles are observed between the mucosa and luminal content of the small and large intestine, and that *C. jejuni* colonization is associated with an alteration of the gut microbiota, which confirms that the *Campylobacter* carrier state in chickens is characterized by multiple changes in the intestinal ecology within the host.

CONCLUSION

Recent researches indicate that genomic polymorphism, restricted catabolic capacity, self-regulation or deregulation of genes, bacterial cooperation and unknown contamination routes may all be connected to the specificity of pathogenic species of *Campylobacter*.

AUTHOR CONTRIBUTIONS

OT prepared the content. OT, IC, and AA wrote the paper.

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ORIGINAL RESEARCH
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Campylobacter coli in Organic and Conventional Pig Production in France and Sweden: Prevalence and Antimicrobial Resistance

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The purpose of the study was to evaluate and compare the prevalence and antimicrobial resistance of *Campylobacter coli* in conventional and organic pigs from France and Sweden. Fecal or colon samples were collected at farms or at slaughterhouses and cultured for *Campylobacter*. The minimum inhibitory concentrations of ciprofloxacin, nalidixic acid, streptomycin, tetracycline, erythromycin, and gentamicin were determined by microdilution for a total of 263 French strains from 114 pigs from 50 different farms and 82 Swedish strains from 144 pigs from 54 different farms. Erythromycin resistant isolates were examined for presence of the emerging rRNA methylase *erm*(B) gene. The study showed that within the colon samples obtained in each country there was no significant difference in prevalence of *Campylobacter* between pigs in organic and conventional productions [France: conventional: 43/58 (74%); organic: 43/56 (77%) and Sweden: conventional: 24/36 (67%); organic: 20/36 (56%)]. In France, but not in Sweden, significant differences of percentages of resistant isolates were associated with production type (tetracycline, erythromycin) and the number of resistances was significantly higher for isolates from conventional pigs. In Sweden, the number of resistances of fecal isolates was significantly higher compared to colon isolates. The *erm*(B) gene was not detected in the 87 erythromycin resistant strains tested.

Keywords: *Campylobacter*, pig, organic production, conventional production, antimicrobial resistance

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INTRODUCTION

The organic pig sector is still a minority production in Europe, accounting in 2010 for approximately 0.33% (livestock units) in the EU-27. Organic production reached 0.9 million heads in 2011 and 0.6% of the total pig production of the EU-15 (European-Commission, 2013). France was the third largest producer (165,518 heads) behind Germany and Denmark (European-Commission, 2013). Previous studies have shown that the European organic production systems may differ considerably between countries with regards to housing,

management, animal health, and welfare issues (Dippel et al., 2014; Edwards et al., 2014; Früh et al., 2014; Prunier et al., 2014a,b). However, the EU regulations for organic farming (Council Directives 2007/834/EC and 2008/889/EC) require that animals have outdoor access. Moreover, the preventive use of chemically synthesized allopathic medicinal products is not permitted. For treatment of sick animals, chemically synthesized allopathic veterinary medicinal products including antibiotics, may be used when necessary. However, this is only allowed when the use of phytotherapeutic, homeopathic, and other products is inappropriate[1]. In addition, if an animal or a group of animals receive more than one course of treatment and if their productive lifecycle is less than 1 year, the livestock concerned, or produce derived from them, may not be sold as organic products.

It may be possible that organic pigs may receive fewer antibiotics than conventionally raised animals and therefore harbor antimicrobial resistant bacteria less frequently (Young et al., 2009). This could indeed represent an interesting advantage as antimicrobial resistance is recognized as a major hazard for farmers, technicians, or veterinarians potentially exposed by direct or indirect contact with animals (Alali et al., 2010; Huijbers et al., 2015), or for consumers exposed via the food chain (Verraes et al., 2013). Thus, two aims of the SafeOrganic project (restrictive use of antibiotics in organic animal farming – a potential for safer, high quality products with less antibiotic resistant bacteria[2]) were to compare antimicrobial resistance of intestinal *Escherichia coli* (Osterberg et al., 2016) and of intestinal *Campylobacter coli* from conventional and organic pigs.

Campylobacteriosis in humans was the most commonly reported zoonosis in the European Union in 2015 (EFSA, 2016). When the species information was provided, *Campylobacter jejuni* accounted for 81.0% and *C. coli* for 8.4% of the human cases although in France the *C. coli* percentage was 15.25% (Sifre et al., 2015). Poultry and pigs are recognized as reservoirs of *Campylobacter* (Quintana-Hayashi and Thakur, 2012) and *C. coli* is the main species in pigs (Avrain et al., 2004). When treatment of human campylobacteriosis is needed, quinolones, macrolides and tetracyclines are the drugs of choice (Sifre et al., 2015).

In this paper we present the results from the SafeOrganic project investigating *C. coli* from pigs sampled in two different countries (Sweden and France) and evaluate differences possibly linked to production type and country. In addition, as macrolide-resistant *C. coli* were detected, the presence of the macrolide-resistance mechanism encoded by the rRNA methylase *erm*(B) gene, which has recently been identified in *Campylobacter* isolates (Zhang et al., 2016), was investigated.

MATERIALS AND METHODS

Sampling

In France, colon contents of pigs from 31 organic and 31 conventional batches (group of pigs from the same farm, sampled on the same day at slaughterhouse or at farm) were collected in one slaughterhouse during April to October 2012. From each batch, as far as possible, two pigs were sampled, in total 58 conventional and 56 organic ones. As some French farms sent pigs to slaughterhouse on different dates, the samples originated from 29 and 21 unique conventional and organic farms respectively.

In Sweden, colon content of approximately 6 months old pigs from 18 organic and 18 conventional batches were collected at four different slaughterhouses from August 2012 to October 2013. From each batch two pigs were sampled, in total 72 different pigs. In addition, from each organic farm and from 18 conventional farms (not the same as sampled at slaughter), fresh feces were collected on farm from two pigs within 1 week before slaughter, in total 72 different pigs. Sampling feces from live animals without any manipulation of the animals requires no ethical permission according to Swedish legislation (SJVFS 2015:24). As the organic farms were sampled both at farm and at slaughterhouses the samples originate from 18 and 36 unique organic and conventional farms, respectively.

Campylobacter Collection

In France, colon contents were diluted 10-fold in tryptone salt and streaked on Karmali plates (Oxoid, Thermo Fisher Scientific, France). Plates were incubated at 37°C in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) (Air Liquide, France) for 48 h. Presence of colonies with small curved bacilli and spiraling "corkscrew" motility under phase contrast microscopy was checked on Karmali plates and isolates were then sub-cultured on blood agar plates for 24 h at 37°C for *Campylobacter* confirmation, as described in the ISO 10272 method (ISO, 2006), and for species identification. A few colonies from the bacterial culture were suspended in 200 μL of TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 7.6) for species identification by PCR. DNA was extracted by placing the samples at 95°C for 10 min. After low centrifugation (5,000 *g* for 2 min), 10 μL of the supernatant was diluted in 90 μL TE buffer. Multiplex-PCR was used to confirm the genus of the bacterial isolates and to identify them at the species level as described by Wang et al. (2002). This multiplex-PCR was used in our study for identification of the following five *Campylobacter* species: *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, and *C. upsaliensis*. Five μL of DNA were used for amplification. PCR products were visualized by the electrophoresis of 10 μL aliquots of each amplification product, for 3 h at 100 V, in a 2% agarose gel stained with GelRed (Thermo Fisher Scientific). As far as possible, up to four isolates per pig were stored at −80°C pending further analysis, resulting in a maximum of eight isolates per sampled batch.

In Sweden, one loop (10 μL) of colon contents or feces was spread on Preston selective agar (SVA, Sweden) and cultured at 42°C for 48 h in a microaerophilic atmosphere (6–13% O_2, 3–10% CO_2, 85% N_2) generated using the Campygen system (Oxoid, United Kingdom). Colonies on Preston plates with a morphology suggesting *Campylobacter* were examined for presence of small curved bacilli and spiraling

[1]https://ec.europa.eu/agriculture/organic/eu-policy/eu-rules-on-production/livestock_en

[2]http://www.coreorganic2.org/

"corkscrew" motility under phase contrast microscopy and for production of oxidase and catalase by standard procedures. Colonies meeting the morphologic criteria on microscopy and producing oxidase and catalase were sub-cultured on blood agar plates for 24 h at 37°C. Before antimicrobial susceptibility testing, all isolates were identified to species by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany). Mass spectra were compared against 4613 spectra in the MALDI Biotyper database using the MALDI Biotyper 3.0 real-time classification software (Bruker Daltonics). One isolate of *C. coli* per pig was stored at −80°C pending further analysis.

Antimicrobial Susceptibility Testing

Minimal inhibitory concentrations (MICs) of antimicrobials were determined using broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) document M31-A3 with Sensititre® plates (Biocentric, Bandol, France) in France or M45-A2 with VetMIC panels (SVA, Uppsala, Sweden) in Sweden.

In France, after a 24-h culture on blood Mueller Hinton plates, a few colonies were suspended in saline to obtain an optical density equivalent to a 0.5 McFarland standard. The suspension was diluted in blood Mueller Hinton broth to obtain approximately 5×10^5 CFU/mL and distributed in a custom-made Sensititre® plate. Plates were read after incubation at 41.5°C for 24 h in a microaerophilic atmosphere.

In Sweden, after a 48-h culture on blood agar, one loop (1 μL) of colony material was suspended in 2 mL saline to a density of $\approx 10^8$ CFU/mL. Subsequently, 50 μL was added to 10 mL of Mueller Hinton broth with 3% lysed horse blood and from this suspension VetMIC panels were inoculated with 100 μL in each well. Plates were read after incubation at 37°C for 48 h in a microaerophilic atmosphere. In both countries, *C. jejuni* ATCC 33560 was used for quality control.

Antimicrobials tested were gentamicin, streptomycin, ciprofloxacin, nalidixic acid, tetracycline, and erythromycin. MICs were interpreted according to epidemiological cut-off values issued by EUCAST[3] as recommended by the EURL-AMR[4]. Epidemiological cut-offs for *C. coli* were >2 μg/mL for gentamicin, >4 μg/mL for streptomycin, >0.5 μg/mL for ciprofloxacin, >16 μg/mL for nalidixic acid, >2 μg/mL for tetracycline, and >8 μg/mL for erythromycin. Isolates with a non-wild-type phenotype, i.e., with a MIC above the epidemiological cutoff, were considered microbiologically resistant, for brevity hereafter referred to as resistant. The number of resistances of each isolate was calculated taking into account resistance to gentamicin, streptomycin, ciprofloxacin, tetracycline, and erythromycin.

Detection of the *erm*(B) Gene

For erythromycin resistant strains, the presence of the *erm*(B) gene was determined by the SYBR Green PCR method. The primers ermB-F amplify a 364 bp product (**Table 1**). The test was performed in a volume of 25 μL containing 5 μL of DNA, primers (200 nM) (Huerta et al., 2013) and reaction buffer (iQ™ SYBR® Green, Bio-Rad, Marnes-la-Coquette, France). The qPCR detection limit of the *erm*(B) gene was 50 copies (Le Devendec et al., 2016). The *Enterococcus faecalis* JH2-2:Tn1545 *erm*(B) strain obtained from the EURL-AMR was used as a positive control.

Statistical Analysis

As the protocols for sampling, transport of samples, and laboratory methods were different in France and in Sweden no comparison between countries was made. However, differences in *Campylobacter* isolation rate between different matrices (colon contents and feces), and production types within each country were evaluated using the chi-square test or Fisher's exact test. Logistic regressions were performed to evaluate the impact of the production within France and Sweden, and of the matrix type and their second-order interactions (for Sweden only) on each of the resistances under study. As up to 16 isolates per farm were analyzed, farm is considered as a random effect in

[3]www.eucast.org

[4]http://www.crl-ar.eu/data/images/reports/rapport-eurl%20salm-camp%20eqas%202010.pdf

TABLE 1 | Primer sequences and the expected size of the amplicons.

Primer	Size (in bp)	Sequence (5′–3′)	Target gene	Reference
CJF	323	ACTTCTTTATTGCTTGCTGC	*C. jejuni hipO*	Wang et al., 2002
CJR		GCCACAACAAGTAAAGAAGC		
CCF	126	GTAAAACCAAAGCTTATCGTG	*C. coli glyA*	Wang et al., 2002
CCR		TCCAGCAATGTGTGCAATG		
CLF	251	TAGAGAGATAGCAAAAGAGA	*C. lari glyA*	Wang et al., 2002
CLR		TACACATAATAATCCCACCC		
CUF	204	AATTGAAACTCTTGCTATCC	*C. upsaliensis glyA*	Wang et al., 2002
CUR		TCATACATTTTACCCGAGCT		
CFF	435	GCAAATATAAATGTAAGCGGAGAG	*C. fetus sapB2*	Wang et al., 2002
CFR		TGCAGCGGCCCCACCTAT		
ermB-F	364	GATACCGTTTACGAAATTGG	*ermB*	Huerta et al., 2013
ermB-R		GAATCGAGACTTGAGTGTGC		

the logistic model. The analyses were fitted using the geeglm() function of the geepack package in the R software[5] (R version 3.3.1).

Multinomial logistic regression was performed to evaluate the impact of the production (for France and Sweden), and of the matrix type and their second-order interactions (for Sweden only) on the number of resistances (considered as an ordinal response). The analyses were fitted using the polr() function of the MASS package and the vglm() functions of the VGAM package, respectively, to perform the analysis and to check the proportional odds assumption.

For all tests, values of $p < 0.05$ were considered statistically significant differences.

RESULTS

Isolation Rate

Isolation rates of *Campylobacter* in samples collected from pigs in organic and conventional production were not significantly different: 77% (43/56) of the organic pigs and 74% (43/58) of the conventional pigs carried the bacteria in their colon content (Chi2, $p > 0.05$). At the batch level, 27 out of 31 (87%) organic and 28 out of 31 (90%) conventional batches were positive (Fisher exact test, $p = 1$) and at the farm level, 19 out of 21 (90%) and 24 out of 29 (83%) were positive (Fisher exact test, $p = 0.68$). A total of 263 isolates were obtained from positive samples, 140 from organic and 123 from conventional pigs. All were identified as *C. coli*.

In Sweden, there was also no statistically significant difference in isolation rates between samples obtained from organic and conventional pigs. In fecal samples, the isolation rate was 61% (22/36) in organic pigs and 44% (16/36) in conventional pigs (Chi2, $p > 0.05$). In samples of colon content, the isolation rate was 56% (20/36) in organic pigs and 67% (24/36) in conventional pigs (Chi2, $p > 0.05$). There was no statistically significant difference in isolation rate between samples of feces or colon content, 53% (38/72) and 61% (44/72), respectively (Chi2, $p > 0.05$). Twelve out of 18 organic farms (67%) yielded *Campylobacter*-positive fecal samples and 12 out of 18 organic farms (67%) yielded positive colon samples; 16 out of 18 (89%) organic farms had at least one positive sample. For fecal samples collected from conventional pigs, isolates were obtained from 12 out of 18 farms (67%) and for colon samples, 16 farms out of 18 (89%) were positive. All 18 conventional farms (100%) had at least one *Campylobacter*-positive sample. All 82 isolates were identified as *C. coli*.

[5]https://cran.r-project.org/web/packages/geepack/index.html

Antimicrobial Resistance

The percentages of antimicrobial-resistant *C. coli* isolates are shown in **Table 2**. Resistance to erythromycin was not detected in the isolates from Sweden, but in France 62 (50%) and 25 (18%) of the isolates from conventional and organic pigs, respectively, were resistant to this antimicrobial. However, the *erm*(B) gene was not detected among these 87 erythromycin resistant isolates. Similarly, resistance to tetracycline was rare in Sweden, and was only detected in isolates from conventional pigs, with one isolate (4%) from colon content and one isolate (6%) from feces being tetracycline resistant. In contrast, resistance to tetracycline was common in France, present in 108 (77%) and 114 (93%) isolates from organic and conventional pigs, respectively. Resistance to streptomycin was frequently observed in both countries, with more than 70% of the isolates from both conventional (88 resistant isolates out of 123) and organic (104 resistant isolates out of 140) pigs in France being resistant to this antimicrobial. In Sweden, 10 out of 20 (50%) and 12 out of 24 (50%) of the isolates from colon content of both conventional and organic pigs were resistant to streptomycin and 7 out of 16 (44%) and 17 out of 22 (77%) of the isolates from feces of conventional and organic pigs, respectively. Resistance to quinolones and fluoroquinolones was common, 20–50%, in both conventional and organic pigs in France as well as in Sweden. The levels were lowest (20–30%) for isolates from French organic pigs and for isolates from colon contents of Swedish pigs of both production types. Resistance to gentamicin was not detected in Sweden and in only one of 123 isolates (1%) from conventional pigs in France. Resistance to chloramphenicol was not tested in Sweden and was not detected in the French isolates (data not shown).

In France, there was a significant impact of the production type on resistance only for tetracycline (logistic regression, $p = 0.007$) and erythromycin (logistic regression, $p = 0.005$) with resistance to these antimicrobials being more common in isolates from conventional pigs. For Sweden, no significant associations related to production system were observed for the different antimicrobials, but there was a tendency for isolates from fecal samples to be more often resistant to quinolones and fluoroquinolones (logistic regression, $p = 0.06$) compared to isolates from colon.

TABLE 2 | Number and percentages of resistance of *Campylobacter coli* isolates from samples of feces and colon content from conventional and organic pigs.

Country	Sample type	Number of isolates	Tetracycline	Erythromycin	Nalidixic acid	Ciprofloxacin	Streptomycin	Gentamicin
France	Colon Conv.	123	114 (93%)	62 (50%)	53 (43%)	53 (43%)	88 (72%)	1 (1%)
	Colon Org.	140	108 (77%)	25 (18%)	41 (29%)	39 (28%)	104 (74%)	0 (0%)
Sweden	Colon Conv.	24	1 (4%)	0 (0%)	7 (29%)	7 (29%)	12 (50%)	0 (0%)
	Colon Org.	20	0 (0%)	0 (0%)	4 (20%)	4 (20%)	10 (50%)	0 (0%)
	Fecal Conv.	16	1 (6%)	0 (0%)	8 (50%)	8 (50%)	7 (44%)	0 (0%)
	Fecal Org.	22	0 (0%)	0 (0%)	9 (41%)	9 (41%)	17 (77%)	0 (0%)

Conv., conventional pig; Org., organic pig; ND, not determined.

For French isolates, the number of resistances of individual isolates was significantly associated with the production type (multinomial logistic regression, $p = 1.8e\text{-}07$) with higher number of resistances in isolates from conventional pigs. For Swedish isolates, the number of resistances of fecal isolates was significantly higher compared to the colon isolates (multinomial logistic regression, $p = 0.04$).

In France, when several isolates from the same pig were studied, various resistance profiles were found: thus for pigs for which four isolates were analyzed, the mean number of resistance profiles was 1.8. Similarly, when several isolates from the same farm were analyzed, several resistance profiles were usually observed: for example, when four or more isolates from a French farm were studied, 2–6 and 1–5 different resistance profiles were detected for organic and conventional farms, respectively. Likewise, when three or four isolates were obtained from a Swedish farm, a minimum of two different profiles were detected. In France, the most frequent resistance profiles were streptomycin–tetracycline (27/123, 22%), streptomycin-ciprofloxacin-tetracycline-erythromycin (19/123, 15%), streptomycin-ciprofloxacin-tetracycline (18/123, 15%) and streptomycin-tetracycline-erythromycin (17/123, 14%) for conventional production and streptomycin–tetracycline (46/140, 33%), streptomycin-ciprofloxacin-tetracycline (22/140, 16%) and tetracycline (17/140, 12%) in organic production (**Table 3**). Multidrug-resistance (resistance to three or more antimicrobial families) was more frequently observed for isolates from conventional pigs (68/123, 55% of the isolates) than for isolates from organic pigs (37/140, 26%) (Chi2, $p < 0.001$). In Sweden, the two most frequent profiles were wild-type (13/40, 32.5%) and streptomycin (12/40, 30%) for conventional production and streptomycin (17/42, 40.5%) and wild-type (12/42, 29%) for organic production. No isolate from Sweden was multidrug-resistant.

DISCUSSION

Occurrence of *C. coli*

Campylobacter coli was the main species isolated and the prevalence in colon contents was over 50% in both countries and in both production types. These results are in accordance with the metagenomic data for a subset of our samples reported from another study within the SafeOrganic project. Gerzova et al. (2015) found the 16S rRNA gene of the genus *Campylobacter* in all six French samples of pig colon contents investigated and in three out of five individual samples of colon contents and one pooled sample from Sweden. Such a high prevalence of *Campylobacter* in pigs is frequently observed in many countries such as France (Avrain et al., 2004), Great Britain (Milnes et al., 2008), the United States (Tadesse et al., 2011), or Canada (Huang et al., 2015) but slightly lower prevalences have sometimes been reported, as in Japan (Haruna et al., 2013) and in Sweden (Bywater et al., 2004). Levels from 0 to 92.7% were reported for European member and non-member states (EFSA and ECDC, 2015). These differences in prevalence of *Campylobacter* in pigs are probably related to true differences between countries but also to differences in the protocols used in the different studies, such as farm or slaughterhouse sampling, type and number of samples, individual or pooled samples, enrichment or direct plating and number

TABLE 3 | Antimicrobial resistance profiles.

	France			Sweden						
	Conventional	Organic	Total	Conventional			Organic			
Resistance profile	Feces			Feces	Colon	Total	Feces	Colon	Total	Total
C				4	2	6	1	2	3	9
CT	2	2	4	1	1	2				2
CTE	13	2	15							
E	1	1	2							
GCT	1		1							
S	4	15	19	4	8	12	9	8	17	29
SC		5	5	3	4	7	8	2	10	17
SCT	18	22	40							
SCTE	19	8	27							
SE	3	3	6							
ST	27	46	73							
STE	17	5	22							
T	8	17	25							
TE	9	6	15							
W	1	8	9	4	9	13	4	8	12	25
Total	123	140	263	16	24	40	22	20	42	82

S, streptomycin; C, ciprofloxacin; T, tetracycline; E, erythromycin; W, wild-type.

and type of media. However, these data clearly indicate that maintaining pig herds free of *Campylobacter* is likely to be challenging. Nevertheless, *Campylobacter*-free pig herds were described in Norway, although these herds were located in remote areas far away from conventional pig herds (Kolstoe et al., 2015).

In the current study there was no difference in the prevalence of *C. coli* in organic and conventional production, neither in France nor in Sweden. This is consistent with the metagenomic study of Gerzova et al. (2015) on the samples from this study where there was no significant difference in the composition of the microbiota of organic and conventional pigs. In the study reported by Rollo et al. (2010), concerning 60 conventional farms and 35 antibiotic-free farms in Midwestern United States, *Campylobacter* was detected in 35.8 and 36.4% of pigs, respectively, with no significant difference between these two production types. A similar observation was made by Gebreyes et al. (2005), with prevalences of 55 and 56.3% for extensive and intensive pig rearing systems, respectively, and by Quintana-Hayashi and Thakur (2012), who monitored the presence of *Campylobacter* in the environment of conventional and antimicrobial-free pig production systems and found no difference. Likewise, in other studies no significant differences could be detected for prevalence of *Campylobacter* in conventional and organic dairy productions (Sato et al., 2004), in meat from free-range and conventional broilers (Economou et al., 2015), in organic, antibiotic-free and conventional retail chicken breasts (Mollenkopf et al., 2014) and in conventional and organic layer hens (Schwaiger et al., 2008). However, *C. jejuni* was significantly less frequently found in organically raised than in conventionally raised layer hens (Kassem et al., 2017). Inversely the meta-analysis of Young et al. (2009) showed that at slaughter, *Campylobacter* was more common in organic than in conventional broilers. Various factors such as access to outdoor runs, animal density, age at slaughter or slaughterhouse processing conditions may partially explain these differences.

Antimicrobial Resistance

The resistance ratios registered in this study for conventional pigs can be compared with data reported for *C. coli* from pigs by France in the 2011 mandatory monitoring of production animals in the EU (EFSA-ECDC, 2013) and for Sweden in the 2011 and 2015 monitorings (EFSA-ECDC, 2013, 2017). In the mandatory monitoring scheme, the herds sampled are representative of the national production, which means that, for both France and Sweden, the samples originate almost exclusively from conventional herds. The data on resistance presented in this study were similar to the results of the EFSA and ECDC monitorings, although data on streptomycin and multidrug resistance were not included in the latter.

Several studies have investigated the occurrence of antimicrobial resistance in organic or antibiotic-free production systems in comparison to conventional systems. Although several factors may differ between conventional and organic productions, such as animal density, access to outdoor areas, and age at slaughter, the most important factor for selection and persistence of antimicrobial resistance is probably use of antimicrobials as recognized in other situations (WHO, 2012). To investigate possible differences in antimicrobial resistance between production types, the susceptibility of commensal *Enterobacteriaceae* or *E. coli* is sometimes the chosen criteria as these bacteria are present in most fecal samples, are easy to isolate and analyze, and are considered a major resistance gene reservoir. Thus in the course of the SafeOrganic project, Osterberg et al. (2016) showed that, in Denmark, Italy, France and Sweden, resistance to ampicillin, streptomycin, sulphonamides, and trimethoprim was significantly less frequent in *E. coli* strains from organic than from conventional pigs. This was also observed in France for chloramphenicol. Several antimicrobials were tested for both the *E. coli* and the *C. coli* isolates collected during this project (ciprofloxacin, gentamicin, nalidixic acid, streptomycin, and tetracycline). The lower ratio of resistance to tetracycline reported for *E. coli* from organic compared to conventional pigs in France, but not in Sweden, was also observed for *Campylobacter* in France. For quinolones and fluoroquinolones, no differences between production types were registered for either *Campylobacter* or *E. coli* in both countries. Streptomycin resistance was significantly different in the two production types in both countries for *E. coli* but not for *C. coli*. Thus, within a given country, the result of a comparison of the antimicrobial susceptibility between two animal production types will depend on the bacterial species tested, which may differ in their evolution and the persistence of their antimicrobial resistance. Such differences in the susceptibility of bacterial species are probably related to various factors including the biological cost of resistance, which will influence the rate at which a bacterial species persists or regains wild-type status after reduced use of a specific antimicrobial. It was previously demonstrated that for example fluoroquinolones, not always impose a biological cost in *Campylobacter* (Luo et al., 2005; Zeitouni and Kempf, 2011). This phenomenon could explain the rather high levels of quinolone and fluoroquinolone resistance in *Campylobacter* strains from organic pigs observed in both France and Sweden. Indeed, in the United States, Zawack et al. (2016) showed that the withdrawal of enrofloxacin for use in poultry 10 years ago had no effect on the occurrence of ciprofloxacin resistance in *Campylobacter* from chickens, and El-Adawy et al. (2015) reported surprisingly high levels of resistance to ciprofloxacin in *Campylobacter* isolates from organically raised turkeys in Germany, which they attributed to the persistence of strains during the transition from conventional to organic production. High levels of fluoroquinolone resistance was also detected in *Campylobacter* isolates from chicken in organic productions in Portugal (Fraqueza et al., 2014).

The persistence of resistance can also be linked to co-selection whereby the use of one antimicrobial selects for resistance also to other antimicrobials. In Japan Ozawa et al. (2012) used logistic regression to demonstrate that the use of tetracyclines within the 6 months prior to the survey

was associated with chloramphenicol resistance of *C. coli* in pigs. This co-selection was explained by the fact that all chloramphenicol-resistant isolates were also resistant to tetracycline.

Besides the analysis of various indicator or zoonotic bacteria (Young et al., 2009), another criteria that can be analyzed is the abundance of antimicrobial resistance genes. However, in the SafeOrganic project, the abundance of a few selected genes [*strA, sul1, sul2, tet*(A), and *tet*(B)] was found to be linked to the country but not to the production type (Gerzova et al., 2015). This rather unexpected result may be due to a lower discriminatory power of the qPCR method compared to culturing. Moreover, the resistance genes that were analyzed may be harbored by many different bacterial species, e.g., *tet*(A) can be found in *Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae, Vibrionaceae* and others, and each bacteria may contain different genes encoding for the same antimicrobial family. For example, *Escherichia* can contain *tet*(A), *tet*(B) *tet*(C), *tet*(D), *tet*(E), *tet*(I), and others (Chopra and Roberts, 2001), which may explain why for a given antimicrobial, results may be different in terms of the susceptibility ratios of *E. coli* or *Campylobacter*, or the abundance of resistance genes.

Our results show associations between antimicrobial resistance of *C. coli* in France and type of production, in particular for tetracycline and macrolides. Different studies have previously focused on the link between antimicrobial consumption and resistance of *C. coli* in pigs. In Japan, Ozawa et al. (2012) found significant associations, with odds ratios around 10, between the use of macrolides and phenicols and resistance to erythromycin and chloramphenicol, respectively. In the United States, the *C. coli* strains from antibiotic-free pigs were less often resistant to tetracycline and to clindamycin than those from conventional pigs (Quintana-Hayashi and Thakur, 2012) and the most common profile (resistance to ciprofloxacin, nalidixic acid, and tetracycline) observed in conventional pigs was found in only one antibiotic-free pig. Rollo et al. (2010) had similar findings with significant differences for resistance to macrolides (erythromycin and azithromycin) and tetracycline between farm types. They noted that resistance to these antimicrobials decreased with the number of years that a farm had been antibiotic-free.

The results of the current study are globally consistent with the significant positive associations between consumption of macrolides in animals in different European countries and resistance in *C. coli* and *C. jejuni* in animals in these countries, as reported in the joint ECDC/EFSA/EMA report on integrated analysis of antimicrobial consumption and occurrence of antimicrobial resistance (ECDC et al., 2015). However, the authors of the report note that there are limitations to their study since it is based on consumption data for all species of food-producing animals and on only a small number of countries reporting resistance data for *Campylobacter*.

Notably, in the current study erythromycin resistance was not found in isolates from Sweden. In a study evaluating data on resistance from the 2011 EU summary report in relation to use of antimicrobials as reported to EMA, the absence of macrolide-resistance in *C. coli* from pigs in Sweden was attributed to withdrawal of the macrolide tylosin as growth promoter as early as 1986 and to the restricted therapeutic use of this antimicrobial (Garcia-Migura et al., 2014). In contrast, many *C. coli* strains from French conventional or organic pigs were resistant to erythromycin. Previous studies have shown that resistance to macrolides in French isolates of *C. coli* from pigs was associated with mutation in the 23S rRNA genes and efflux pumps (Payot et al., 2004). However, the recent emergence of macrolide-resistance linked to the transmissible *erm*(B) gene, encoding an rRNA methylase, prompted us to screen for this gene in our recent strains. None contain the *erm*(B) gene. To our knowledge, this gene has only been detected in Chinese isolates from humans, swine, chickens, and ducks and in one *C. coli* isolated from a broiler in Spain (Florez-Cuadrado et al., 2016). The absence of *erm*(B) in our strains is reassuring, but surveillance must be maintained.

CONCLUSION

The protocol of this study enabled us to evaluate the prevalence and antimicrobial resistance in *C. coli* from conventional and organic pigs in Sweden and France. We could show that within each country there was no significant difference in prevalence of *C. coli* between organic and conventional production. We also showed that in France there were significant differences between production types in occurrence of resistance to tetracycline and erythromycin and the number of resistances of *C. coli* isolates, resistance being more common in conventional than in organic production. In Sweden, no differences in resistance between the two systems were detected.

AUTHOR CONTRIBUTIONS

IK: contributed to the design of the work, contributed to the acquisition, analysis and the interpretation of the data, wrote the paper. AK: contributed to the design of the work, participated to the field study, contributed to the acquisition, analysis and the interpretation of the data, wrote the paper. SB: contributed to the acquisition, analysis and the interpretation of the data, wrote the paper. BN: participated to the field study, contributed to the acquisition, analysis and the interpretation of the data, final approval of the submitted version. VR: participated to the field study, contributed to the acquisition, analysis and the interpretation of the data, final approval of the submitted version. GM: contributed to the acquisition, analysis and the interpretation of the data, final approval of the submitted version. JO: contributed to the design of the work, participated to the field study, contributed to the acquisition, analysis and the interpretation of the data, wrote the paper. MD: contributed to

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No Clear Differences between Organic or Conventional Pig Farms in the Genetic Diversity or Virulence of *Campylobacter coli* Isolates

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To evaluate the impact of pig farm management on the genetic diversity and on the virulence of *Campylobacter coli*, we characterized isolates from 19 organic pig farms (62 isolates) and from 24 conventional pig farms (58 isolates). The 120 *C. coli* isolates were typed using pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) and the presence of nine virulence genes was screened using real-time PCR. The capacity of adhesion and invasion of 61 isolates (32 from organic and 29 from conventional farms) were then tested on human intestinal *Caco-2* cells. A total of 59 PFGE types and of 50 sequence types (STs) were identified. Twelve PFGE types and nine STs, accounting for 34 and 41.6% of the isolates, respectively, were common between the two production systems with ST854 dominating (18.3% of the isolates). Twenty-nine PFGE types and 25 STs were only found in isolates from organic farms, and 18 PFGE types and 16 STs from conventional farms. No significant differences were found in diversity despite the differences in rearing systems, except at the locus level for the *glnA*, *gltA*, and *uncA* genes. All isolates, regardless of their origin, carried the *ceuE*, *iam*, *ciaB,* and *flaA* genes and more than 95% of the isolates carried the *cadF* and *cdtABC* genes. No significant differences were found in pathogenicity between the two farming systems. The pathogenicity of the *C. coli* isolates was low compared to *C. jejuni* control strains tested. The plasmid gene *virb11* was detected in only 13 isolates from organic farms; these isolates showed greater invasion capacity than those without this gene. Our study indicates that pig farm management does not significantly affect the diversity and the virulence of *Campylobacter coli* isolated from pigs. The common genotypes between conventional and organic farms may indicate that some genotypes are adapted to pigs.

Keywords: *Campylobacter*, pig, organic, PFGE, MLST, virulence

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INTRODUCTION

New consumer trends focus on products derived from systems that promote good animal welfare conditions and a high safety level. Organic pig production differs in many ways from conventional pig production, particularly in terms of antibiotic use, herd structure, feeding regimes, access to outdoor areas, and space allowance per pig. More specifically, the European Union regulations for organic farming (Council Directives 2007/834/EC and 2008/889/EC) require that animals have

access to an outdoor area. In addition, the preventive use of chemically-synthesized allopathic veterinary medicinal products is not authorized, and may be used only for treatment of sick animals when necessary. If more than one treatment, derived products are no longer considered organic.

Recently, we investigated the carriage of antibiotic-resistant *Escherichia coli* in colons at the slaughterhouse and in feces on organic and conventional pig farms in four European countries (SafeOrganic project, Österberg et al., 2016). We also studied the carriage of resistant *Campylobacter* from the same samples in two European countries (SafeOrganic project, Kempf et al., 2017). In France, the level of antibiotic resistance in *E. coli* and *Campylobacter coli* is lower for organic pig production than for conventional production, suggesting that practices such as little or no use of antibiotics on organic pig farms can affect the level of bacterial resistance. Several studies (Saini et al., 2013; Garcia-Migura et al., 2014) indicate that extensive use of antibiotics produces a selection pressure favoring resistance among commensal bacteria from animals.

Because the management of conventional and organic pig farms has an impact on *Campylobacter* resistance to antibiotics (Kempf et al., 2017), we assumed that the management of these two types of pig production systems (in terms of antibiotic use and access to the outdoors) may also have an impact on the diversity of *Campylobacter* isolates excreted by pigs and on the virulence of these isolates. The more frequent use of antibiotics and confinement of pigs in a building in conventional farming may reduce the number of *Campylobacter* genotypes. In contrast, in organic farming, little or no use of antibiotics and access to an outdoor area may promote the presence of a higher number of *Campylobacter* genotypes. Access to an outside area increases exposure of animals to environmental sources of different microorganisms including *Campylobacter jejuni* (Greig et al., 2015).

Thus in this study, the *C. coli* previously isolated from pigs from organic and conventional farms to test their resistance to antibiotics (Kempf et al., 2017) were typed using two molecular typing methods, and tested for their virulence.

MATERIALS AND METHODS

Origin of the Isolates

The *Campylobacter* isolates considered in this study were isolated by our laboratory, which is also the French National Reference Laboratory for *Campylobacter* as part of the SafeOrganic project. Sampling and isolation methods for *Campylobacter* are described in Kempf et al. (2017). Briefly, colon contents were sampled at one slaughterhouse from 114 pigs. These pigs came from 31 organic pig batches (56 pigs) and 31 conventional pig batches (58 pigs). These batches involved 21 organic farms and 29 conventional farms, all located within 200 km of the slaughterhouse. Out of the 50 sampled farms, 43 farms were positive for *Campylobacter*: 19 organic farms and 24 conventional farms.

The isolates were kept at −80°C in peptone glycerol broth. They were all identified as *C. coli* and tested for their antibiotic resistance (Kempf et al., 2017). Here, we randomly selected two to three isolates per positive farm for a total of 120 isolates: 62 isolates from 19 organic farms and 58 isolates from 24 conventional farms.

DNA Extraction

The 120 isolates were cultured on blood agar plates (Oxoid, Dardilly, France) for 48 h at 37°C in a micro-aerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). A few colonies from the bacterial culture were used for DNA extraction using the InstaGene® Matrix (BioRad Laboratories, Marnes-la-Coquette France) according to the manufacturer's recommendations. DNA was adjusted to 10 ng/µl and intended for use in PCRs for virulence gene detection, and multilocus sequence typing (MLST) as described below. The remaining colonies were used for genotyping by pulsed-field gel electrophoresis (PFGE).

Pulsed-Field Gel Electrophoresis (PFGE) and Analysis of Electrophoretic Profiles

DNA preparation, restriction endonuclease digestion with the *Kpn*I enzyme and PFGE were carried out as described by the Campynet protocol (Rivoal et al., 2005). One *Kpn*I restriction profile was obtained for each isolate.

Electrophoretic patterns were compared using BioNumerics v. 6.5 software (Applied Maths, Sint-Martens-Latem, Belgium). Similarities between profiles, based on band positions, were determined by calculating the Dice correlation coefficient, with a maximum position tolerance of 1%. A dendrogram based on the *Kpn*I restriction profiles was constructed to represent the similarities between the isolates in the matrix. Isolates were clustered by the unweighted pair-group method using the arithmetic mean (UPGMA) (Struelens, 1996). Isolates displaying high levels of similarity were clustered together using a threshold of 80% (Denis et al., 2008) and considered as the same PFGE type.

The Simpson's index (D) was determined as described by Hunter (1990), and was given with a 95% confidence interval, as described by Grundmann et al. (2001). This index was used to assess the genetic diversity of the *Campylobacter* populations.

Multilocus Sequence Typing (MLST)

The seven housekeeping genes for MLST (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt,* and *uncA*) were amplified and sequenced according to previously developed experimental conditions (Dingle et al., 2001; Miller et al., 2005). PCR products were cleaned up using the ExoSAP-IT treatment (GE Healthcare), and sequence extension reactions were carried out in BigDye Ready reaction mix according to the manufacturer's instructions. Unincorporated dye terminators were removed using an ethanol precipitation method before the products were analyzed on an ABI Prism 3130 sequencer (Applied Biosystems). The sequences were assembled using the assembler implemented in BioNumerics v. 6.5 software. All allelic sequences were queried against the *C. jejuni* MLST database (http://pubmlst.org/campylobacter). Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the MLST database and assigned new numbers. STs were assigned into genetically related clusters called clonal complexes (CCs), based on the sharing of four or more alleles with the

central genotype that had been identified in previous studies using the BURST algorithm and UPGMA cluster analysis (Dingle et al., 2002).

Detection of Virulence Genes

Table 1 shows the nine virulence genes screened in the present study. Eight of the genes are localized on the bacterial chromosome and one on the plasmid (*virB11*). These genes are involved in adhesion/invasion of epithelial cells (*flaA, ciaB, cadF, iam, virB11),* in the acquisition of iron (*ceuE),* and in the production of the cytholethal distenting toxin (CDT) (*cdtA*, *cdtB*, *cdtC).*

The presence of these nine virulence genes in the 120 isolates was checked using real-time PCR developed for this study using primers published by Gonzalez et al. (1997), Denis et al. (1999), Konkel et al. (1999), Carvalho et al. (2001), Moore et al. (2002), Bang et al. (2004), or by Zheng et al. (2006) or using primers designed by pour laboratory for this study (**Table 1**).

Some published primers were slightly modified (one or two bases added or removed) to obtain primers with the same (or very similar) melting temperature (indicated by * in **Table 1**). We used also the reverse sequence of the initial primer (*virB11*) published by Bang et al. (2004).

We designed eight primers from a sequence alignment using Multalin v. 5.4.1 (INRA, France) for the detection of the three *cdt* genes (GenBank accession numbers: AB562905, AB274801, AB274800, AB274799, AB274798, AB274797, AB274796, AB274795, AB274794, AB274793, AB182109) and the *ciaB* gene (GenBank accession numbers: HG326877, CP006702, AB433217, CP004066).

The size of the PCR products was estimated by *in silico* PCR (FastPCR online v. 2.07, PrimerDigital) and confirmed after electrophoresis on an agarose gel.

Each PCR was carried out in a total volume of 25 µl with the mix SYBR® Green JumpStart™ Taq ReadyMix™ from Sigma-Aldrich, in which 1 µl of each primer at 10 µM was added. All nine amplifications were done using the same PCR conditions: 35 cycles, each cycle with a first step at 95°C for 1 min, a second step at 56°C for 1 min and a final step at 72°C for 1 min 40 s. The PCRs finished with an incremental step from 60 to 95°C, increasing by 0.5°C every 5 s to obtain the fusion curve.

Three isolates of human origin were used as positive controls (**Table 2**): *C. jejuni* 81–176 (with pVir plasmid) and *C. jejuni* NCTC11168 (without the pVir plasmid) (purchased from the Pasteur Institute Collection, Paris) and *C. coli* 04FM842 (purchased from the French National Reference Center) genetically close to *C. coli* from pigs by PFGE and with all the virulence genes except *virB11.*

Adhesion and Invasion Assay on Human Intestinal Epithelial Cells

For this assay, we selected 61 isolates (32 from the 19 organic farms and 29 from the 24 conventional farms) on the basis of their PFGE profile and ST to ensure good representativeness of the isolates from each farm.

Capacity of adhesion and invasion of the isolates was tested *in vitro* on *Caco-2* human intestinal epithelial cells following the protocol developed in our laboratory by Guyard-Nicodème et al. (2013). *Caco-2* cells (ECACC 86010202) were obtained from the European Collection of Cell Culture (ECACC, Salisbury, UK). Capacity of adhesion and invasion of the isolates was expressed as the percentage of adhesives cells (p_adh) and of invasive cells (p_inv), respectively. For each isolates, the results were the mean of at least two separate determinations.

C. jejuni and *C. coli* strains isolated from humans and poultry were also tested to compare the invasiveness of our isolates with other *Campylobacter*. The three human strains were *C. jejuni* 81–176 (with pVir plasmid), *C. jejuni* NCTC11168 (without pVir plasmid), and *C. coli* 04FM842 (without pVir plasmid). The poultry strains were Plouf12 (*C. jejuni* from poultry) and Plouf13 (*C. coli* from poultry), both previously tested on pig in a previous study (Leblanc Maridor et al., 2011), 17MD18, 47MD12 (two *C. coli* from poultry), 54MD16, and 27MD13 (two *C. jejuni* from poultry) isolated from a previous study (Denis et al., 2008), CRL204-08 (*C. coli* from poultry) purchased from the European Reference Laboratory, Uppsala, Sweden. We added also a *C. coli* reference strain isolated from a pig (CIP70.80T) purchased from the collection of Pasteur Institute, Paris, France.

Data Analysis

The distribution of the PFGE types or STs on organic farms was compared to that of conventional farms using the chi-square test of independence in R software (version 3.2.5). For each typing method, we considered the number of isolates from one production system that shared types common with the other system, and the number of isolates found in only one of the two production systems. The distribution was considered statistically different between the two production systems when *p*-values were lower than 0.05.

Using the "comparing partitions" method (http://www.comparingpartitions.info), we compared the distribution of the PFGE types of the 120 isolates with the distribution of the STs of these isolates. We also compared the distribution of the antibiotic resistance (ATB) profiles of the 120 isolates with the distribution of the PFGE types or STs of these isolates. A *p*-value was calculated using the jackknife pseudo-values method. We considered that the PFGE/ST, ATB/PFGE, or ATB/ST associations were weak if their distributions were significantly different ($p < 0.05$).

Results of adhesion to or invasion of *Caco-2* cells were analyzed using the Mann-Whitney test in R software version 3.2.5. A *p*-value lower than 0.05 was considered significant. *C. coli* were also classified into three classes of pathogenicity (low, intermediate and high) using hierarchical clustering with the method "hclust ward D2" implemented in R.

RESULTS

Genetic Diversity

We observed high genetic diversity for our 120 *C. coli* whatever the typing method. A total of 110 *Kpn*1 PFGE profiles were associated with 59 PFGE types (**Figure 1**) when clustered at 80%

TABLE 1 | Primer sequences for the detection of the nine virulence genes in *Campylobacter coli*.

Gene	Primer	Sequence (5′ →3′)	Size (bp)	References
flaA	flaA2-F flaA1-R	GCTTCAGGGATGGCGATAGCAGAT TTGATCTCTTCAGCCAAAGCTCCAAGT	533	Moore et al., 2002
cdtA	cdtA-cF cdtA-cR	TGTCCCACCTGTAATCACTCC CTCTTGCATCTCCAAAAGGTCT	245	This study
cdtB	cdtB-cF cdtB-cR	GAGTGGATGTAGGAGCAAATCG CGTAGAAGAAGGCGGAACAAC	332	This study
cdtC	cdtC-cF cdtC-cR	AGCTTGGATGAATTAGCAGACT TGGCGATACTAGAGTCAGGAAA	403	This study
cadF	F2B* R1B*	CTTTGAAGGTAATTTAGATATG AACTAATACCTAAAGTTGAAAC	401	Konkel et al., 1999
virB11	virB11-235 rev-virB11-F**	TGTGAGTTGCCTTACCC GCTAGTTTTTCCACTTCCTG	240	Zheng et al., 2006 Bang et al., 2004;
ceuE	COL3 MDCOL2	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG	462	Gonzalez et al., 1997 Denis et al., 1999
iam	Car-F Car-R	GCGCAAAATATTATCACCC TTCACGACTACTATGCGG	519	Carvalho et al., 2001
ciaB	ciaB-cF ciaB-cR	GAAAGAAGCTATGGTGTTTTGGT GGATGACCTACTTGYAATGGAGA	284	This study

**Primer modified from the initial primer reported in the Reference.*

***Reverse sequence of the initial primer (virB11) published by Bang et al. (2004).*

TABLE 2 | Presence (+) of the nine genes in the three strains used as positive PCR controls.

Strain	Species	*flaA*	*cdtA*	*cdtB*	*cdtC*	*cadF*	*virB11*	*ceuE*	*iam*	*ciaB*
NCTC 11168	*C. jejuni*	+	−	−	−	+	−	−	−	−
81−176	*C. jejuni*	+	−	−	−	+	+	−	−	−
04FM842	*C. coli*	+	+	+	+	+	−	+	+	+

of similarity. The 62 isolates from organic farms showed 41 PFGE types and the 58 isolates from conventional farms showed 30 PFGE types. Organic farms and conventional farms shared 12 PFGE types (12/59) representing 34.16% of the isolates (41/120). Among the 59 PFGE types, 29 were found only from organic farm isolates (40 isolates) and 18 from conventional farm isolates (39 isolates). The diversity of the *Campylobacter* populations was slightly higher in organic isolates with an index of diversity $D = 0.98$ $_{CI95\%}[0.97–0.99]$ than in conventional isolates with $D = 0.96$ $_{CI95\%}[0.94–0.98]$. The distribution of the PFGE types in the two production systems was not significantly different (χ^2, $p = 0.345$).

Almost all isolates (91%) were from the ST828 CC; the other isolates (11) had no identified CC. Isolates were distributed among 50 STs (**Figures 1**, **2**). The 62 isolates from organic farms were distributed into 34 STs and the 58 isolates from conventional farms into 26 STs. Nine STs (9/50), representing 41.6% of the isolates (50/120), were common to both organic and conventional farms, with a ST854 dominating (18.3% of the isolates, with 10 isolates from 8 organic farms and 12 isolates from 12 conventional farms). Among the 50 STs, 25 STs were only found in organic farm isolates (38 isolates) and 16 in conventional farm isolates (32 isolates), where ST1096 was found in 9 isolates. The diversity of the *Campylobacter* populations was higher in organic production systems with an index of diversity $D = 0.96$ $_{CI95\%}[0.94–0.99]$ than in conventional production ($D = 0.93$ $_{CI95\%}[0.89–0.97]$). The distribution of the STs in the two production systems was not significantly different (χ^2, $p = 0.496$).

The distribution of the STs was significantly different from the distribution of PFGE types ($p = 0.003$), indicating that STs are only weakly related to PFGE types.

Genetic diversity at individual MLST loci is shown in **Table 3**. The mean genetic diversity was higher in organic farms (0.435 ± 0.154) than in conventional farms (0.333 ± 0.206). Allelic diversity was higher for the *pgm* and *tkt* loci in both systems with a greater number of alleles for these two genes (**Table 3**). Between the two production systems, we observed a significant difference in genetic diversity for the *glnA*, *gltA*, and *uncA* locus, with higher genetic diversity for the organic system.

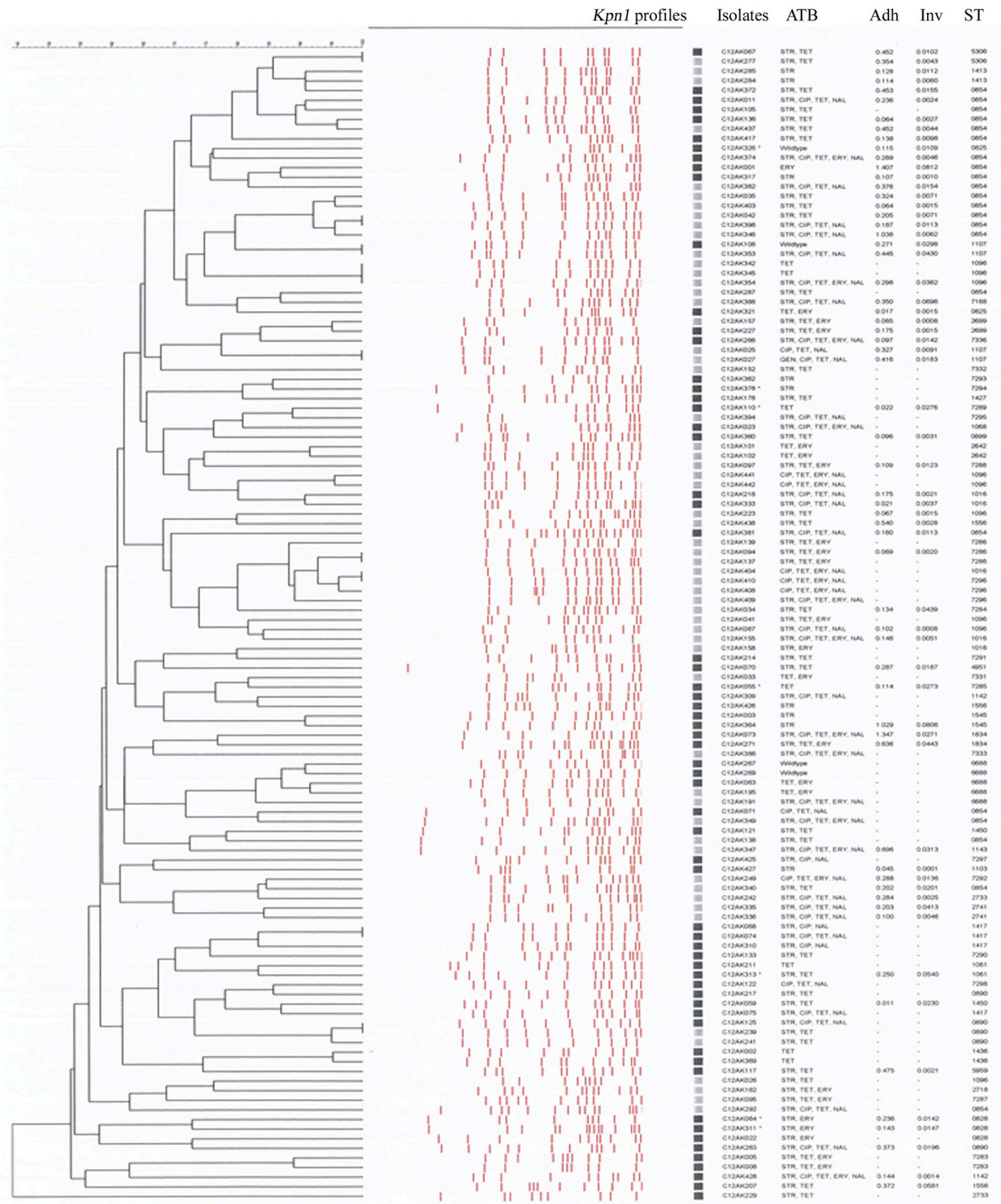

FIGURE 1 | Dendrogram of the *Kpn*1 profiles of the 120 *Campylobacter coli* isolates from organic and conventional pig farms (optimization, 1%; tolerance, 1%; active zones, [7.0–87.0%]). Black squares, isolates from organic pig farm; Gray squares, isolates from conventional pig farms; ATB, profile of antibiotic resistance; Adh, percentage of adhesion on *Caco-2* cells; Inv, percentage of invasion on *Caco-2* cells; ST, sequence type.

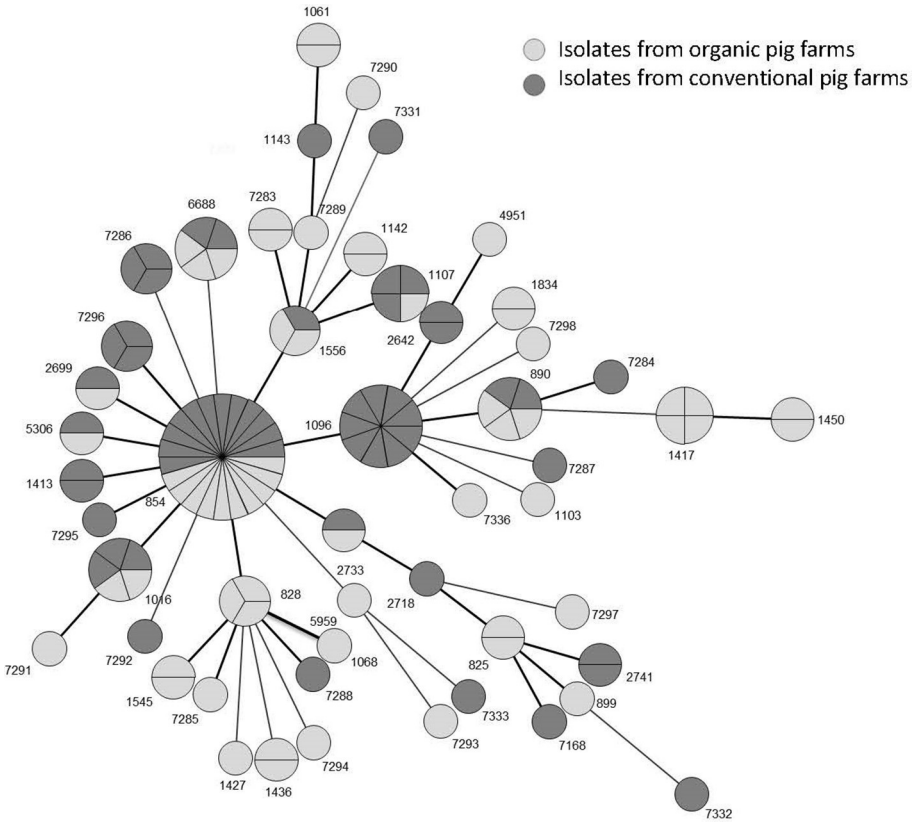

FIGURE 2 | Distribution of the 120 isolates according their sequence type (ST) in a phylogenetic tree drawn using BioNumerics software.

TABLE 3 | Genetic diversity at individual loci of the multilocus sequence type for the 120 *C. coli* isolates from organic and conventional pig farms.

Locus	Organic			Conventional		
	No. of allele	D	CI 95%	No. of allele	D	CI 95%
aspA	4	0.211	0.08–0.35	4	0.134	0.01–0.25
glnA	4	0.544	0.48–0.61	3	0.220	0.08–0.35
gltA	4	0.472	0.35–0.60	3	0.133	0.01–0.25
glyA	5	0.240	0.10–0.38	7	0.391	0.23–0.55
pgm	8	0.439	0.29–0.59	8	0.492	0.34–0.65
tkt	8	0.617	0.51–0.72	10	0.693	0.60–0.79
uncA	5	0.527	0.44–0.62	2	0.267	0.14–0.40
Mean D	0.435 ± 0.154			0.333 ± 0.206		

D, Simpson's index with 95% of confidence interval (CI 95%).

Association between Genetic Profiles and Antibiotic Resistance Profiles

The 120 *Campylobacter coli* were previously tested for their resistance to antibiotics. Resistant to tetracycline and erythromycin, and the number of resistances were significantly higher in isolates from conventional farms (Kempf et al., 2017).

The distribution of the ATB profiles was significantly different from the distribution of the PFGE types ($p = 0.009$) or the distribution of the STs ($p = 0.012$), indicating that ATB profiles are weakly related to PFGE types or STs.

However, the ST854 *C. coli* isolates predominantly showed resistance to tetracycline (90.9% of the ST854 isolates), streptomycin (90.9%), and susceptibility to erythromycin (86.3%).

Moreover, we noted that 76% of the isolates resistant to tetracycline were isolates with the allele 38 of the *glnA* gene (χ^2, $p = 0.007$).

Presence of the Virulence Genes

Regardless of pig origin, all the isolates carried the *ceuE*, *iam*, *ciaB,* and *flaA* genes. Moreover, all isolates from organic pigs carried also the *cadF* gene and the three *cdt* genes. *One* conventional isolate did not have the *cadF* gene and three conventional isolates lacked the three CDT genes. The plasmid gene *virB11* was detected only in eight isolates (7%), all from organic pigs. The presence of the plasmid was neither associated with a particular PFGE profile (see * in **Figure 1**) nor with a particular ST [ST 7285, 1450, 828 (2 isolates), 729, 1061, 825, and 724].

Capacity of the Isolates to Adhere and Invade Caco-2 Cells

The percentage of adhesion and invasion of the 61 *C. coli* are indicated on the dendrogram in **Figure 1**. Adhesion was 0.30 and

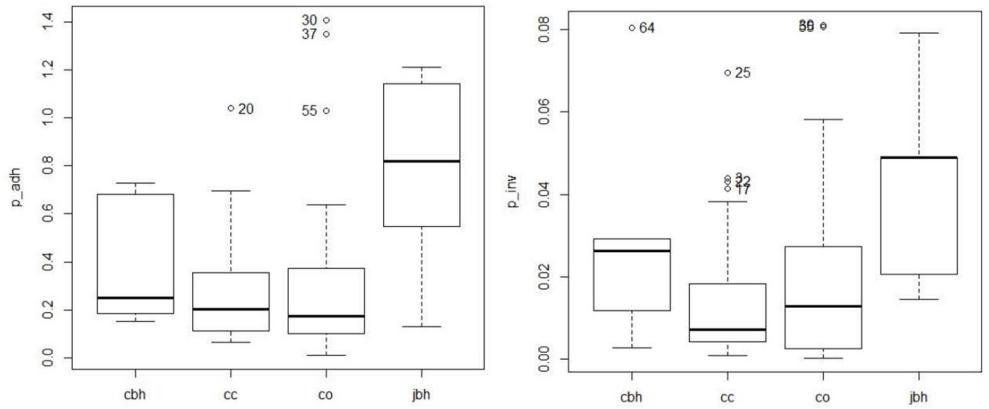

FIGURE 3 | Distribution of the percentage of adhesion (p_adh) and the percentage of invasion (p_inv) according the origin of the isolates. cc, *C. coli* from conventional pig farm; co, *C. coli* from organic pig farm; cbh, *C. coli* from poultry and humans; jbk, *C. jejuni* from poultry and humans.

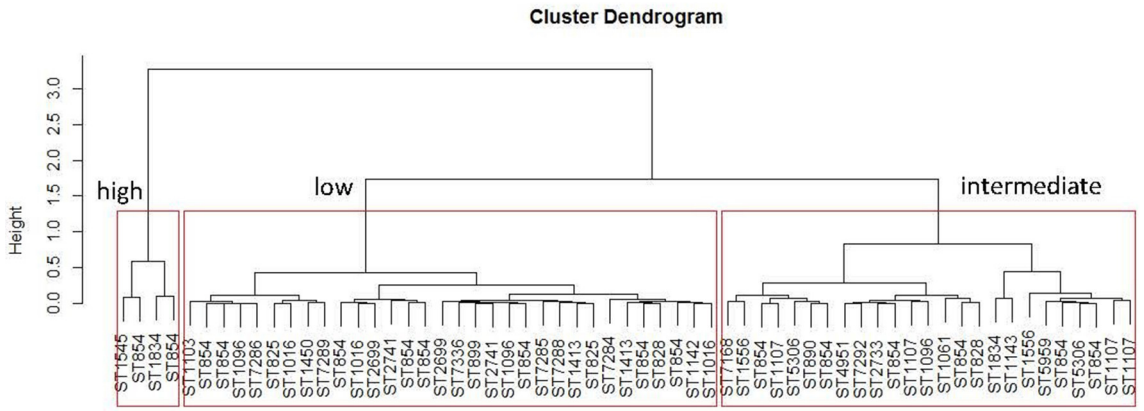

FIGURE 4 | Distribution of the sequence types of the 61 *C. coli* isolates tested on *Caco2*-cells according their percentages of adhesion and invasion. The hierarchical clustering was done with R software using the method "hclust ward D2". Three clusters were obtained for low, intermediate and high level pathogenicity for the *C. coli* tested.

0.28% on average for organic pig and conventional pig isolates, respectively, and invasion was 0.019 and 0.015% on average for organic pig and conventional pig isolates, respectively. There were no significant differences between the isolates from organic pigs and isolates from conventional pigs for adhesion (Mann-Whitney test, $p = 0.523$) or invasion (Mann-Whitney test, $p = 0.590$) (**Figure 3**).

The *C. coli* isolates' capacity to adhere and invade *Caco-2* cells was significantly lower than *C. jejuni* isolates isolated from poultry or humans (Mann-Whitney test, *p*-value for p_adh = 0.018 and *p*-value for p_inv = 0.014), but not significantly different from those of *C. coli* isolates isolated from poultry or humans ($p >$ to 0.05) (**Figure 3**).

Among these isolates, eight had the plasmid gene *virB11*. There was no significant difference for adhesion between isolates with or without this gene (Mann-Whitney test, $p = 0.058$), but a significant difference was observed for invasion. Isolates with the *virB11* gene had a higher capacity to invade *Caco-2* cells than isolates without this gene (Mann-Whitney test, $p = 0.040$). We also observed that the human *C. jejuni* strain 81–176 carrying the *virB11* gene had a higher invasion capacity (0.079%) than the human *C. jejuni* strain NCTC 11168 without the *virB11* gene (0.015%).

The 61 *C. coli* were classified into three classes of pathogenicity (low, intermediate and high) from the hierarchical clustering done on the adhesion and invasion values (**Figure 4**). It was difficult to associate a ST with a virulence profile because there were too few isolates representing each ST, with the exception of ST854 for which 16 isolates were tested on *Caco-2* cells. These ST854 isolates were distributed among the three classes of pathogenicity.

DISCUSSION

In this study, the *C. coli* previously isolated from organic and conventional pig farms to test their resistance to antibiotics (Kempf et al., 2017) were typed by PFGE and MLST and tested for their virulence. We wanted to determine if the production system

has an impact on the genetic diversity and on the virulence of these isolates excreted by the pigs.

First, our study focused only on *C. coli,* the only species that was isolated from our pigs. We expected that outdoor pigs would be more exposed to *C. jejuni* from the environment because wild animals, particularly birds, can shed *Campylobacter* species other than *C. coli* (Greig et al., 2015). However, although Jensen et al. (2006) showed that the paddock environment of organic pigs was contaminated by non-pig strains, e.g., by wild fauna strains, they did not demonstrate that these strains could contaminate pigs, at least not *C. jejuni* strains. Pigs show a dominance of *C. coli* (Nielsen et al., 1997; Alter et al., 2005; Boes et al., 2005) and *C. jejuni* may co-exist with *C. coli* in pigs, but *C. jejuni* is typically present at in numbers that are 10–100-fold lower than *C. coli* (Madden et al., 2000; Jensen et al., 2005). In our study, we tested only two to three isolates per farm which may explain also why we never detected *C. jejuni*.

PFGE and MLST revealed high genetic diversity in our *C. coli* populations, as previously observed in French pig farms using PFGE (Denis et al., 2011). Some of the STs have been already described in other countries: ST854 and ST2718 from pig livers in Germany (von Altrock et al., 2013), ST854, ST890, ST1068, ST1096, ST1142, ST1413, ST1417, ST1436, and ST1450 from pig farms in USA (Thakur et al., 2006), and ST828, ST854, ST890, ST1016, ST1061, ST1096, ST1413, ST1556, ST2733, and ST4951 from pig feces at slaughterhouses in Switzerland (Egger et al., 2012). The most prevalent ST in our study was ST854. This ST was also reported to be predominant in pig farms in the USA (Quintana-Hayashi and Thakur, 2012) and has been detected all along the production chain, from pig farms to retail pork meat products (Abley et al., 2012). It is also the most frequently recovered ST from surface of pig livers in Germany (von Altrock et al., 2013). In our study, the ST854 isolates predominantly exhibited the TET+STR+ERY− phenotype whereas the CIP/NAL+TET+ phenotype was mainly found for this ST in the USA study (Quintana-Hayashi and Thakur, 2012).

We also noted that allelic diversity was higher at the *pgm* and *tkt* loci than the other virulence loci, with a greater number of alleles for these two loci. Quintana-Hayashi and Thakur (2012) also observed higher allelic diversity for the *tkt* and *glyA* loci in *C. coli* populations from swine farms.

Although the genetic diversity was higher in organic production than in conventional production for both typing methods, PFGE and MLST, there were no significant differences, except at the locus level for the *glnA*, *gltA*, and *uncA* genes. There were a higher number of alleles for these genes when isolates were from organic pigs. Finally, we were not able to clearly demonstrate that organic production practices with little or no use of antibiotics and outdoor access for pigs promotes a higher number of *Campylobacter* genotypes. There were common *Campylobacter* genotypes shared between both production systems. We already identified these PFGE types on French pig production farms in 2008 (Denis et al., 2011) and two STs (ST584, ST890) were also isolated by Thakur et al. (2006) from conventional and antimicrobial-free pig farms in the USA. Our results suggest that these common genotypes are adapted to the pig and that other genotypes are likely specific to the farm environment where the pigs are grown.

The production system does not select for specific virulence gene profiles, with the exception of the plasmid gene *virB11*. Almost all the isolates carried the *ceuE*, *iam*, *ciaB, flaA, cadF* genes, and the three *cdt* genes. High prevalence for these genes has been obtained on *C. coli* in many studies (Bang et al., 2003; Rozynek et al., 2005; Wieczorek and Osek, 2010, 2013; Andrzejewska et al., 2011; Acik et al., 2013; Khoshbakht et al., 2013) except for the *ciaB* gene. The detection of *ciaB* varies with species and study, ranging from 20% for *C. coli* (Wieczorek and Osek, 2010; Acik et al., 2013) up to 100% for *C. jejuni* (Datta et al., 2003; Feodoroff et al., 2010). This discrepancy may be due to the primers used; we designed new primers that facilitated the detection of the *ciaB* gene in all our *C. coli* isolates.

The plasmid gene *virB11*was detected in eight isolates, all from organic pigs. This gene has also been detected in 28% of the *C. jejuni* strains isolated from free-range broiler flocks (Hanning et al., 2010) and with a low prevalence for *C. coli* from various origins (Wieczorek and Osek, 2010, 2013; Acik et al., 2013). The low frequency of these isolates did not allow us to conclude that their presence in organic farms is related to the management of this type of production system.

There was no difference in the pathogenicity between organic and conventional *C. coli* pig isolates when tested on *Caco*-2 human intestinal cells. Moreover, different levels of pathogenicity were observed for the ST854 isolates, the most prevalent ST, regardless of the production system. Our *C. coli* isolates have low adhesion and invasion capacities, similar to *C. coli* from poultry and humans, compared with *C. jejuni* strains from poultry and humans. Guyard-Nicodème et al. (2013) reported similar results between the two species in a comparison of *C. jejuni* and *C. coli* strains isolated from poultry.

We showed that the isolates with the plasmid gene *virB11* had a higher invasion capacity than isolates without this gene. Moreover, the human *C. jejuni* 81–176 strain carrying the plasmid also showed higher invasion capacity than the human *C. jejuni* NCTC 11168, which does not possess the vi*rB11* gene. This relationship between high invasion and presence of the plasmid gene *virB11* has previously been reported (see Bacon et al., 2002).

CONCLUSION

Our study could not conclusively demonstrate that the type of pig production system influences the *C. coli* population. We confirmed the high genetic diversity of *C. coli* in pigs in France, and showed that isolates sharing the same ST may show different levels of pathogenicity. This study helped improve the detection of virulence genes in *C. coli*, a species less studied than *C. jejuni,* and provided data on the virulence of this species, and more particularly of *C. coli* isolated from pigs.

AUTHOR CONTRIBUTIONS

MD: Conception of the study, analysis and interpretation of data, and drafting of the manuscript; BN, VR, KB, MC, AK: Acquisition of the isolates and data; MD, AK: Critical revision

of important intellectual content; All authors: Final approval of the version to be published and accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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Comparative Proteomics Reveals Differences in Host-Pathogen Interaction between Infectious and Commensal Relationship with *Campylobacter jejuni*

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Campylobacter jejuni is the leading food-borne poisoning in industrialized countries. While the bacteria causes disease in humans, it merely colonizes the gut in poultry or pigs, where seems to establish a commensal relationship. Until now, few studies have been conducted to elucidate the relationship between *C. jejuni* and its different hosts. In this work, a comparative proteomics approach was used to identify the underlying mechanisms involved in the divergent outcome following *C. jejuni* infection in human and porcine host. Human (INT-407) and porcine (IPEC-1) intestinal cell lines were infected by *C. jejuni* for 3 h (T3h) and 24 h (T24h). *C. jejuni* infection prompted an intense inflammatory response at T3h in human intestinal cells, mainly characterized by expression of proteins involved in cell spreading, cell migration and promotion of reactive oxygen species (ROS). Proteomic analysis evidenced significantly regulated biofunctions in human cells related with engulfment and endocytosis, and supported by canonical pathways associated to infection such as caveolar- and clathrin-mediated endocytosis signaling. In porcine IPEC-1 cells, inflammatory response as well as signaling pathways that control cellular functions such as cell migration, endocytosis and cell cycle progression resulted downregulated. These differences in the host response to infection were supported by the different pattern of adhesion and invasion proteins expressed by *C. jejuni* in human and porcine cells. No marked differences in expression of virulence factors involved in adaptive response and iron acquisition functions were observed. Therefore, the results of this study suggest that both host and pathogen factors are responsible for commensal or infectious character of *C. jejuni* in different hosts.

Keywords: human, pig, intestinal epithelial cells, SWATH-MS, immunity, infection

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INTRODUCTION

Human campylobacteriosis is leading the rank of food-borne diseases in developed countries, with *Campylobacter* being the most regularly reported zoonotic pathogen in the European Union since 2005 (EFSA, 2015). The disease is usually associated with self-limiting mild diarrhea, which in particular cases such as immunocompromised patients can progress to a severe systemic infection (Clark et al., 2016). In addition to diarrhea, campylobacteriosis has been linked to inflammatory bowel disease (Spiller, 2007) and reactive arthritis or Guillain-Barré syndrome (Yuki et al., 2004).

Human *Campylobacter* infections are caused mainly by species *Campylobacter jejuni* and *C. coli*, although the former accounts for more than 90% of the human infections (Wilson et al., 2008). Several environmental and animal reservoirs may become the origin of human infections (Young et al., 2007), although the primary source of human campylobacteriosis is contaminated chicken and pork meat (Wilson et al., 2008). *Campylobacter* spp. usually colonizes the gut of chickens and pigs, where it seems to establish a commensal relationship, harboring concentrations of 10^7–10^{10} CFU/gram of feces in their intestines but lacking the development of clinical signs in the host (Battersby et al., 2016). Despite the importance of the disease in humans, our understanding of the virulence mechanisms of *Campylobacter* is still relatively poor (Dasti et al., 2010; Aguilar et al., 2014). Moreover, little is known about the mechanisms that determine the pathogenic of commensal character of *Campylobacter* in different hosts. In a previous *in vitro* study, we revealed that *C. jejuni* preferentially interacted with human intestinal epithelial cells in which the level of bacterial invasion was up to 10-fold higher than in porcine intestinal cells (Aguilar et al., 2014). This study also showed that a strong inflammatory response occurred in human cells after bacterial infection while no response was observed in intestinal epithelial cells of porcine origin. Despite these results, much is still unknown about why *Campylobacter* is pathogenic to humans and not to other species such as pigs. Here, we surveyed the proteome of human and porcine intestinal epithelial cells after *C. jejuni* infection in order to elucidate the molecular mechanisms underlying the pathological or commensal behavior of *Campylobacter* in different hosts.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Porcine intestinal epithelial cell line IPEC-1, derived from the small intestine of a newborn unsuckled piglet, was cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS, PAA Laboratories GmbH, Pasching, Austria), epidermal growth factor (5 μg/mL) (Sigma–Aldrich, St. Louis, MO, USA), insulin (10 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL) (ITS Premix, Sigma) and 2 mM L-glutamine (Life Technologies). Human intestinal epithelial cells INT-407 (human embryonic intestine, ATCC CCL-6) were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland) and supplemented with 10% FBS (PAA Laboratories GmbH) and 2 mM L-glutamine (Life Technologies). All cell lines were seeded in multi-well tissue culture plates (Thermo Fischer Scientific, Waltham, USA) the day before the assay, and allowed to reach confluence for the *in vitro* infection. The cells were maintained in an atmosphere of 5% CO_2 at 37°C.

Bacterial Strain and *In vitro* Infection

A confirmed isolate of *C. jejuni* from chicken feces was recovered from stocks kept at −80°C by plating on Columbia sheep blood agar (Oxoid, Basinstoke, Hampshire, UK) for 48 h at 37°C under microaerobic conditions (AnaeroGen system, Oxoid). The bacteria were harvested from plate and resuspended in fresh cell culture media. The optical density (OD_{600}) was adjusted to 1 to achieve 10^8 CFU/ml for their straight inoculation into INT-407 and IPEC-1 cells at a multiplicity of infection (MOI) of 100/1. All *in vitro* cell infections with bacteria were performed in triplicate as previously described (Aguilar et al., 2014) using two points in the study time course: 3 h (early time, T3h) and 24 h (late time, T24h). The same time course was used with the uninfected controls.

Protein Extraction and Trypsin Digestion

The cells were centrifuged at 10,000 × g for 3 min, and cell pellets were frozen in liquid nitrogen until used for protein extraction. Approximately 10^7 cells were pooled from each time point and homogenized with a needle (27G) in 500 μl lysis buffer [1% Triton X-100 supplemented with Complete protease inhibitor cocktail (Roche, Basel, Switzerland)]. The samples were sonicated for 1 min in an ultrasonic cooled bath, followed by 10 s of vortexing. After 3 cycles of sonication-vortexing, total cell extracts were centrifuged at 200 × g for 5 min to remove cell debris. The supernatants were collected and protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as standard. Protein extracts (150 μg) were on-gel concentrated by SDS-PAGE as previously described (Villar et al., 2014). The unseparated protein bands were visualized by staining with GelCode Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA), excised, cut into 2 × 2 mm cubes and digested overnight at 37°C with 60 ng/ml of sequencing grade trypsin (Promega, Madison, WI, USA) at 5:1 protein:trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8 containing 10% (v/v) acetonitrile (Shevchenko et al., 2006). The resulting tryptic peptides were extracted by 30 min-incubation in 12 mM ammonium bicarbonate, pH 8.8. Trifluoroacetic acid was added to a final concentration of 1% and the peptides were finally desalted onto OMIX Pipette tips C18 (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at –20°C until mass spectrometry analysis.

Proteome Analysis by SWATH-MS

The desalted protein digest was resuspended in 5% acetonitrile with 0.1% formic acid and analyzed by reverse phase liquid chromatography coupled online with mass spectrometry (RP-LC-MS/MS) using an Ekspert nLC 415 system coupled to a 6,600 TripleTOF mass spectrometer (AB SCIEX, Framingham, US)

through Information-Dependent Acquisition (IDA) followed by SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra). Approximately 4 μg of each protein digest from each of the replicate samples were pooled together as a mixed sample for each condition (control and infected cells) and each cellular type. Pooled mixed samples were then used for the generation of the reference spectral ion library as part of SWATH-MS analysis. The peptides were concentrated using a 0.1 × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075 × 250 mm C18 RP column (New Objetive, Woburn, MA, USA) operating at 0.3 ml/min. Peptides were eluted using a 120-min gradient from 5 to 40% solvent B followed by 15-min gradient from 40 to 60% solvent B (Solvent A: 0.1% formic acid in water, solvent B: 0,1% formic acid in acetonitrile) and directly injected into the mass spectrometer for analysis. Three technical replicates of each mixed sample were analyzed. For IDA experiments, the mass spectrometer was set to scanning full spectra (350–1,400 m/z) using 250 ms accumulation time per spectrum, followed by up to 50 MS/MS scans (100–1,500 m/z). Candidate ions with a charge state between +2 and +5, and counts per second above a minimum threshold of 100, were isolated for fragmentation. One MS/MS spectrum was collected for 100 ms, before adding those precursor ions to the exclusion list for 15 s (mass spectrometer operated by Analyst® TF 1.6, AB SCIEX). Dynamic background subtraction was turned off. MS/MS analyses were recorded in high sensitivity mode with rolling collision energy on and a collision energy spread of 5. For SWATH quantitative analysis, 8 μg of each mixed sample were subjected in triplicate to the cyclic data independent acquisition (DIA) of mass spectra using the SWATH variable windows calculator (V 1.0, AB SCIEX) and the SWATH acquisition method editor (AB SCIEX), following previously established methods (Gillet et al., 2012). A set of 50 overlapping windows was constructed (containing 1 m/z for the window overlap), covering the precursor mass range of 400–1,250 m/z. For these experiments, a 50 ms survey scan (350–1,400 m/z) was acquired at the beginning of each cycle, and SWATH-MS/MS spectra were collected from 100 to 1,500 m/z for 70 ms at high sensitivity mode, resulting in a cycle time of 3.6 s. Collision energy for each window was determined according to the calculation for a charge +2 ion-centered upon the window with a collision energy spread of 15.

Library Generation/Protein Identification, Data Processing, and Relative Quantitation

To create a spectral library with those peptides that have been detected and identified in the experimental time course, the IDA MS raw files were combined and subjected to database searches in unison using ProteinPilot software v. 5.0.1 (AB SCIEX) with the Paragon algorithm. Spectra identification was performed by searching against the *Homo sapiens* proteome for INT samples, Sus scrofa taxa for IPEC-1 samples and the *C. jejuni* taxa for INT and IPEC infected samples (Uniprot Databases: 70,826, 34,409, and 41,050 entries respectively, in December 2015) with the following parameters: iodoacetamide cysteine alkylation, trypsin digestion, gel-based ID as special factor, identification focus on biological modification and evolutionary variants and thorough ID as search effort. The detected protein threshold was set at 0.05. An independent False Discovery Rate (FDR) analysis, using the target- decoy approach provided by ProteinPilot, was used to assess the quality of identifications. Positive identifications were considered when identified proteins reached a 1% global FDR. For SWATH processing, up to 10 peptides with seven transitions per protein were automatically selected by the SWATH Acquisition MicroApp2.0 in the PeakView2.2 software (AB SCIEX) with the following parameters: 15 ppm ion library tolerance, 5 min XIC extraction window, 0.01 Da XIC width, and considering only peptides with at least 99% confidence and excluding those which were shared or contained modifications. However, to ensure reliable quantitation, only proteins with 3 or more peptides available for quantitation were selected for XIC peak area extraction and exported for analysis in the MarkerView 1.3 software (AB SCIEX). Global normalization was performed according to the Total Area Sums of all detected proteins in the samples. A Student's *T*-test was used to perform two-sample comparisons between the averaged area sums of all the transitions derived for each protein across the three replicate runs for each sample under comparison, in order to identify proteins that were significantly differentially represented between infected and uninfected samples.

Systems Biology Analysis

Ingenuity Pathway Analysis (IPA) web-based application (Ingenuity Systems Inc., Redwood City, CA, USA) was used to assess the biological meaning in the host proteome datasets. IPA retrieves biological information from the literature and then integrates the differentially expressed proteins into functions and pathways with biological meaning and significance ($p < 0.05$). Functional association networks of the potentially interacting proteins were generated using STRING (Search Tool for the Retrieval of Interacting Genes, v.10 web server, http://stringdb.org/), a database of known and predicted protein interactions (Szklarczyk et al., 2011). Virulence factors expressed in *C. jejuni*-infected INT-407 cells were used as dataset framework for mapping functional pathways. The model was enriched up to 100 partners with different sorts of associations to the proteins included in the model.

RESULTS

Intestinal Epithelial Cell Proteins

Differentially expressed (DE) proteins were identified both in human and porcine intestinal epithelial cells after *C. jejuni* infection (**Figure 1**, **Table S1**). A total of 366 and 485 DE proteins were identified at T3h and T24h time points of infection, respectively, in INT-407 cell line. We then used Ingenuity Pathway Analysis (IPA) to examine the predicted biological effects of the protein expression differences seen between control and infected cells. The results of this analysis (**Table S2**) predicted a significant activation of the inflammatory response, increased at T3h compared to T24h, and associated to the differential expression of many proteins involved in cell migration and infiltration, cell movement, cytoskeleton organization and promotion of reactive oxygen species (ROS)

(**Figure 2A**). IPA analysis also predicted that infection of cells, molecular transport and synthesis of proteins were functions activated at T3h and reduced at T24h. Similarly, INT-407 infected cells showed increased endocytosis, proliferation and viability at T3h compared to T24h. Cell death as well as gene expression, mainly represented by mRNA expression and translation process, were significantly decreased at early and late time points (**Figure 2A**, **Table S2**). Additionally, we found changes in the host energy metabolism at T24h characterized by upregulation of key glycolytic enzymes and transporters for glucose uptake, and downregulation of enzymes participating in the pyruvate metabolism and oxidative phosphorylation (**Figure 2A**, **Table S2**). The canonical signaling and metabolic pathways of all differentially expressed proteins in *C. jejuni*-infected cells at 3 and 24 h are shown in **Table S3**. **Table 1** lists the top 10 most significantly enriched pathways. As shown in **Table 1**, diverse and complex signals related to cell immune/inflammatory transduction pathways, cell death, cell growth and proliferation, and metabolic pathways are involved in the intestinal mucosa after *C. jejuni* infection. The most significant canonical pathway both at early and late time post-infection was eukaryotic initiation factor-2 (eIF2) signaling, which is involved in eukaryotic protein synthesis and plays a central role in antibacterial response. Other pathways involved in translational regulation such as regulation of eIF4 and p70S6K signaling and mTOR signaling, as well as protein ubiquitination and mitochondrial dysfunction were also induced at both times of infection in human cells. Also in INT-407 cells, Ras related nuclear protein (RAN) signaling, caveolar-mediated endocytosis, tRNA charging and granzyme B signaling were induced only at earlier stages of infection, while remodeling of epithelial adherens junction and phagosome maturation were induced only at later times.

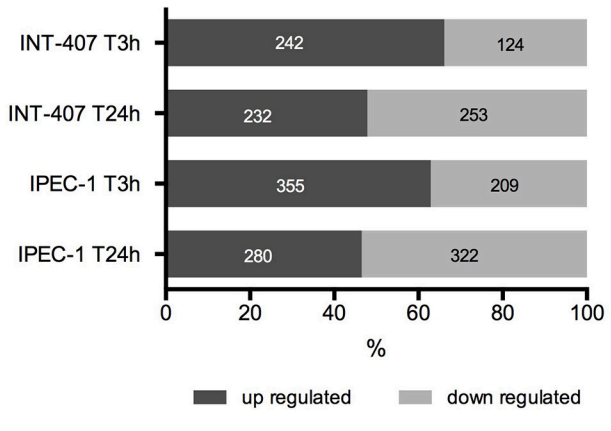


FIGURE 1 | Percentage and number of up and downregulated proteins in human (INT-407) and porcine (IPEC-1) intestinal epithelial cells at early (T3h) and late (T24h) stages of *C. jejuni* infection.

In the porcine epithelial cells, we found 355 upregulated and 209 downregulated proteins shortly after *C. jejuni* infection (**Figure 1**). Later, we observed upregulation of 280 molecules and downregulation of 322. The response proteins DISP2 and NAA15 were among the most upregulated molecules, finding higher levels of expression at later stages of the infection (**Table S1**). When exploring the biological functions altered (**Figure 2B**, **Table S2**), we observed a predicted inhibition of apoptosis signaling during early infection. This decreased apoptosis and subsequent increase in cell proliferation was mainly IL-8 and

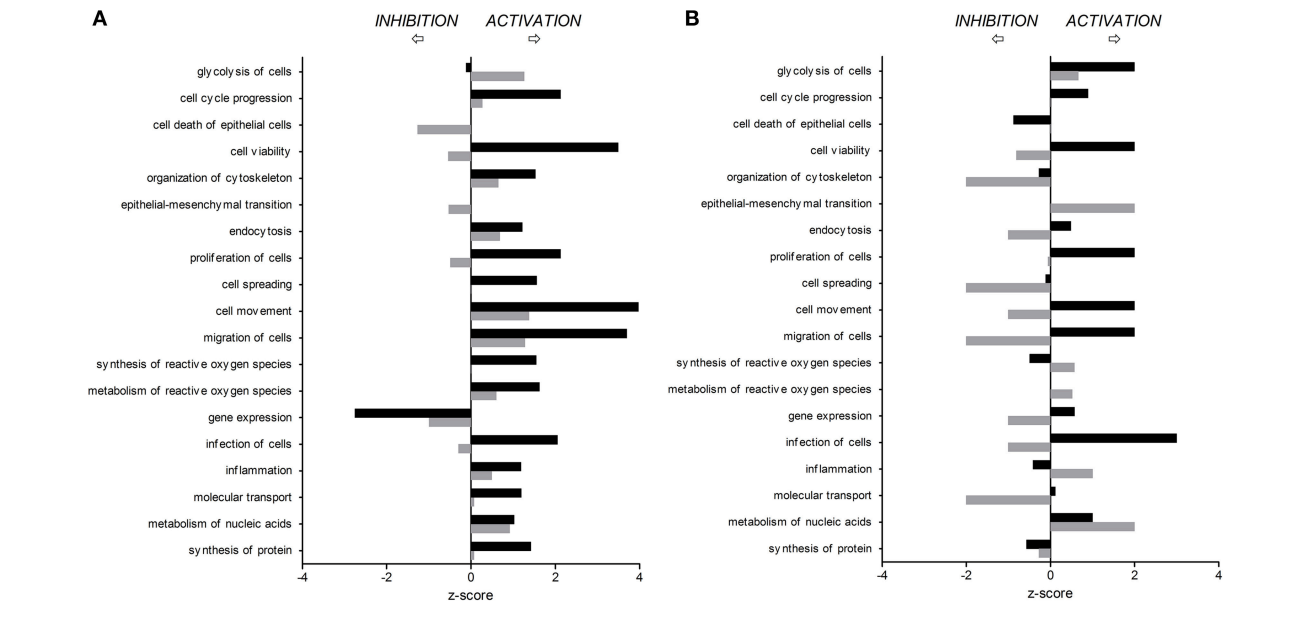


FIGURE 2 | Predicted activation or inhibition of selected biological functions affected by *C. jejuni* infection in human (A) and porcine **(B)** intestinal epithelial cells 3 h (T3h, black bars) and 24 h (T24h, gray bars) after *in vitro* infection, compared to non-infected cells.

TABLE 1 | The top 10 canonical pathways induced by *Campylobacter jejuni* in INT-407 and IPEC-1 cells at 3 and 24 h post-infection.

Canonical pathways	Proteins Up	Proteins Down	−log (*p*-value)
INT-407 T3h			
EIF2 signaling	47/184 (25.4%)	5/184 (2.6%)	4.78E01
Regulation of eIF4 and p70S6K signaling	22/146 (15.1%)	4/146 (2.7%)	1.84E01
mTOR signaling	21/187 (11.2%)	4/187 (2.2%)	1.47E01
Protein ubiquitination pathway	9/255 (3.5%)	10/255 (3.9%)	6.96E00
RAN signaling	3/16 (18.75%)	3/16 (18.75%)	6.75E00
Caveolar-mediated endocytosis signaling	5/71 (7.1%)	4/71 (5.6%)	5.44E00
Unfolded protein response	4/54 (7.4%)	4/54 (7.4%)	5.42E00
tRNA charging	6/39 (15.4%)	1/39 (2.5%)	5.38E00
Granzyme B signaling	5/16 (31.2%)	0/16 (0%)	5.25E00
Mitochondrial dysfunction	7/171 (4.1%)	6/171 (2.5%)	5.04E00
INT-407 T24h			
EIF2 signaling	32/187 (17.1%)	5/187 (2.6%)	2.43E01
Protein ubiquitination pathway	17/259 (6.5%)	15/259 (5.8%)	1.48E01
Regulation of eIF4 and p70S6K signaling	13/150 (8.7%)	6/150 (4%)	9.22E00
Remodeling of epithelial adherens junctions	5/68 (7.3%)	7/68 (10.3%)	7.68E00
Mitochondrial dysfunction	16/188 (8.5%)	3/188 (1.6%)	7.57E00
mTOR signaling	13/193 (9.7%)	6/193 (3.1%)	7.39E00
Phagosome maturation	7/127 (5.5%)	8/127 (6.3%)	7E00
Epithelial adherens junction signaling	9/148 (6.1%)	5/148 (3.4%)	5.4E00
Germ cell-sertoli cell junction signaling	6/163 (3.7%)	8/163 (4.9%)	4.91E00
Unfolded protein response	3/54 (5.6%)	5/54 (9.25%)	4.72E00
IPEC-1 T3h			
EIF2 signaling	18/187 (9.6%)	15/187 (8%)	2.03E01
Regulation of eIF4 and p70S6K signaling	15/150 (10%)	8/150 (5.3%)	1.29E01
Integrin signaling	15/208 (7.2%)	9/208 (4.3%)	1.07E01
Protein ubiquitination pathway	18/259 (6.9%)	8/259 (3.1%)	1.02E01
Actin cytoskeleton signaling	12/220 (5.45%)	11/220 (5%)	9.44E00
mTOR signaling	13/193 (6.7%)	7/193 (3.6%)	8.22E00
Germ cell-sertoli cell junction signaling	9/163 (5.5%)	9/163 (5.5%)	7.89E00
Virus entry via endocytic pathways	8/95 (8.4%)	6/95 (6.3%)	7.88E00
Remodeling of epithelial adherens junctions	6/68 (8.8%)	6/68 (8.8%)	7.74E00
ILK signaling	7/187 (3.7%)	12/187 (6.4%)	7.7E00
IPEC-1 T24h			
EIF2 signaling	34/187 (18.2%)	18/187 (9.6%)	4.05E01
Regulation of eIF4 and p70S6K signaling	12/150 (8%)	18/150 (12%)	1.9E01
mTOR signaling	10/193 (5.2%)	16/193 (8.3%)	1.22E01
Protein ubiquitination pathway	11/259 (4.3%)	14/259 (5.4%)	8.65E00
Actin cytoskeleton signaling	11/220 (5%)	9/220 (4.1%)	6.62E00
Glycolysis I	9/41 (21.3%)	0/41 (0%)	6.47E00
Caveolar-mediated endocytosis signaling	4/73 (5.5%)	7/73 (9.6%)	6.03E00
ILK signaling	3/187 (1.6%)	14/187 (7.5%)	5.72E00
RAN signaling	1/18 (5.6%)	5/18 (27.8%)	5.65E00
Germ cell-sertoli cell junction signaling	5/163 (3.1%)	10/163 (6.2%)	5.18E00

PI3K/AKT mediated (**Table S3**). In both human and porcine epithelial cell lines, cell survival was activated in early stages, but tended to be inhibited at later stages of infection, as apoptosis increased.

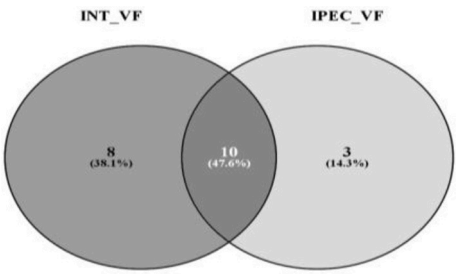
Infection of this porcine epithelial cell line was demonstrated by upregulation of proteasome subunits PSMA1, PSMA2, PSMA5, PSMC5, and PSMD12, as well as other molecules such as CLTA, BSG, IDH1, KPNB1, MAP4, PCBP2, PGM1, RPL10A, RPL12, SF3A1, SF3B2, SNRPD3, TAGLN2, and TFRC (**Table S1**). However, we did not find upregulation of canonical inflammatory pathways at any time point after infection, and acute phase response was downregulated (**Table S3**). Actin polymerization was activated at early but inhibited in late stages of infection (**Table S3**). The endocytic

process (membrane ruffling) mediated by beta integrins (e.g., CDC42 and RAC1) was mildly increased at T3h, but inhibited T24h after infection. In IPEC-1 cells, eIF2 signaling pathway was predictably inhibited at early infection, and activated later (**Table 1**, **Table S3**). Similarly, cholesterol biosynthesis metabolic process was downregulated at early stages, while it was increased at later stages, thus inhibiting NFκB-mediated transcription of inflammatory mediators. Glycolisis and gluconeogenesis were upregulated 24 h after infection.

C. jejuni Proteins

The label-free quantification by SWATH-MS allowed the identification of 53 and 31 *C. jejuni* proteins upon infection of INT-407 (**Table S4**) and IPEC-1 cells (**Table S5**). Some of these proteins are related to virulence and others are involved in bacteria survival, metabolism, DNA replication and genetic information processing. The virulence factors expressed by *C. jejuni* in human and porcine intestinal epithelial cells are shown in **Table 2**. Eight virulence proteins were exclusively detected in INT-407 cells, including two type VI secretion system effectors (pebA and hcP), two adaptive response proteins (the chaperone dnaK and the histidine kinase cheA), the p19 iron acquisition protein, the thiol-peroxidases Tpx that plays a role in oxidative stress protection, and the two-component regulator Cj0355c. Ten *C. jejuni* virulence proteins associated to adherence, adaptive response, oxidative stress and iron acquisition were expressed both in human and porcine intestinal epithelial cell lines. Interestingly the expression of some of these proteins in each cell type was markedly different. For example, at early infection, the flagellin protein flaA, the methyl accepting chemotaxis protein cstIII ($p < 0.01$) and the chaperonin groL ($p < 0.01$) expression was upregulated in INT-407 cells (**Table S4**). In contrast, flaA and groL expression was downregulated in porcine IPEC-1 cells after *C. jejuni* infection whereas cstIII ($p = 0.33$) remained unchanged (**Table S5**). Iron captation proteins such as iron deficiency induced protein cfbpA and the ferric enterobactin receptor cfrA expression resulted upregulated at T24h in both cell lines compared to T3h ($p < 0.001$).

TABLE 2 | *Campylobacter jejuni* virulence factors identified in INT-407 and IPEC-1 infected cells using SWATH-MS label-free.



Protein	Gene	Type of function	INT-407	IPEC-1
Adhesin pebA	*PebA*	Adherence	Detected	–
Chaperonin GroEL	*groL*	Adherence	Detected	Detected
Methyl-accepting chemotaxis protein CstIII	*cstIII*	Adherence	Detected	Detected
Flagellin FlaA	*flaA*	Motility	Detected	Detected
Chaperone protein DnaK	*dnaK*	Heat shock response	Detected	–
Chemotaxis histidine kinase cheA	*cheA*	Heat shock response	Detected	–
Chaperone protein HtpG	*htpG*	Heat shock response	Detected	Detected
Chemotaxis protein	*N218_03105*	Heat shock response	Detected	–
Peroxidase	*ahpC*	Oxidative stress	Detected	Detected
Periplasmic protein p19	*P19*	Iron captation	Detected	–
Iron deficiency-induced protein A	*cfbpA*	Iron captation	Detected	Detected
Ferric enterobactin receptor cfrA	*cfrA*	Iron captation	Detected	Detected
Superoxide dismutase	*sodB*	Oxidative stress	Detected	Detected
Thiol peroxidase tpx	*tpX*	Oxidative stress	Detected	–
Two-component regulator Cj0355c	*Cj0355c*	Oxidative stress	Detected	–
Putative membrane protein CjaE	*cjaE*	Oxidative stress	Detected	Detected
Type VI secretion system protein	*hcP*	Virulence factor	Detected	–
Peptidoglycan-associated essential protein cjaD	*cjaD*	Virulence factor	Detected	Detected
Thioredoxin	*trxB*	Oxidative stress	–	Detected
Hydrogenase 2 large subunit	*M635_02020*	Oxidoreduction process	–	Detected
Lytic transglycosylase	*mltE*	Cell wal murein synthesis	–	Detected

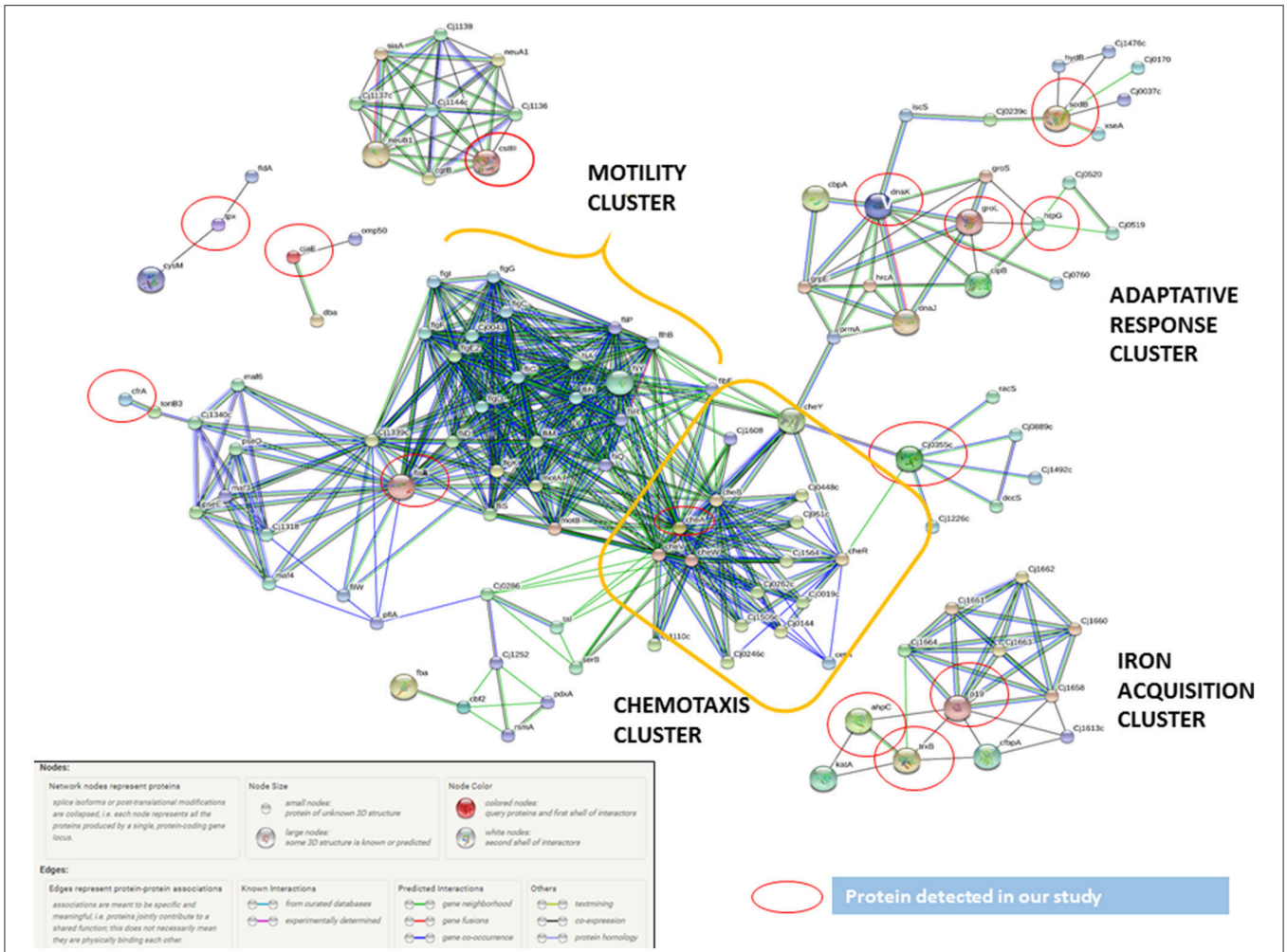


FIGURE 3 | *C. jejuni* virulence proteins interaction network. The network allocated the *C. jejuni* detected proteins in INT-407 cell line (red circles) together with functional partners. The biggest cluster was associated to chemotaxis and related to cluster including all motility proteins. The other proteins included were associated to adaptive response (heat shock and oxidative stress proteins) and iron acquisition proteins.

A relevant number of *C. jejuni* proteins identified in this study were involved in metabolism. Thus, 33 proteins associated to metabolic processes were detected in INT-407 infected cells, 16 of these proteins showed significantly ($p < 0.05$) reduced levels at T24h compared to T3h and only three proteins showed a moderate increase in their levels at 24 h post-infection. This *C. jejuni* metabolic downshift at T24h of infection was not observed in IPEC-1, where from 12 *C. jejuni* identified proteins associated to metabolism, only hisF ($p < 0.01$) and acnB ($p < 0.05$) resulted significantly downregulated after 24 h of infection.

By using STRING tool we built an enriched model to predict associations among observed *C. jejuni* proteins and functional partners (**Figure 3**, **Table S6**). The predicted model organized proteins in different clusters. The main cluster was built around cheA chemotaxis protein, surrounded by 15 functional proteins in total. This "chemotaxis cluster" was closely linked to the "motility cluster," where the adhesion protein flaB was associated to flagellar proteins such as fliD, fliY, or motA as well as the protein pseE, involved in the glycosylation of the flagella (**Figure 3**). The heat-shock proteins dnaK and htpG were clustered together and with the chemotaxis protein groL. Finally, two "iron uptake clusters" were conformed. The siderophor cbpA and iron captation protein p19 were joined to the oxidative stress protein ahpC and other functional partners, which include the permease cfbpB, the lipotrotein cj0176c, and the tioredoxin trxB. On the other hand, iron captation protein cfrA was included in other iron uptake cluster, among its partners are included the protein exB1 associated to iron uptake from the host and the protein atpD, involved in the synthesis of ATP, which was detected in our study, although not included as virulence factor.

DISCUSSION

Despite the fact that *Campylobacter* continues to be the leading cause of bacterial foodborne diarrheal disease in EU (EFSA, 2015), little is known about the molecular mechanisms

underlying the intestinal infection caused by bacteria. Much less is known about why *Campylobacter* is pathogenic to humans but acts as commensal bacterium in the gastrointestinal tract of pigs (Horrocks et al., 2009; Bratz et al., 2013). In a previous work we demonstrated that *C. jejuni* adheres preferentially to human intestinal epithelial cells and that this cellular tropism was accompanied by a strong inflammatory response (Aguilar et al., 2014). In this work, using a next generation proteomic approach, we sought to understand better the duality of behavior from commensal to pathogen that *Campylobacter* manifests in the porcine and human host, respectively.

As expected, *C. jejuni* infection prompted an intense inflammatory response in human intestinal cells, mainly characterized by expression of proteins involved in cell spreading, cell movement or cell migration and promotion of reactive oxygen species (ROS). In addition, the eIF2 signaling pathway, involved in inflammatory response, was the most representative canonical pathway, with major activation state at T3h, when bacterial invasion was more intense. eIF2 signaling, critical for stress-induced regulation of translation in eukaryotic cells, is activated by pathogens and is part of a general antibacterial defense system (Shrestha et al., 2012). In agreement with previous studies (Skjolaas et al., 2007; Horrocks et al., 2009; Bratz et al., 2013; Aguilar et al., 2014), we found just a slight inflammatory response in porcine intestinal cells after *Campylobacter* infection, indicating a potential commensal behavior.

C. jejuni uses different strategies to enter intestinal epithelial cells such as receptor binding or membrane ruffling (Krause-Gruszczynska et al., 2007; Croinin and Backert, 2012; Eucker and Konkel, 2012). In this work, proteomic analysis highlighted membrane ruffling associated with bacterial entry in INT-407 cells, evidenced by biofunctions related with engulfment and endocytosis, and supported by canonical pathways associated to infection such as caveolar- and clathrin-mediated endocytosis signaling. In porcine IPEC-1 cells, the endocytic process was mildly increased at T3h but inhibited 24 h after infection. The presence of membrane ruffling commonly involves the activation of small Rho family GTPases to induce cellular responses during the infection process (Krause-Gruszczynska et al., 2011). The cell division control protein 42 homolog (CDC42) is a prominent member of this protein family, whose role is to regulate signaling pathways that control cellular functions such as cell migration, endocytosis and cell cycle progression. Here we found upregulation of CDC42 in the early stages of the *C. jejuni* infection in INT-407 cells but downregulation in porcine intestinal epithelial cells, according with the lower levels of bacterial invasion previously observed in the porcine cells (Aguilar et al., 2014). The decrease of cytoskeletal reorganization observed in IPEC-1 cells, probably due to the downregulation of the cytokeratins KRT18/KRT8 and filamin A (FLNA), both activators of the organization and cross linking of actin filaments (Shi et al., 2013), can also be an indicator of reduced cell invasion.

In addition to invasion, pathogenesis of enteric *Campylobacter* infection depends on the ability of bacteria to adhere the intestinal epithelial barrier by adhesins, chemotaxis proteins, and binding proteins (Dasti et al., 2010), causing epithelial damage, loss of cellular function, liberation of electrolytes and finally diarrhea (Everest et al., 1992; Carvalho et al., 2001). In this study, a number of *C. jejuni* proteins related to motility, adhesion and invasion were detected at the time of infection of intestinal epithelial cells. Among these factors, it was included flaA, which is a major component of the *C. jejuni* flagellum filament, involved in motility and secretion (Sulaeman et al., 2012), and a major factor in adherence and invasion of the host cells (Yao et al., 1994). Other adherence and invasion factors found were the periplasmic invading protein pebA (Sulaeman et al., 2012) and the methyl-accepting chemotaxis protein (CstIII) (Hendrixson and DiRita, 2004). The overexpression of all these proteins in INT-407-infected cells at T3h was one of the major insights of this study, showing the success of the *C. jejuni* adhering and invading INT-407 cells in early stages of the infection. In contrast, in IPEC-1 cells, these proteins were either not detected or their expression was downshifted evidencing the existence of clear differences in expression of *C. jejuni* adherence and invasion factors between susceptible and commensal hosts, and confirming the insights stated by Aguilar et al. (2014). However, no marked differences were observed in the expression of other virulence proteins grouped in adaptive response and iron acquisition functions, both required to colonize and invade the host cell (Miller et al., 2009).

Also, a relevant number of proteins and enzymes related to *C. jejuni* metabolic routes were detected in both cell lines, but particularly in INT-407 cells. Our major finding was that a relevant number of metabolism factors resulted downregulated in the INT-407 cells at 24 h after infection. Liu et al. (2012) already described a remodeling of the bacterial proteome after infection of COS-1 cells in which metabolic factors were downshifted. This transition to a stationary stage could presumably associated with the *C. jejuni* adaptation to the intracellular environment.

In conclusion, the present study reveals marked differences between infective and commensal behavior of *C. jejuni* in human and porcine intestinal cell lines. Inflammatory response, endocytosis and cellular stress observed in human INT-407 cells because of the bacterial infection were not evidenced in IPEC-1 cells. These differences in the translational cell response to infection was supported by the different expression program triggered by *C. jejuni* in human and porcine cells which could be a key factor in successful cell invasion of human intestinal epithelial cells. However, the fact that no marked differences in expression of some virulence proteins required to colonize host cells were observed in INT-407 and IPEC-1 cells, suggests that certain host factors, such as cell receptors, should also be responsible for differences in host cell invasion and consequently for differences in the relationships that *Campylobacter* establishes with the different hosts.

AUTHOR CONTRIBUTIONS

NA, ÁJ, and CA carried out the experimental infections. JD and MV performed the proteomic quantification and analysis.

ÁJ, AM, HA, and SZ performed the biological functions analyses and interpretation of results, drafted and edited the manuscript. JG conceived and designed the project, and participated in the interpretation and discussion of the results, as well as in the writing of the manuscript. NA, ÁJ, HA, SZ, CA, MV, AM, JD, and JG read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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

Table S1 | Differentially expressed proteins in both cell lines.

Table S2 | Biological functions IPA results in both cell lines.

Table S3 | Canonical pathways IPA results in both cell lines.

Table S4 | *C. jejuni* proteins identified in INT-407 cell line.

Table S5 | *C. jejuni* proteins identified in IPEC-1 cell line.

Table S6 | String proteins information.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Effect of Feed Additives on Productivity and *Campylobacter* spp. Loads in Broilers Reared under Free Range Conditions

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The poultry reservoir, especially broiler meat, is generally recognized as one of the most-important sources for human Campylobacteriosis. The measures to control *Campylobacter* targeted essentially the primary production level. The aim of this work was to evaluate the effectiveness of different treatments against natural *Campylobacter* colonization in a French experimental farm of free-range broilers during the whole rearing period. Five commercial products and a combination of two of them were tested and all the products were added to feed or to water at the dose recommended by the suppliers. *Campylobacter* loads in caeca and on carcasses of broilers at the slaughter were determined by culture methods. Natural contamination of the flock occurred at the end of the indoor rearing period between day 35 and day 42. At day 42, the multispecies probiotic added to the feed reduced the contamination of 0.55 $\log_{10}$ CFU/g ($p = 0.02$) but was not significant ($p > 0.05$) at the end of rearing at day 78. However, another treatment, a combination of a cation exchange clay-based product in feed and an organic acid mixture (formic acid, sodium formate, lactic acid, propionic acid) in water, led to a slight but significant reduction of 0.82 ± 0.25 $\log_{10}$ CFU/g ($p = 0.02$) compared to the control group at day 78. Testing this combination in field conditions in several flocks is needed to determine if it is biologically relevant and if it could be a valuable measure to reduce *Campylobacter* in broiler flocks.

Keywords: *Campylobacter*, control measure, feed additive, broiler, free-range

INTRODUCTION

Campylobacteriosis is the most commonly reported zoonosis in the EU since 2005 and 229 213 confirmed cases were reported in 2015 (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC], 2016). The infectious agent is *Campylobacter* spp. mainly *Campylobacter jejuni* (90%) and *Campylobacter coli* (10%), which cause an acute gastrointestinal infection in humans. The poultry reservoir, especially broiler meat, is generally

recognized as one of the most-important source for human campylobacteriosis (European Food Safety Authority [EFSA], 2010b). In Europe, the mean prevalence of *Campylobacter* in primary poultry production is very high, up to 70% of broiler batches being contaminated with large differences ranging between 2 and 100% observed between countries (European Food Safety Authority [EFSA], 2010a). Moreover high numbers, up to 8 log CFU/g, of *Campylobacter* can be enumerated from broiler caecal contents (Hansson et al., 2010; Hue et al., 2010).

However, to date, no criteria have been established in the European legislation for *Campylobacter* spp. load in foodstuffs, and then a preventative approach is considered. Indeed, according to the study of Romero-Barrios et al. (2013), reducing *Campylobacter* spp. loads by 3-$\log_{10}$ in broilers' gut would reduce the public health risk by at least 90%.

Evaluation of different potential interventions to prevent or to reduce *Campylobacter* colonization in broilers is still in progress, as there is no effective, reliable and practical strategy available so far. Some of them have been reviewed recently (Robyn et al., 2015; Sahin et al., 2015; Meunier et al., 2016a; Saint-Cyr et al., 2016a). Vaccination and the use of bacteriocins are not currently available, but they could represent promising measures in the future (Svetoch and Stern, 2010; Meunier et al., 2016a,b). Feed additives with non-antibiotic products such as probiotic bacteria, prebiotics, plant extracts or organic acids against *Campylobacter* colonization are still extensively studied. They give some promising results in experimental trials leading to at least 2 $\log_{10}$ CFU/g reduction or more in *Campylobacter* colonization for some of them (Skanseng et al., 2010; Ghareeb et al., 2012; Guyard-Nicodeme et al., 2016; Saint-Cyr et al., 2016b). In these studies testing feed additives, trials were generally performed using conventional production conditions: indoor rearing, broiler breeds, whole rearing (Hilmarsson et al., 2006; Thibodeau et al., 2015; Gracia et al., 2016; Guyard-Nicodeme et al., 2016), or shorter periods (Solis de Los Santos et al., 2008; Skanseng et al., 2010; Ghareeb et al., 2012). However, there is an increasing consumer interest in free-range poultry. In France, the free-range Label Rouge traditional poultry, accounted for 15% of the production and 60% of the consumption of whole broiler carcasses by French household (Salvat et al., 2017). Breeding conditions of free–range broilers are different from those of conventional production and vary according to the European Member States. According to the French Label Rouge specifications, slow growing breeds of broilers are reared with a lower breeding density indoor from 1 to 42 days old, and have access to an outdoor range from 6 weeks until depopulation at 81 days old at least. As for the conventional broilers, the free-range broiler flocks can be colonized by *Campylobacter* (Rivoal et al., 2005; Huneau-Salaün et al., 2007; Allen et al., 2011; Economou et al., 2015; Salvat et al., 2017). However, to the best of our knowledge the effect of feed additives against *Campylobacter* in free-range broilers has not been yet studied. The aim of this work was to evaluate the effectiveness of different additives against natural *Campylobacter* colonization in a French experimental farm of free-range broilers.

MATERIALS AND METHODS

Ethics Statements

This study was carried out in an approved establishment for animal experimentation under the "Label Rouge" program specifications for rearing conditions by the « Direction Départementale de la Cohésion Sociale et de la Protection des Populations des Landes » (agreement number A-40-037-2). The protocol was designed and all practices were performed according to the 2010/63/EU regulation about animal welfare.

Experimental Design

One day-old male chicks of strain T44 N x SA 51 ($n = 1440$), purchased from a commercial hatchery, were reared in the facilities of the Nutricia experimental farm (Benquet, Southwest of France). This facility is designed to replicate rearing conditions according to the Label Rouge program. At the hatchery, the birds were vaccinated against Marek's Disease, Bronchitis infectious, Gumboro and Newcastle diseases and Coccidiosis. A booster vaccination for Bronchitis infectious was carried out at 21 days. Rearing temperature was held constant at 28°C during the first 3 days and then, it was gradually reduced until the fourth week to reach 20°C. This temperature was maintained until access to outdoor range after 42 days (according to the criteria of the Label Rouge specifications). A continuous light was applied during the first 48 h and was then reduced to 12 h per day.

Upon arrival, chicks were randomly allocated to one of the 36 pens ($n = 40$ chicks per pen). Six pens were randomly assigned to the control group, without any treatment (T1), and five pens were randomly assigned per treatment (T2–T7).

Five commercial products and a combination of two of them were tested and all the products were added to feed or to water at the dose recommended by the suppliers (**Table 1**). According to suppliers' recommendations, treatments T3, T4, and T7 were distributed throughout the trial; treatments T2, T5, and T6 were distributed only from day 71 to day 78 (**Table 1**). Food and water were available *ad libitum*. Individual feeders and drinkers were displayed in each pen, avoiding feed contamination from one pen to the others. The birds were fed from day 1 to day 28 with a starter crumble, from day 29 to 49 with a grower mash and from day 50 to day 78 with a finisher mash (Supplementary Table S1). Formulation of the different diets were iso-caloric and iso-nitrogenous. Birds were slaughtered at D79 in a conventional slaughterhouse where skin sampling was performed (first broiler batch of the day).

Sampling and Microbiological Analyzes

Different types of samples were collected and analyzed during the course of the trial according to **Figure 1**. The sampling included the collection of cardboards at the bottom of the transport crates, fresh fecal material (pool of feces), caecal material (caeca or pool of caeca) after euthanasia (electronarcosis followed by bleeding) and neck skin samples. Until day 71, treatments T1, T2, T5, and T6 were not distributed, therefore, animals in these groups were confounded in a single control group called treatment T0.

TABLE 1 | Tested products, doses, and period of distribution.

Treatment	Composition	Mode	Dose	Period
T1 (Control)	None	None	None	None
T2	Cation exchange clay based additive	Feed	0.25 kg/ton	D71–D78
T3	Multi-species Probiotic	Feed	1 kg/ton	D1–D78
T4	Prebiotic-like	Feed	1.25 kg/ton	D1–D78
T5	Organic acid mixture (formic acid, sodium formate, lactic acid, propionic acid)	Water	1 ml/L	D71–D78
T6	Clay based additive (T2) + Organic acid mixture (T5)	Feed + Water	0.25 kg/ton + 1 ml/L	D71–D78
T7	Fermented plant extract	Water	2 ml/L	D1–D78

The caecum was separated from the rest of the intestinal package through sterile scissors and placed in hermetically sealed plastic bags. Neck skin samples were collected from carcasses taken from the processing line after chilling at the slaughterhouse. After collection, samples were shipped in an insulated box to the ANSES laboratory (Ploufragan, Northwest, France) within 24 h with a cooler carrier (4°C). Samples were processed and analyzed upon arrival depending on the sample as following:

Absence of *Campylobacter* from cartons on the bottom of transport crates was assessed after enrichment according to part 1 of the ISO 10272 (Anonymous, 2006). Samples were weighed and diluted 1:10 (wt:vol) in Bolton broth and the mix was homogenated in a peristaltic homogenizer (AES, Bruz, France). For detection purposes, 10 ml of the homogenate was added to 90 ml of Bolton broth (Oxoid, Dardilly, France). The inoculated broth was then incubated under microaerophilic conditions for 4 h at 37°C and then for 44 ± 4 h at 41.5 ± 1°C.

Fecal materials were weighed and diluted 1:10 (wt:vol) in tryptone salt broth and the mix was homogenized in a peristaltic homogenizer (AES, Bruz, France). Presence or absence of *Campylobacter* was assessed after direct plating of the homogenate on mCCDA plates (modified Charcoal, Cefoperazone, Desoxycholate Agar, Oxoid, Dardilly, France), and incubation as above. Characteristic colonies were confirmed with optical microscopy analysis.

Campylobacter from caecal contents were recovered using direct plating and/or enumeration. Direct isolation of *Campylobacter* was assessed by direct seeding of the caecal content on mCCDA and 44 ± 4 h of incubation at 41.5 ± 1°C in a microaerophilic atmosphere (85% N_2, 10% CO_2, 5% O_2). In order to assess *Campylobacter*'s counts, caecal contents were weighed, diluted in tryptone salt broth, and homogenized in a peristaltic homogenizer (AES, Bruz, France). Serial dilutions of the homogenate in tryptone salt broth, were plated on selective mCDDA plates and enumeration was assessed after incubation as above. The threshold for enumeration was 2×10^2 CFU/g (2.3 $\log_{10}$ CFU/g) of caecal content.

For the neck skin samples, detection after enrichment and enumeration of *Campylobacter* was performed according to part 1 and 2 of the ISO 10272 (Anonymous, 2006) respectively. Samples were weighed and diluted 1:10 (wt:vol) in tryptone salt broth and the mix was homogenated in a peristaltic homogenizer (AES, Bruz, France). For detection purposes, 10 ml of the homogenized was added to 90 ml of Bolton broth (Oxoid, Dardilly, France). The inoculated broth was then incubated under microaerophilic conditions for 4 h at 37°C and then for 44 ± 4 h at 41.5 ± 1°C. The culture in Bolton broth was subsequently plated onto mCCDA and Butzler agar (Virion N°2) (Oxoid, Dardilly, France) and incubated for 44 ± 4 h at 41.5 ± 1°C. Characteristic colonies were confirmed with optical microscopy analysis. For the quantification, *Campylobacter* were enumerated by plating 1 ml of the homogenate onto three plates of mCCDA. Tenfold serial dilutions of the homogenate in tryptone salt broth were also prepared and plated onto mCCDA plates. All plates were incubated under microaerophilic conditions for 44 ± 4 h

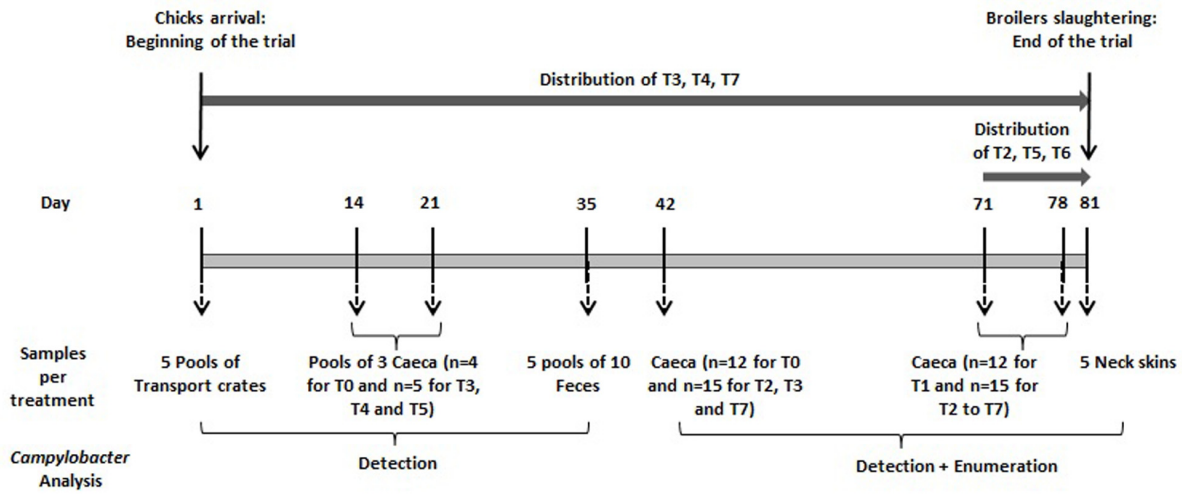

FIGURE 1 | Timeline of sampling and microbiological analyzes performed during the trial.

at 41.5 ± 1°C. The threshold for enumeration was 10 CFU/g (1 $\log_{10}$ CFU/g) of neck skin.

Performance

Animal weights were recorded individually at days 14, 28, 53, 72, 79 and at the slaughterhouse. The record of food consumption took place weekly and at the day of weighing. Daily consumption, daily gain and feed conversion ratio (FCR) were calculated for 3 periods: from D1 to D28, D29 to D72 and D73 to slaughter. Mortality was recorded daily and dead animals were weighted individually.

Statistical Analysis

Campylobacter loads in caeca, weight and feed consumption were analyzed using an ANOVA model including the treatment as a fixed effect and the pen as a random effect; *post hoc* tests were carried out for mean comparisons (Tukey test, $P < 0.05$). For comparison of *Campylobacter* loads on neck skin, the ANOVA model only included the treatment as a fixed effect.

RESULTS

Effect of Treatments on *Campylobacter* Colonization

No *Campylobacter* was detected on chick transport crate. From day 1 to day 35, *Campylobacter* was not recovered from the samples, whatever the treatment was (data not shown). *Campylobacter* was detected in samples from day 42 onward. Therefore, natural contamination of the flock occurred between day 35 and day 42, and the treatments T3, T4, and T7 did not prevent colonization of the broilers.

At day 42, enumeration of *Campylobacter* in caecal contents was performed to determine if the treatments T3, T4, and T7 distributed from day 1 impacted *Campylobacter* loads in caeca, compared to the control group T0 (constituted of samples from the T1, T2, T5, and T6 groups). As shown in **Table 2**, broilers that received the treatment T3 (Multispecies probiotic) had significantly lower *Campylobacter* counts than the control group ($P = 0.02$). However, the observed mean reduction was less than 1 $\log_{10}$ CFU/g. Treatments T4 (prebiotic-like) and T7 (fermented product) did not lead to a significant reduction compared to the control group.

A second sampling was carried out at day 71 to determine the contamination levels in the groups T2, T5, and T6 before application of the treatments and to check the effect of the treatments T3, T4, and T7 compared to the control group T1. At day 71, the contamination level was not significantly different in the groups T2, T5, and T6 before application of treatments compared to the control group T1. *Campylobacter* loads in the three treated groups T3, T4, and T7 were not significantly different than the one observed in the control group T1.

Otherwise, the mean *Campylobacter* loads in all treatment decreased from 8.08 $\log_{10}$ CFU/g ($CI_{95\%}$ [7.93–8.22]) at D42 to 6.69 $\log_{10}$ CFU/g ($CI_{95\%}$ [6.50–6.88]) at D71. The decrease was also observed in the control treatment (8.43 $\log_{10}$ CFU/g ($CI_{95\%}$ [8.15–8.72]) at D42 vs. 6.83 $\log_{10}$ CFU/g ($CI_{95\%}$ [6.24–7.42]) at D71).

Results at day 78 revealed that the three groups receiving a treatment since the beginning of the trial (T3, T4, and T7) did not show a significant reduction of the colonization compared to the control group. Among the three groups receiving a product only during the last week of rearing (T2, T5, and T6), only T6 (combination T2 + T5: a clay-based product in feed, and an organic acid mixture in water, respectively) showed a significant reduction estimated at 0.82 ± 0.25 $\log_{10}$ CFU/g ($p = 0.02$) compared to the control group.

Effect of Treatments on *Campylobacter* Contamination of Carcasses (Neck Skin)

At slaughter, carcasses from treatment T6 showed a slight but significant ($p = 0.01$) reduction estimated at 0.68 ± 0.24 $\log_{10}$ CFU/g in *Campylobacter* counts on neck skin samples compared to the control group T1 (**Table 3**). No other significant difference was observed between the control group and the other treatments. Nevertheless, these results need to be confirmed using

TABLE 2 | Effect of dietary treatment on *Campylobacter* counts (Log_{10}CFU/g, mean ± standard deviation) in the caeca of broilers at 42, 71, and 78 days of age.

	D42			D71			D78		
Treatment	***n***	**$\log_{10}$CFU/g (Mean ± SD)**	**$CI_{95\%}$**	***n***	**$\log_{10}$CFU/g (Mean ± SD)**	**$CI_{95\%}$**	***n***	**$\log_{10}$CFU/g (Mean ± SD)**	**$CI_{95\%}$**
T0	12	8.43 ± 0.53^{a}	8.15–8.72	–	–	–	–	–	
T1	–	–	–	10	$6.83 \pm 1.00^{a,b}$	6.24–7.42	12	6.71 ± 0.67^{a}	6.34–7.07
T2	–	–	–	15	$6.79 \pm 1.11^{a,b}$	6.25–7.34	15	$6.51 \pm 0.75^{a,b}$	6.14–6.88
T3	15	7.88 ± 0.43^{b}	7.66–8.09	15	$6.70 \pm 0.81^{a,b}$	6.30–7.10	15	$6.16 \pm 0.85^{a,b}$	5.75–6.58
T4	15	$7.96 \pm 0.60^{a,b}$	7.67–8.25	15	6.92 ± 0.80^{a}	6.53–7.31	15	$6.22 \pm 1.16^{a,b}$	5.65–6.79
T5	–	–	–	15	$6.69 \pm 0.69^{a,b}$	6.35–7.02	15	$6.38 \pm 1.00^{a,b}$	5.89–6.87
T6	–	–	–	14	6.86 ± 0.98^{b}	6.63–7.35	15	5.88 ± 0.96^{b}	5.41–6.35
T7	15	$8.11 \pm 0.59^{a,b}$	7.82–8.40	15	$6.10 \pm 1.29^{a,b}$	5.47–6.74	15	$5.71 \pm 1.82^{a,b}$	4.82–6.61

At 42 days, only treatment T3, T4, and T7 were distributed, so T1, T2, T5, and T6 constituted the control group T0. At D71 and D78, T1 corresponded to the control group. ^{a,b}*Means within the same column with different superscript are significantly different ($p < 0.05$).*

TABLE 3 | *Campylobacter* loads (log_{10} CFU/g) on neck skin at D81.

Treatment	*n*	log_{10}CFU/g (Mean ± SD)	$CI_{95\%}$
T1	5	1.70 ± 0.41^{a}	1.20–2.21
T2	5	1.62 ± 0.28^{ab}	1.28–1.97
T3	5	1.46 ± 0.31^{ab}	1.08–1.84
T4	5	1.03 ± 0.65^{ab}	0.22–1.84
T5	5	1.31 ± 0.34^{ab}	0.89–1.74
T6	5	1.02 ± 0.16^{b}	0.83–1.21
T7	5	1.09 ± 0.28^{ab}	0.73–1.45

[a,b]Means within the same column with different superscript are significantly different ($p < 0.01$).

a higher number of samples, as only five carcasses per group were sampled in this study.

Effect of Treatments on Growth Performance

Broilers from T3, T4, and T7 showed a higher daily weight gain in comparison with the ones from treatments with no additive during the first rearing period but their FCR was not significantly improved (**Table 4**). Over the whole rearing period no constant effect of the treatments were observed on daily feed consumption, daily weight gain and FCR. The mean mortality rates varied from 0.07% in T6 (1/240) to 3.8% (7/200) in T1 with no significant difference between treatments (data not shown).

DISCUSSION

Animal welfare is an increasing important issue for the consumers (Napolitano et al., 2010) and therefore there is a growing interest for free range and/or organically ranged broilers. However, the free-range rearing conditions bring together several of the known risk factors favoring *Campylobacter* colonization in broilers with for example the contact of the birds with an open environment and the age of the birds at slaughter (Huneau-Salaün et al., 2007; Newell et al., 2011). In France a representative study conducted in 2008 demonstrated that prevalence of *Campylobacter* in caecal contents of slaughtered batches was 100.0% for the Label chickens compared to 69.7% for the standard chickens (Hue et al., 2010).

During this study, broilers were naturally colonized by *Campylobacter* at the end of the indoor rearing period between day 35 and day 42. These results are in agreement with those of Huneau-Salaün et al. (2007) who reported that 71.2% of French free-range flocks are positive for *Campylobacter* at the end of the indoor rearing period. However, in some cases, broilers become colonized by *Campylobacter* after 6 weeks of rearing inside the building, when they can have access to the outdoor range (Rivoal et al., 2005).

Developing a control strategy against *Campylobacter* in the primary production is needed. Finding an effective product to be added to feed or water among the already marketed products could be a rapid solution. The tested products of this study were chosen based on a claimed activity, such as reducing pathogen, limiting bacterial growth or digestive

TABLE 4 | Daily feed consumption (g), daily body weight gain (g) and feed conversion ratio (FCR) from D1 to D28, from D29 to D72, from D73 to slaughter and over the whole rearing period according to the treatments (Least Squares Mean ± Standard Error).

	D0–D28			D29–D72			D73–D79			D0–D79		
	Cons	Daily gain	F.C.R	Cons	Daily gain	F.C.R	Cons	Daily gain	F.C.R	Cons	Daily gain	F.C.R
T1	37.3^{ab} 0.6	20.0^{a} 0.3	1.86 0.02	114.4 1.7	37.9 0.6	3.03^{ab} 0.04	169.1 5.6	43.3 2.2	3.97 0.26	91.9 1.4	32.1 0.4	2.87 0.04
T2	37.2^{ab} 0.6	20.3^{a} 0.3	1.84 0.02	115.0 1.9	38.0 0.6	3.03^{ab} 0.04	171.8 6.1	43.5 2.4	3.98 0.28	92.5 1.6	32.2 0.5	2.87 0.04
T3	40.0^{b} 0.6	21.9^{bc} 0.3	1.82 0.02	115.7 1.9	37.8 0.6	3.06^{ab} 0.04	180.4 6.1	43.3 2.4	4.17 0.28	94.6 1.6	32.6 0.5	2.90 0.04
T4	39.6^{b} 0.6	22.4^{c} 0.3	1.77 0.02	117.1 1.9	37.1 0.6	3.16^{b} 0.04	172.5 6.1	46.4 2.4	3.76 0.28	94.5 1.6	32.7 0.5	2.90 0.04
T5	37.4^{ab} 0.6	20.2^{a} 0.3	1.86 0.02	112.7 1.9	37.6 0.6	3.00^{ab} 0.04	175.7 6.1	43.5 2.4	4.05 0.28	91.6 1.6	31.9 0.5	2.87 0.04
T6	36.7^{a} 0.6	19.9^{a} 0.3	1.84 0.02	113.7 1.9	38.7 0.6	2.94^{a} 0.04	170.1 6.1	40.2 2.4	4.42 0.28	91.4 1.6	32.2 0.5	2.84 0.04
T7	37.9^{ab} 0.6	20.6^{ab} 0.3	1.84 0.02	113.9 1.9	38.7 0.6	2.95^{a} 0.04	176.9 6.1	45.6 2.4	3.93 0.28	92.5 1.6	32.9 0.5	2.81 0.04

[a,b]Means within the same column with different superscript are significantly different ($p < 0.05$).

pathogens, and/or improving immune functions. Five products and a combination of two of them were evaluated in the same trial. They were added according to the manufacturer's conditions. None of the three treatments (T3, T4, and T7) used from day 1 was able to prevent *Campylobacter* colonization detected at day 42. Similar results were observed in previous works testing several feed additives in experimental facilities with artificial *Campylobacter* contamination on fast-growing broilers (Gracia et al., 2016; Guyard-Nicodeme et al., 2016; Saint-Cyr et al., 2016b). Moreover, no treatment using single product (T2, T3, T4, T5, and T7) led to a significant reduction of *Campylobacter* in caeca, compared to the control group at the end of the rearing period. Treatment T2 (clay-based product) was previously tested in experimental facilities with artificial *Campylobacter* challenge and a mean reduction of 2.5 $\log_{10}$ CFU/g was observed in fast-growing birds after 36 days of rearing, although it failed to reduce the pathogen in slow growing birds in the same conditions (Guyard-Nicodème et al., 2014). It could be hypothesized that product efficacy could be impacted by the broiler breeds. However, Gormley et al. (2014) demonstrated that *Campylobacter* colonization is not affected by the broiler breeds (fast or slow growing breeds).

On the contrary, treatment T6, using the combination of the clay-based product (T2) in feed and an organic acid mixture in water (T5), led to a significant reduction of *Campylobacter* spp. counts in the caeca, and this reduction was also observed on neck skin at the slaughterhouse. Reduction in the caeca was low, as less than 1 $\log_{10}$ CFU/g (0.82 ± 0.25 log10 CFU/g) was observed. Several previous studies presented results of feed or water additives leading to a reduction of *Campylobacter* colonization higher than 2 $\log_{10}$ CFU/g but they were performed in experimental facilities with an artificial *Campylobacter* challenge (Nishiyama et al., 2014; Arsi et al., 2015; Gracia et al., 2016; Guyard-Nicodeme et al., 2016; Saint-Cyr et al., 2016b). However, these controlled conditions cannot reflect the field conditions, especially free-range conditions exposed to multiple sources of contaminations and contaminated with genetically diverse *Campylobacter* isolates (Rivoal et al., 2005). Anyway, the reduction of *Campylobacter* obtained with T6 was less than 1 $\log_{10}$ CFU/g at the flock, and the slight reduction observed at the slaughterhouse, could have an impact on public health. Indeed, reduced colonization in caecal contents of flocks by 1 $\log_{10}$ unit could reduce the number of campylobacteriosis cases from 48 to 83% (Romero-Barrios et al., 2013). Moreover this combination was used only the last week of rearing and had no impact on performance parameters. Therefore, these results need to be confirmed in other field trials using several other flocks to determine if it could be applied as an efficient control measure.

AUTHOR CONTRIBUTIONS

MG-N, AH-S, MQ, and MC contributed to the conception and design of the study. FT, SQ, and TP performed the experiments and MQ supervised the trial in the experimental farm. MG-N, AH-S, and FT analyzed the results and wrote the paper. FS and MC critically analyzed and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00828/full#supplementary-material

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Trans-Cinnamaldehyde, Carvacrol, and Eugenol Reduce *Campylobacter jejuni* Colonization Factors and Expression of Virulence Genes *in Vitro*

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Campylobacter jejuni is a major foodborne pathogen that causes severe gastroenteritis in humans characterized by fever, diarrhea, and abdominal cramps. In the human gut, *Campylobacter* adheres and invades the intestinal epithelium followed by cytolethal distending toxin mediated cell death, and enteritis. Reducing the attachment and invasion of *Campylobacter* to intestinal epithelium and expression of its virulence factors such as motility and cytolethal distending toxin (CDT) production could potentially reduce infection in humans. This study investigated the efficacy of sub-inhibitory concentrations (SICs, concentration not inhibiting bacterial growth) of three GRAS (generally recognized as safe) status phytochemicals namely trans-cinnamaldehyde (TC; 0.005, 0.01%), carvacrol (CR; 0.001, 0.002%), and eugenol (EG; 0.005, 0.01%) in reducing the attachment, invasion, and translocation of *C. jejuni* on human intestinal epithelial cells (Caco-2). Additionally, the effect of these phytochemicals on *Campylobacter* motility and CDT production was studied using standard bioassays and gene expression analysis. All experiments had duplicate samples and were replicated three times on three strains (wild type S-8, NCTC 11168, 81–176) of *C. jejuni.* Data were analyzed using ANOVA with GraphPad ver. 6. Differences between the means were considered significantly different at $P < 0.05$. The majority of phytochemical treatments reduced *C. jejuni* adhesion, invasion, and translocation of Caco-2 cells ($P < 0.05$). In addition, the phytochemicals reduced pathogen motility and production of CDT in S-8 and NCTC 11168 ($P < 0.05$). Real-time quantitative PCR revealed that phytochemicals reduced the transcription of select *C. jejuni* genes critical for infection in humans ($P < 0.05$). Results suggest that TC, CR, and EG could potentially be used to control *C. jejuni* infection in humans.

Keywords: *Campylobacter jejuni*, virulence, attachment, invasion, CDT toxin, phytochemicals, gene expression

INTRODUCTION

The foodborne pathogen *Campylobacter* is the leading cause of bacterial gastroenteritis in humans resulting in an estimated 96 million annual infections globally (Kirk et al., 2015). In the United States, an estimated 1.3 million cases of Campylobacteriosis occur each year largely due to consumption of contaminated poultry products (Newell et al., 2010; CDC, 2014). Chickens act as the reservoir host of *Campylobacter*, wherein the pathogen colonizes the intestine and can persist for the entire lifespan of the birds without causing any disease. This leads to contamination of carcass during slaughter and increases the risk of human foodborne infections (Allen et al., 2007). After gaining entry through contaminated food in humans, *C. jejuni* attaches and invades the epithelial layer of lower intestinal tract (ileum, jejunum, colon) followed by epithelial cytopathy and enteritis (Dasti et al., 2010). In most cases, the infection consists of fever, headache, abdominal pain, vomiting, and diarrhea. However, in a minority of individuals, Campylobacteriosis triggers more serious illnesses such as Guillian-Barre Syndrome and Miller-Fisher syndrome that could lead to inflammatory polyneuropathy and fatal paralysis (EFSA, 2011; Silva et al., 2011). A plethora of virulence factors critical for attachment and invasion of epithelial cells, subsequent cytoplasmic proliferation, and cytopathy have been characterized for *C. jejuni* (Bolton, 2015). Major factors include motility systems (Young et al., 2007), attachment and invasion proteins (CadF, JlpA), and CDT production that causes cellular distension and cell death leading to enteritis (Silva et al., 2011). Thus, reducing the attachment and invasion of *C. jejuni* on intestinal epithelial cells and production of virulence factors such as motility and CDT could potentially control Campylobacteriosis in humans. Antibiotics such as macrolides (erythromycin, clarithromycin), and fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) are commonly used for treating *Campylobacter* infections in humans (Blaser and Engberg, 2008); however, there have been reports of development of resistance to these drugs (Engberg et al., 2001; Payot et al., 2006; Luangtongkum et al., 2009; Cha et al., 2016; Olkkola et al., 2016) and several resistance genes have been discovered in *Campylobacter* spp. (Gibreel et al., 2005; Gibreel and Taylor, 2006; Olkkola et al., 2016). This increase in antibiotic resistance along with reports of adverse drug reactions in patients (Periti et al., 1993; Thong and Tan, 2011) has fueled research exploring the potential of various antibiotic alternatives to combat *Campylobacter* infections in humans.

Since ancient times, plant extracts have been widely used as food preservatives, flavor enhancers, and dietary supplements for preventing food spoilage and improving human health. In addition, plant extracts are used in herbal medicine for treating various diseases. The antibacterial activity of several phytochemicals has been documented (Burt, 2004; Holley and Patel, 2005). A majority of these compounds are secondary metabolites produced during interaction between plants, animals, and microbes (Reichling, 2010). Trans-cinnamaldehyde (TC) is an aldehyde obtained from bark of cinnamon tree (*Cinnamomum zeylandicum*). Carvacrol (CR) or cymophenol is the principal antimicrobial ingredient in oregano oil (*Origanum vulgare*). Eugenol (EG) is yet another polyphenol compound that is the major antimicrobial component present in the oil of cloves (*Syzgium aromaticum*). All the aforementioned phytochemicals are classified as GRAS (Generally Recognized as Safe) by the FDA, and are approved for addition in food products (Food and Drug Administration, 2012, 2013).

Considerable literature exists on the antibacterial properties of phytochemicals that target cellular viability of bacteria; however, limited information is available on their effect in modulating the various aspects of bacterial virulence critical for causing disease in humans. The present study investigated the efficacy of sub-inhibitory concentrations (SICs, concentrations of compounds not inhibitory to bacterial growth) of TC, CR, and EG in reducing *C. jejuni* attachment, invasion, and translocation of human intestinal epithelial cells (Caco-2), and production of virulence factors *in vitro*. In addition, the effect of phytochemicals on the expression of critical virulence genes was investigated using real-time quantitative PCR.

MATERIALS AND METHODS

Campylobacter Strains and Culture Conditions

All culture media were purchased from Difco (Becton Dickinson, Sparks, MD). Three strains of *C. jejuni*, including wild type S-8 (isolated from commercial broilers raised at University of Arkansas), NCTC 11168, and 81–176 (ATCC BAA-2151) were used in the study. Each strain was cultured separately in 10 ml of sterile Campylobacter Enrichment broth (CEB, Neogen, Lansing, MI) and incubated at 37°C for 48 h in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2).

Phytochemicals and Determination of Sub-Inhibitory Concentration (SIC)

The SIC of each phytochemical was determined using a previously published protocol (Amalaradjou et al., 2011) with slight modifications. Sterile 96-well polystyrene plates (Costar, Corning Incorporated, Corning, NY) containing serial dilutions of phytochemicals (Sigma-Aldrich) in CEB (100 μl/well) were inoculated with ∼5.0 log CFU of *C. jejuni* in equal volume of CEB, followed by incubation at 37°C for 24 h. Bacterial growth was determined by culturing on *Campylobacter* Line Agar (CLA) plates (Line, 2001). The highest concentration of phytochemicals that did not inhibit the growth of *C. jejuni* after 24 h of incubation was selected as its respective SIC for the study. Since 100% ethanol was used as a diluent to increase solubility of phytochemicals for the experiments, its effect (at 0.1% concentration) on the various virulence attributes was also studied.

Bacterial Motility Assay

The effect of TC, CR, and EG on *C. jejuni* motility was determined as described previously (Niu and Gilbert, 2004) with modifications. Separate petri dishes containing 25 ml of motility test medium (0.4% agar) with the respective SICs of each phytochemical were prepared. A mid-log culture (8 h) of *C. jejuni*

was centrifuged at 3,600 g for 15 min and washed two times with Butterfield's phosphate diluent (BPD). Five microliters of washed culture (∼7 log CFU/ml) was stab inoculated at the center of the motility medium, incubated in microaerophilic environment at 37°C for 24 h, and the zone of motility (bacterial migration distance from the site of stab) was measured.

Cell Culture

Human intestinal epithelial cells (Caco-2, ATCC HTB-37) were cultured in 25-cm^2 tissue culture flasks (Falcon, Becton and Dickinson Company, Franklin Lakes, NJ) with minimum essential medium (DMEM, Gibco, Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (Invitrogen). The cells were incubated at 37°C in a 5% (v/v) CO_2 atmosphere.

Attachment and Invasion Assay

The effect of SIC of phytochemicals (TC, CR, EG) on *C. jejuni* adhesion to and invasion of human epithelial cells was determined as previously reported (Koo et al., 2012). Monolayers of Caco-2 cells were grown in 24-well tissue culture plates

TABLE 1 | List of primers used for real-time qPCR analysis.

Gene with accession number	Primer	Sequence (5′–3′)
16S-rRNA (NC_002163.1)	Forward	5′-TGAGGGAGAGGCAGATGGAA-3′
Endogenous control (Product length 78)	Reverse	5′-TCGCCTTCGCAATGGGTATT-3′
motA (NC_002163.1)	Forward	5′-AGCGGGTATTTCAGGTGCTT-3′
Flagellar motor protein A (Product length 75 bp)	Reverse	5′-CCCCAAGGAGCAAAAAGTGC-3′
motB (NC_002163.1)	Forward	5′-AATGCCCAGAATGTCCAGCA-3′
Flagellar motor protein A (Product length 51 bp)	Reverse	5′-AGTCTGCATAAGGCACAGCC -3′
fliA (NC_002163.1)	Forward	5′-AGCTTTCACGCCGTTACGAT-3′
Flagellar sigma factor (Product length 56 bp)	Reverse	5′-TCTTGCAAAACCCCAGAAGT-3′
cadF (NC_002163.1)	Forward	5′-CGCGGGTGTAAAATTCCGTC-3′
Campylobacter adhesion to fibronectin (Product length 135 bp)	Reverse	5′-TCCTTTTTGCCACCAAAACCA-3′
ciaB (NC_002163.1)	Forward	5′-TCTCAGCTCAAGTCGTTCCA -3′
Campylobacter invasion antigen B (Product length 50 bp)	Reverse	5′-GCCCGCCTTAGAACTTACAA -3′
jlpA (NC_002163.1)	Forward	5′-AGCACACAGGGAATCGACAG -3′
Jejuni lipoprotein A (Product length 66 bp)	Reverse	5′-TAACGCTTCTGTGGCGTCTT -3′
cdtA (NC_002163.1)	Forward	5′-TTTTGAAAATCGCCCTGCGG-3′
Cytolethal distending toxin A (Product length 57 bp)	Reverse	5′-GCTCCGCTAGGGCCTAAAAT-3′
cdtB (NC_002163.1)	Forward	5′-CTAGCGCAACTCAAGCAAGC-3′
Cytolethal distending toxin B (Product length 98 bp)	Reverse	5′-AATCGCAGCTAAAAGCGGTG-3′
cdtC (NC_002163.1)	Forward	5′-TAGCCCCTTGCACCCTAGAT-3′
Cytolethal distending toxin C (Product length 80 bp)	Reverse	5′-AGCAGCTGTTAAAGGTGGGG-3′
cetA (NC_002163.1)	Forward	5′-CCTACCATGCTCTCCTGCAC -3′
Campylobacter energy taxis protein (Product length 78 bp)	Reverse	5′-CGCGATATAGCCGATCAAACC-3′
cetB (NC_002163.1)	Forward	5′-GCCTTGTTGCTGTTCTGCTC -3′
Campylobacter energy taxis protein (Product length 88 bp)	Reverse	5′-TTCCGTTCGTCGTATGCCAA -3′
racS (NC_002163.1)	Forward	5′-AGACAAGTTGCCGAAGTTGC -3′
Reduced ability to colonize system Sensor (Product length 79 bp)	Reverse	5′-AGGCGATCTTGCCTACTTCA -3′
racR (NC_002163.1)	Forward	5′-AGAGAACAGCTTGTAAGTCGCT-3′
Reduced ability to colonize system Response regulator (Product length 83 bp)	Reverse	5′-ACCCTTAAGCGACCGATGAT -3′

(Costar) at $\sim 10^5$ cells per well and inoculated with a mid-log culture (8 h) of *C. jejuni* ~6 log CFU/well (multiplicity of infection-10:1) either alone (control) or in combination with SIC of phytochemicals. The inoculated monolayers were incubated at 37°C for 1.5 h in a microaerophilic environment. For the adhesion assay, the inoculated monolayers (after 1.5 h of incubation) were rinsed three times in BPD and lysed with 1 ml of 0.1% Triton X-100 (Invitrogen, Carlsbad, CA). The number of adherent *C. jejuni* was determined by serial dilution and plating on CLA plates. For the invasion assay, the Caco-2 monolayer after 1.5 h of incubation (post-inoculation) was rinsed three times in minimal media and incubated for another 2 h in cell culture media containing gentamicin (100 μg/ml) to kill extracellular bacteria. Subsequently, the monolayer was treated with 0.1% triton X-100 as described above. The number of *C. jejuni* that invaded the epithelial cells was enumerated by serially diluting and plating the cell lysate on CLA plates followed by incubation at 37°C for 48 h for bacterial enumeration.

Epithelial Translocation Assay

Bacterial epithelial translocation assay was performed as described previously (Koo et al., 2012). Caco-2 cells were cultured on transwell filter inserts (5-μm pore filter, Corning) placed in sterile 24-well tissue culture plates for 10–12 days to form a monolayer. Two hundred microliters of mid-log culture (8 h) of *C. jejuni* (6 log CFU/ml) inoculum was added to the apical well of the insert and incubated at 37°C for 3 h in a microaerophilic environment. Culture media (600 μl) from the basal well was plated on CLA agar for enumerating the number of *C. jejuni* that crossed the epithelial barrier.

Cell Viability Assay for Cytolethal Distending Toxin Quantification

CDT activity in the supernatant of *C. jejuni* culture grown either alone (control) or in the presence of SICs of phytochemicals was quantified by cell viability assay (AbuOun et al., 2005; Lin et al., 2011) with modifications. Briefly, 1 ml of the 48 h culture of *C. jejuni* (grown with or without phytochemicals) was centrifuged at 10,000 g and the supernatant was collected. Serial 2-fold dilutions (100, 50, 25, 12.5, 6.25, 3.125, 1.56%) of the supernatant were made by mixing 100 μl of supernatant with 100 μl of cell culture medium and were added to Caco-2 cells followed by incubation for 9 days at 37°C. Negative control (0% cytotoxicity, obtained by addition of 200 μl of cell culture medium), media control (obtained by addition of appropriate volumes of CEB), and positive control (100% cytotoxicity, obtained by exposing the Caco-2 cells to cell lysis reagent) were also included for comparison. After 9 days, the amount of LDH release (indicator of cell cytotoxicity) was quantified using CytoTox-ONE homogeneous membrane integrity kit according to the manufacturer's instruction (Promega Inc., Madison, WI). Fluorescence was recorded (excitation wavelength of 560 nm and emission wavelength of 590 nm) on a Synergy 2 plate

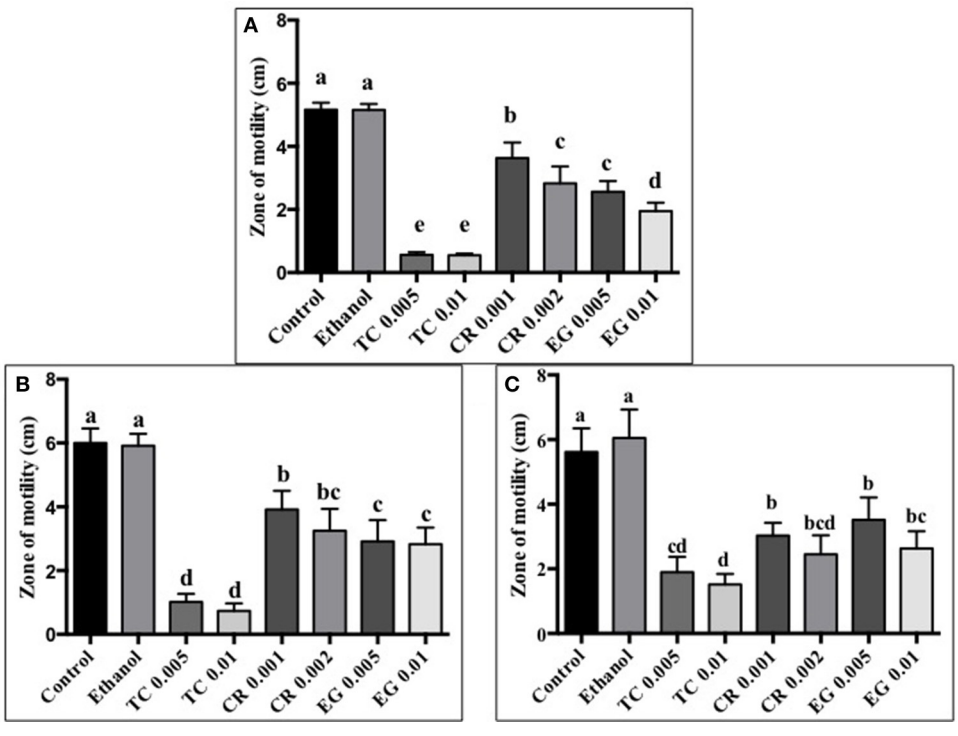

FIGURE 1 | Effect of phytochemicals on *Campylobacter jejuni* (A) S-8 **(B)** NCTC 11168 **(C)** 81–176 motility. The treatments include control, ethanol, TC (0.005, 0.01%), CR (0.001, 0.002%), and EG (0.005, 0.01%). Error bars represent SEM ($n = 6$). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

reader (BioTek, Higland Park, Winooski, VT) and percentage cytotoxicity was calculated according to the formula [Percent cytotoxicity = 100 × (LDH_T/LDH_C) where LDH_T refer to the difference in fluorescence between the sample and negative control (background fluorescence from media], and LDH_C refers to the difference in fluorescence between the positive control and negative control respectively.

Real-Time Quantitative PCR (RT-qPCR)

The effect of phytochemicals on the expression of *C. jejuni* virulence genes was investigated using real-time quantitative PCR, as described previously (Upadhyay et al., 2012). Each *C. jejuni* strain was cultured separately with the higher SIC of phytochemicals at 37°C in CEB to mid-log phase (8 h) and total RNA was extracted using RNeasy RNA isolation kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using the Superscript Reverse transcriptase kit (Invitrogen). The cDNA synthesized was used as the template for RT-qPCR. The primers for each gene (**Table 1**) were designed from published GenBank *C. jejuni* sequence using NCBI Primer design software. The amplification specificity was tested using NCBI-Primer BLAST, melt curve analysis and *in silico* PCR amplification (Bikandi et al., 2004). The amplified product was detected using SYBR Green reagent. Relative gene expression was determined using the comparative critical threshold (Ct) method on a Quant Studio 3 Real Time PCR system. Data were normalized to the endogenous control (16S rRNA gene), and the level of candidate gene expression between control and phytochemical treated sample was analyzed.

Statistical Analysis

A completely randomized designed was used for the study. The bacterial counts were logarithmically transformed before analysis to achieve homogeneity of variance (Byrd et al., 2001). All experiments had duplicate samples and were replicated three times on three strains (wild type S-8, NCTC 11168, 81–176) of *C. jejuni*. Data from independent trials were pooled and analyzed using ANOVA with Fisher LSD test for multiple comparisons on GraphPad Prism ver 6.0. Differences were considered significant with P-values < 0.05.

RESULTS

The two SICs of phytochemicals that did not reduce *C. jejuni* (S-8, NCTC 11168, 81–176) growth as compared to respective control were 0.005, 0.01% for TC and EG, and 0.001, 0.002% for CR (data not shown). The SICs of phytochemicals did not change the pH of the culture media ($P > 0.05$).

Effect of Phytochemicals on *C. jejuni* Motility

The effect of TC, CR, and EG on *C. jejuni* S-8 motility is presented in **Figure 1A**. All three phytochemicals at their respective SICs reduced *C. jejuni* S-8 motility ($P < 0.05$). TC was found to be

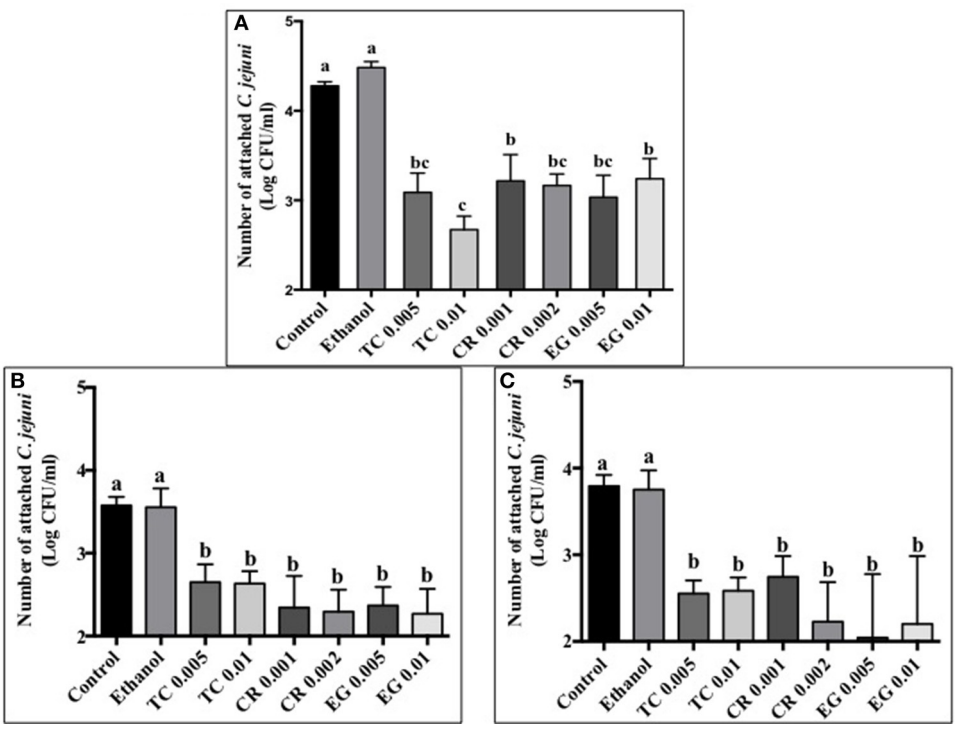

FIGURE 2 | Effect of phytochemicals on attachment of *Campylobacter jejuni* (A) S-8 **(B)** NCTC 11168 **(C)** 81–176 to Caco-2 cells. The treatments include control, ethanol, TC (0.005, 0.01%), CR (0.001, 0.002%), and EG (0.005, 0.01%). Error bars represent SEM ($n = 6$). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

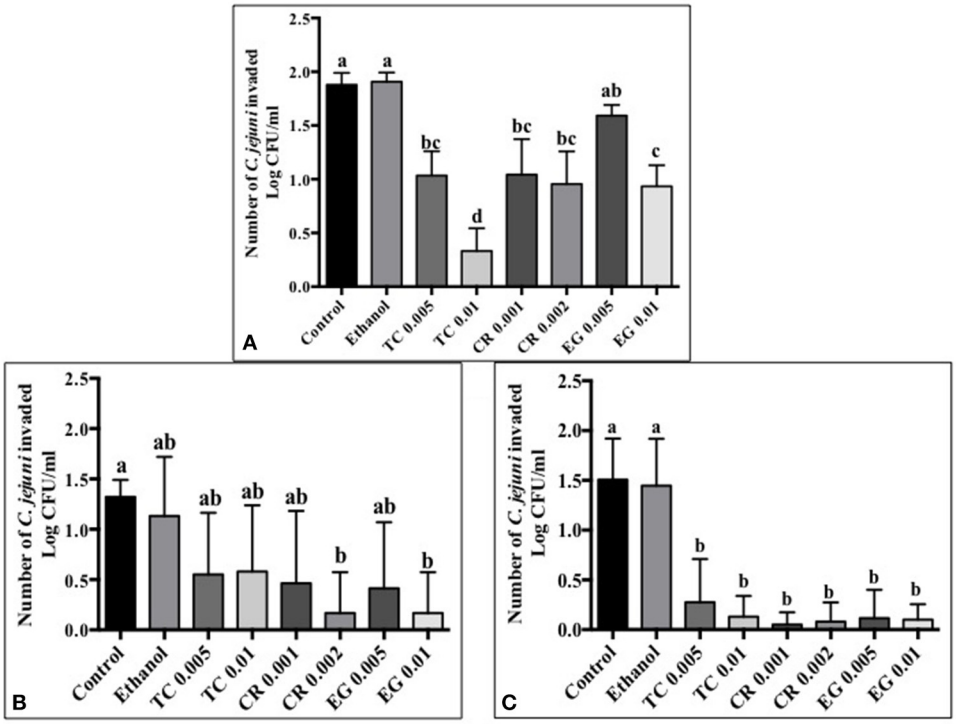

FIGURE 3 | Effect of phytochemicals on *Campylobacter jejuni* (A) S-8 **(B)** NCTC 11168 **(C)** 81–176 invasion of Caco-2 cells. The treatments include control, ethanol, TC (0.005, 0.01%), CR (0.001, 0.002%), and EG (0.005, 0.01%). Error bars represent SEM ($n = 6$). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

the most effective treatment and decreased *C. jejuni* S-8 motility by >70% resulting in less than 1.0 cm zone as compared to control (5 cm) after 24 h incubation. Similar results were obtained with *C. jejuni* NCTC 11168 (**Figure 1B**) and *C. jejuni* 81–176 (**Figure 1C**) where all the phytochemical treatments significantly reduced pathogen motility.

Effect of Phytochemicals on *C. jejuni* Attachment, Invasion, and Translocation of Caco-2 Cells

Tissue culture studies showed that the majority of phytochemical treatments decreased attachment, invasion and translocation of *C. jejuni* on Caco-2 cells in comparison to controls (**Figures 2–4**). For example, the SICs of TC, CR, and EG decreased attachment of *C. jejuni* S-8 by ∼1–1.5 log CFU/ml as compared to control which had an attachment of ∼4.2 log CFU/ml (**Figure 2A**). All phytochemicals except EG 0.005% reduced *C. jejuni* S-8 invasion as compared to controls (**Figure 3A**; $P < 0.05$). TC 0.01% was the most effective treatment and reduced the number of invading *C. jejuni* S-8 by ∼1.5 log CFU/ml ($P < 0.05$). For TC and EG treatments, a concentration dependent reduction in *C. jejuni* S-8 invasion was observed. For translocation assay, all phytochemical treatments were able to significantly reduce *C. jejuni* S-8 translocation of Caco-2 cells (**Figure 4A**). The phytochemical treatments also reduced the attachment, invasion, and translocation of *C. jejuni* NCTC 11168 (**Figures 2B**,**3B**,**4B**) and *C. jejuni* 81–176 (**Figures 2C**,**3C**,**4C**). In case of *C. jejuni* NCTC 11168, a reduction of ∼0.5–1 log CFU/ml was observed in adhesion to Caco-2 cells when exposed to phytochemicals (**Figure 2B**). Similarly, all phytochemical treatments reduced the attachment of *C. jejuni* 81–176 by at least 1 log CFU/ml as compared to control (**Figure 2C**). In case of invasion assay, both TC treatments, and lower SIC of CR and EG were not effective in reducing *C. jejuni* NCTC 11168 invasion ($P > 0.05$). However, the higher SIC of CR and EG reduced the invasion of all three strains of *C. jejuni* as compared to controls ($P < 0.05$). EG 0.01% treatment also exerted significant anti-translocation efficacy and completely inhibited the transfer of all three *C. jejuni* strains across the epithelial barrier (**Figure 4**; $P < 0.05$). None of the phytochemical treatments affected the health or integrity of Caco-2 cells ($P > 0.05$; data not shown).

Effect of Phytochemicals on *C. jejuni* Induced Cytotoxicity of Caco-2 Cells

All phytochemicals except CR 0.001% reduced *C. jejuni* S-8 toxin induced cytotoxicity by at least 30% as compared to control (**Figure 5A**). A concentration dependent reduction in cell cytotoxicity was observed in TC treatments. The lower concentration of TC (0.005%) reduced cell cytotoxicity by ∼30%, whereas the higher concentration (0.01%) reduced cell cytotoxicity by ∼60% as compared to controls ($P < 0.05$). In case of *C. jejuni* NCTC 11168, only CR 0.002% and EG 0.01%

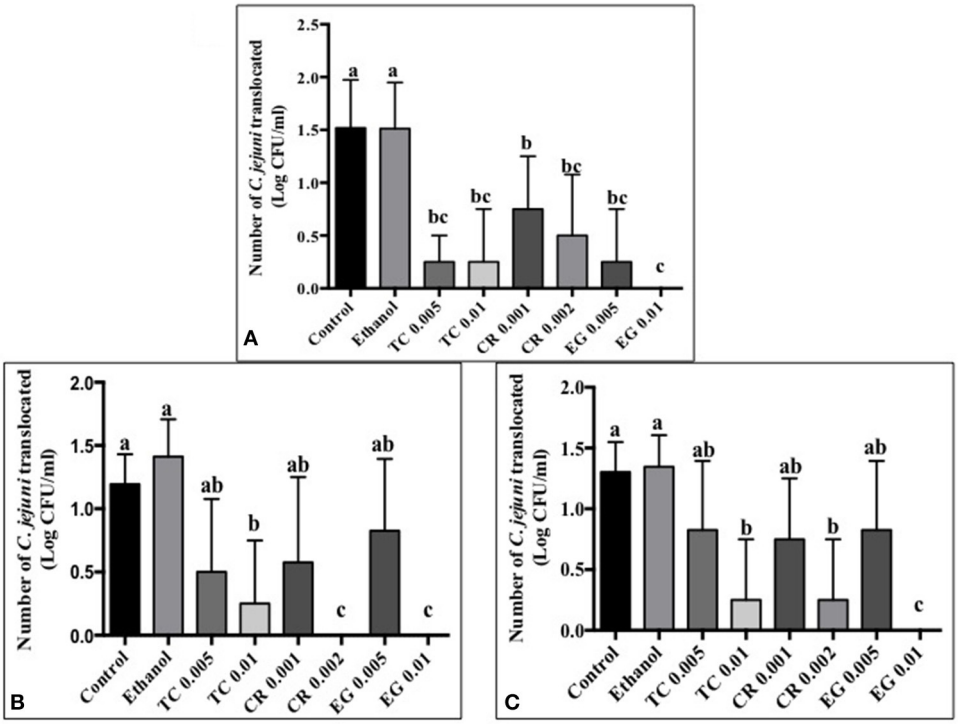

FIGURE 4 | Effect of phytochemicals on *Campylobacter jejuni* (A) S-8 **(B)** NCTC 11168 **(C)** 81–176 translocation of Caco-2 cells. The treatments include control, ethanol, TC (0.005, 0.01%), CR (0.001, 0.002%), and EG (0.005, 0.01%). Error bars represent SEM ($n = 6$). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

were effective in reducing cell cytotoxicity by ∼30% (**Figure 5B**; $P < 0.05$). None of the phytochemical treatments were able to reduce cell cytotoxicity mediated by *C. jejuni* 81–176 (**Figure 5C**; $P > 0.05$).

Effect of Phytochemicals on Expression of *C. jejuni* Virulence Genes

Effect of phytochemicals on expression of virulence genes in the three strains of *C. jejuni* is presented in **Table 2**. Real-time quantitative PCR results revealed that TC 0.01% down-regulated the expression of virulence genes coding for motility (*fliA*), attachment (*cadF*) and toxin production (*cdtA, cdtB, cdtC*) in *C. jejuni* S-8. CR and EG did not significantly reduce the expression of the tested virulence genes in *C. jejuni* S-8 except for *racR* by EG. Interestingly, many of the virulence genes were upregulated by CR and EG treatments ($P < 0.05$). The ethanol treatments did not affect the expression of the tested virulence genes in *C. jejuni* S-8 ($P > 0.05$). In case of *C. jejuni* NCTC 11168, CR 0.002%, and EG 0.01% treatments reduced the expression of *motA, cadF, cdtB,* and *motA, cdtA* genes respectively ($P < 0.05$). The expression of the tested genes was not affected by ethanol treatment ($P > 0.05$). None of the phytochemical treatments were able to significantly reduce the expression of tested genes in *C. jejuni* 81–176 ($P > 0.05$). Interestingly, the expression of select genes in *C. jejuni* NCTC 11168 (*motB*, *jlpA*) and *C. jejuni* 81–176 (*motA, motB, cadF, jlpA, cdtA,B,C*) was upregulated by 0.01% TC treatment and CR 0.002, EG 0.01% treatments respectively ($P < 0.05$).

DISCUSSION

The colonization of enteric pathogens in the gut and their interaction with the human intestinal cells is central to most infections and illnesses. The poultry associated foodborne pathogen *C. jejuni* expresses an array of virulence factors that orchestrate its pathophysiology in humans. In this study we investigated the effect of phytochemicals trans-cinnamaldehyde, carvacrol, and eugenol in reducing the expression of virulence factors of *C. jejuni in vitro* as a first step before conducting future *in vivo* studies.

The motility imparted by the polar flagella of *C. jejuni* is essential for colonization of the mucus lining of the intestinal tract and subsequent attachment and invasion of intestinal epithelium (Guerry, 2007). Our results revealed that TC, CR, and EG significantly decreased *C. jejuni* motility (**Figures 1A–C**). In addition, majority of phytochemical treatments at their higher SIC also reduced attachment, invasion and translocation of *C. jejuni* through intestinal epithelial cells (**Figures 2–4**). (van Alphen et al., 2012) had similar findings and observed that pre-exposure of *C. jejuni* to low concentrations of carvacrol modulated motility and invasion of INT-407 intestinal epithelial cells without affecting intracellular ATP levels or epithelial function. Reduced motility and attachment efficiency in *C. jejuni* on exposure to clove oil (Kovács, 2014), thyme (Pogacar et al., 2016), herbal extracts (Bensch et al., 2011), and campynexins (Johnson et al., 2015) have also been observed.

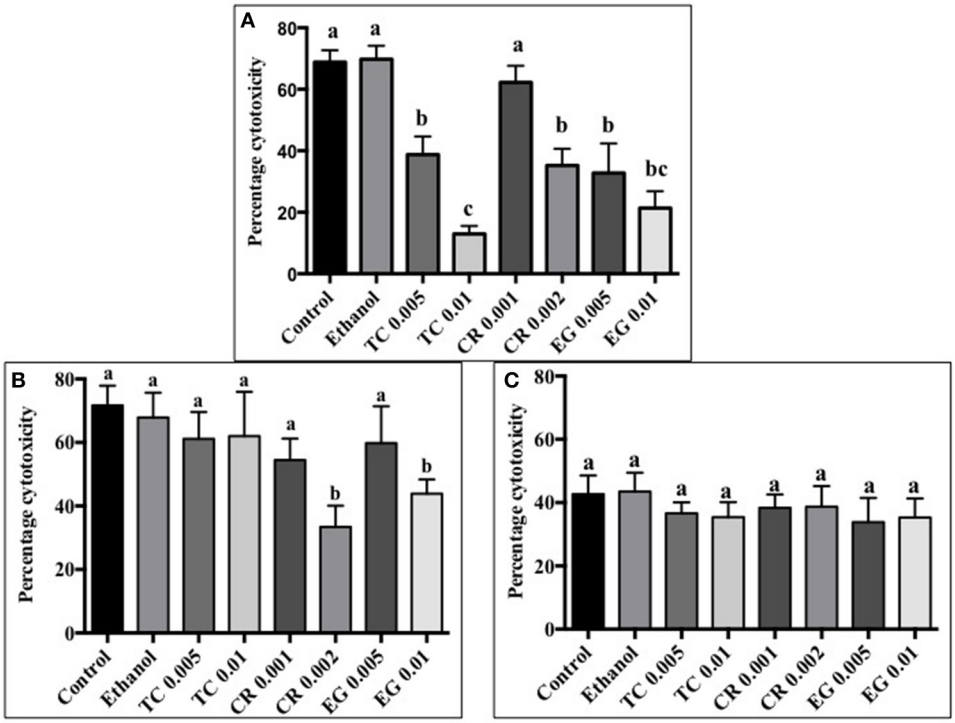

FIGURE 5 | Effect of phytochemicals on *Campylobacter jejuni* (A) S-8 **(B)** NCTC 11168 **(C)** 81–176 induced cytotoxicity on Caco-2 cells. The treatments include control, ethanol, TC (0.005, 0.01%), CR (0.001, 0.002%), and EG (0.005, 0.01%). Error bars represent SEM ($n = 6$). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

After invading the human intestinal epithelium, *C. jejuni* produces the tripartite CDT toxin. The catalytic domain (CdtB) localizes in the nucleus leading to DNA damage, cell-cycle arrest, and cytotoxicity (Pickett et al., 1996; Lara-Tejero and Galán, 2000; Young et al., 2007). The activity of CDT was first described in culture supernatants that caused eukaryotic cells to slowly distend over a period of 2–5 days, eventually leading to cell death (Johnson and Lior, 1988). We observed that majority of phytochemical treatments significantly reduced the *C. jejuni* S-8 CDT mediated cell cytotoxicity in Caco-2 cells (**Figure 5A**). Previously, Castillo et al. (2011) demonstrated that extracts from *A. farnesiana* and *A. ludoviciana* inhibited cytotoxin production in *C. jejuni* and *C. coli*. In another study, Gillespie et al. (2013) screened 30,000 small molecules for toxin inhibition activity and observed that 4-bromobenzaldehyde N-(2, 6-dimethyphenyl) semicarbazone (EGA) inhibited intoxication by CDT derived from *Haemophilus ducreyi* and *Escherichia coli*. Although the phytochemical treatments were found to be effective against *C. jejuni* S-8 mediated cell cytotoxicity, considerable strain based variation in their efficacy was observed in our assay (**Figures 5B,C**). We believe that this result is due to variations in the genomic composition of the three strains tested that could potentially alter the molecular targets of phytochemicals. For example, Jeon et al. (2005) reported that a mutation in *luxS* gene (coding for quorum sensing) affected the transcription of CDT operon. Since phytochemicals are known to exert their effect by modulating *luxS* and quorum sensing (Koh and Tham, 2011; Nazzaro et al., 2013; Upadhyay et al., 2013), a disruption of their efficacy due to changes in target genes coding for quorum sensing is possible.

It has been previously shown that sub-inhibitory or sub-lethal concentrations of antimicrobials modulate the transcription of genes in various bacterial pathogens (Fonseca et al., 2004; Tsui et al., 2004; Upadhyay et al., 2012) including *C. jejuni* (Arambel et al., 2015; Oh and Jeon, 2015). Since the sub-inhibitory concentrations of phytochemicals did not inhibit the growth of *C. jejuni*, the reduction observed in *C. jejuni* virulence attributes could be due to the effect of phytochemicals on the transcription of virulence genes. Therefore, we used RT-qPCR to determine the effect of phytochemicals on the expression of genes, which have been previously reported to contribute to the *C. jejuni* infection process in humans. We selected an incubation time of 8 h since we wanted to investigate the effect of phytochemicals on the expression of virulence genes of *C. jejuni* over extended exposure time, as would be the case in the human gut. 16S rRNA gene was selected as endogenous control since it was stable in its expression between control and treatment groups. Several other researchers have also used 16S rRNA gene as endogenous control for studying the expression of virulence genes in pathogens (McKillip et al., 1998; Tasara and Stephan, 2007; Xue et al., 2008; Hays, 2009; Atshan et al., 2013; Schroeder et al., 2015) including *C. jejuni* (Koolman et al., 2016). The flagellar biosynthesis gene,

fliA regulates several genes involved in *Campylobacter* motility. Genes *motA* and *motB* code for flagella motor function and aid in motility and colonization (Hermans et al., 2011). *cetA* and *cetB* are energy taxis genes that contribute to directional motility of *Campylobacter* in response to changes in external environment. The *Campylobacter* surface protein CadF in combination with CiaB (Young et al., 2007) and JlpA mediates binding of the pathogen to host epithelial cells through fibronectin-mediated attachment thereby facilitating colonization (Konkel et al., 1999, 2005; Monteville and Konkel, 2002; Monteville et al., 2003). RacS-RacR is another important two-component regulatory system that plays a role in temperature dependent growth and colonization in *C. jejuni* (Brás et al., 1999). In addition to the aforementioned attachment and motility factors, *C. jejuni* produces CDT encoded by *cdtA, cdtB,* and *cdtC* genes. The subunits CdtA and CdtC associate with the nuclease CdtB to form a tripartite complex that translocate CdtB into the host cell leading to arrest in cell cycle, cell death and enteritis (Young et al., 2007). We observed that phytochemicals modulated the expression of some of the virulence genes tested and this varied among the three strains of *C. jejuni*. For example, SIC of TC decreased the expression of genes coding for motility *(fliA)* and attachment (*cadF*) in strain S-8 (**Table 2**). In addition, TC down-regulated the transcription of *cdtA, cdtB,* and *cdtC* genes that contribute to CDT-mediated cell lysis in *C. jejuni* S-8. Thus, the reduced cell cytotoxicity observed in response to TC (**Figure 5A**) could potentially be due to the effect of TC on the expression of related genes in *C. jejuni* S-8. We did not observe significant down-regulation in the expression of *cdtA,B,C* genes in response to TC in *C. jejuni* NCTC 11168 and *C. jejuni* 81–176 (**Table 2**). This was reflected in the phenotypic assay as well where TC did not significantly reduce cell cytotoxicity in the two strains (**Figures 5B,C**). Similarly, CR down-regulated the expression of genes critical for motility (*motA*), attachment (*cadF*), and CDT production (*cdtB*) in *C. jejuni* NCTC 11168, whereas it did not reduce the expression of tested genes in wild type S-8 or 81–176

TABLE 2 | Effect of TC 0.01%, CR 0.002%, EG 0.01%, and ethanol on the expression of *Campylobacter jejuni* (S-8, NCTC 11168, 71–176) virulence genes.

Gene	Relative fold change ($Log_{10}RQ$)					
	TC 0.01%			CR 0.002%		
	S-8	11168	81–176	S-8	11168	81–176
motA	-0.2 ± 0.2^{a}	0.05 ± 0.4^{ab}	0.6 ± 0.2^{b}	-0.2 ± 0.07^{a}	$-0.7 \pm 0.1^{b*}$	$1.5 \pm 0.09^{c*}$
motB	-0.1 ± 0.1^{a}	$0.5 \pm 0.02^{a*}$	0.5 ± 0.7^{a}	-0.009 ± 0.03^{a}	-0.2 ± 0.3^{a}	$1.7 \pm 0.05^{b*}$
fliA	$-0.3 \pm 0.2^{a*}$	0.3 ± 0.08^{a}	0.1 ± 0.1^{a}	-0.06 ± 0.1^{a}	0.08 ± 0.2^{a}	0.08 ± 0.1^{a}
cadF	$-0.3 \pm 0.2^{a*}$	-0.09 ± 0.1^{a}	0.2 ± 0.6^{a}	-0.2 ± 0.1^{a}	$-0.6 \pm 0.2^{a*}$	$1.2 \pm 0.03^{b*}$
ciaB	0.07 ± 0.04^{a}	0.2 ± 0.06^{a}	0.3 ± 0.07^{a}	-0.1 ± 0.06^{a}	0.1 ± 0.2^{a}	0.2 ± 0.2^{a}
jlpA	-0.09 ± 0.08^{a}	$0.6 \pm 0.1^{a*}$	0.6 ± 0.3^{a}	-0.06 ± 0.04^{a}	$-0.1 \pm 0.1^{a*}$	$1.7 \pm 0.2^{b*}$
cdtA	$-0.4 \pm 0.1^{a*}$	0.3 ± 0.07^{ab}	0.5 ± 0.3^{b}	-0.1 ± 0.06^{a}	-0.3 ± 0.1^{a}	$1.7 \pm 0.06^{b*}$
cdtB	$-0.5 \pm 0.19^{a*}$	0.08 ± 0.1^{ab}	0.6 ± 0.2^{b}	-0.2 ± 0.04^{a}	$-0.4 \pm 0.1^{a*}$	$1.9 \pm 0.1^{b*}$
cdtC	$-0.4 \pm 0.17^{a*}$	0.0004 ± 0.2^{ab}	0.5 ± 0.3^{b}	-0.09 ± 0.1^{a}	-0.1 ± 0.1^{a}	$1.8 \pm 0.2^{b*}$
cetA	-0.05 ± 0.04^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{a}	$0.3 \pm 0.11^{a*}$	0.05 ± 0.05^{a}	0.2 ± 0.2^{a}
cetB	-0.08 ± 0.04^{a}	0.3 ± 0.07^{a}	0.06 ± 0.1^{a}	0.2 ± 0.007^{a}	0.29 ± 0.07^{a}	0.3 ± 0.07^{a}
racS	-0.2 ± 0.07^{a}	0.19 ± 0.06^{a}	0.1 ± 0.1^{a}	$0.4 \pm 0.04^{a*}$	0.3 ± 0.07^{a}	0.3 ± 0.1^{a}
racR	-0.1 ± 0.07^{a}	0.18 ± 0.1^{a}	0.1 ± 0.2^{a}	0.2 ± 0.05^{a}	0.03 ± 0.1^{a}	0.2 ± 0.03^{a}
Gene	**EG 0.01%**			**Ethanol**		
motA	0.2 ± 0.09^{a}	$-0.7 \pm 0.06^{b*}$	$1.2 \pm 0.1^{c*}$	0.04 ± 0.05^{a}	0.03 ± 0.1^{a}	0.06 ± 0.1^{a}
motB	$0.4 \pm 0.07^{a*}$	-0.4 ± 0.2^{b}	$1.4 \pm 0.2^{c*}$	-0.02 ± 0.2^{a}	0.3 ± 0.1^{a}	0.1 ± 0.1^{a}
fliA	0.2 ± 0.07^{a}	0.08 ± 0.05^{a}	0.2 ± 0.1^{a}	-0.03 ± 0.1^{a}	0.4 ± 0.08^{b}	0.08 ± 0.1^{ab}
cadF	$0.3 \pm 0.1^{a*}$	0.12 ± 0.1^{a}	$0.9 \pm 0.1^{b*}$	0.08 ± 0.06^{a}	-0.1 ± 0.01^{a}	-0.1 ± 0.3^{a}
ciaB	0.2 ± 0.08^{a}	0.4 ± 0.1^{a}	0.5 ± 0.2^{a}	0.03 ± 0.04^{a}	0.3 ± 0.01^{a}	0.1 ± 0.09^{a}
jlpA	$0.3 \pm 0.1^{a*}$	-0.2 ± 0.2^{a}	$1.4 \pm 0.1^{a*}$	0.03 ± 0.04^{a}	0.15 ± 0.1^{a}	-0.04 ± 0.1^{a}
cdtA	$0.3 \pm 0.08^{a*}$	$-0.4 \pm 0.08^{b*}$	$1.3 \pm 0.1^{c*}$	0.1 ± 0.03^{a}	0.16 ± 0.08^{a}	-0.04 ± 0.1^{a}
cdtB	0.03 ± 0.07^{a}	0.01 ± 0.09^{a}	$1.4 \pm 0.1^{b*}$	0.06 ± 0.02^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}
cdtC	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	$1.08 \pm 0.1^{b*}$	-0.17 ± 0.1^{a}	-0.1 ± 0.1^{a}	0.03 ± 0.1^{a}
cetA	$0.3 \pm 0.03^{a*}$	0.08 ± 0.06^{a}	0.2 ± 0.07^{a}	-0.02 ± 0.2^{a}	0.09 ± 0.08^{a}	0.1 ± 0.04^{a}
cetB	$0.3 \pm 0.1^{a*}$	0.11 ± 0.1^{a}	0.4 ± 0.05^{a}	0.08 ± 0.06^{a}	-0.03 ± 0.09^{a}	-0.1 ± 0.1^{a}
racS	-0.1 ± 0.14^{a}	0.32 ± 0.1^{b}	-0.1 ± 0.1^{a}	0.16 ± 0.03^{a}	-0.01 ± 0.1^{a}	0.3 ± 0.1^{a}
racR	$-0.3 \pm 0.01^{a*}$	0.06 ± 0.04^{b}	0.2 ± 0.02^{b}	0.03 ± 0.04^{a}	-0.3 ± 0.2^{a}	-0.07 ± 0.1^{a}

*Values represent Mean ± SEM. Control had a basal RQ of 1 (log_{10} RQ = 0). *Relative fold change significantly different from control. Superscripts with different letters in a row represent significant difference between C. jejuni strains for a treatment.*

(**Table 2**). A directly related finding to this gene expression result was observed in the cell cytotoxicity assay where only higher SIC of CR reduced *C. jejuni* NCTC 11168 mediated cell cytoxicity (**Figure 5B**) whereas none of the CR treatments reduced *C. jejuni* 81–176 mediated cell cytotoxicity of Caco-2 cells (**Figure 5C**). As observed with TC and CR, EG also affected the expression of genes in the three strains differently. EG 0.01% treatment significantly reduced the expression of *racR* in *C. jejuni* S-8 and *motA, cdtA* genes in *C. jejuni* NCTC 11168 (**Table 2**), whereas the expression of genes in *C. jejuni* 81–176 was not reduced. These results indicate that phytochemicals exert their anti-virulence effect via only selected genes and this could vary among different strains of the pathogen. Previously, Lee et al. (2012) showed that flavones could exert anti-virulence effect by modulating only selected genes (*sae* and *agr*) in *Staphylococcus aureus*. Expression of genes such as *sigB* (RNA polymerase sigma factor) and *sar* (accessory regulator A) was not affected by flavones. In a recent study, Singh et al. (2016) reported that eugenol has strong binding affinity for surface exposed lysines in proteins. The essential oil inhibited formation of glycation end products by binding to ε-amine group on lysine. In another study, carvacrol was found to inhibit the binding of nicotine to nicotinic acetylcholine receptor (Tong et al., 2013) indicating that these phytochemicals could be exerting their anti-virulence properties through modulating receptor binding of virulence proteins in *C. jejuni* in addition to their effect on gene expression. Moreover, carvacrol has been shown to modulate the expression of HSP60 (GroEL) chaperones and other proteins that affect protein folding in *Escherichia coli* O157:H7 (Burt et al., 2007) and *Listeria monocytogenes* (Guevara et al., 2015). Therefore, it is possible that phytochemicals may be exerting their anti-virulence effect against *C. jejuni* via similar mechanism(s) that affect protein folding.

In conclusion, our study showed that phytochemicals TC, CR, and EG were effective in reducing the virulence attributes of *C. jejuni* critical for causing infection in humans. Since phytochemicals including TC, CR, and EG have been found to be stable in the gastrointestinal tract of monogastric animals (Si et al., 2006; Michiels et al., 2008), these plant compounds could potentially be used as dietary supplements to control *C. jejuni* infection in humans. However, *in vivo* studies in an appropriate mammalian model along with genomic characterization, transcriptomic and proteomic profiling of pathogens are necessary to validate the antimicrobial efficacy, mechanism of action, and safety of the plant compounds.

AUTHOR CONTRIBUTIONS

AU designed the study. AU, KA, BW, IU, and SS conducted the experiments. AU analyzed the data and wrote the manuscript. AD and DD critically analyzed and revised the manuscript.

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Application of β-Resorcylic Acid as Potential Antimicrobial Feed Additive to Reduce *Campylobacter* Colonization in Broiler Chickens

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Campylobacter is one of the major foodborne pathogens that result in severe gastroenteritis in humans, primarily through consumption of contaminated poultry products. Chickens are the reservoir host of *Campylobacter*, where the pathogen colonizes the ceca, thereby leading to contamination of carcass during slaughter. A reduction in cecal colonization by *Campylobacter* would directly translate into reduced product contamination and risk of human infections. With increasing consumer demand for antibiotic free chickens, significant research is being conducted to discover natural, safe and economical antimicrobials that can effectively control *Campylobacter* colonization in birds. This study investigated the efficacy of in-feed supplementation of a phytophenolic compound, β-resorcylic acid (BR) for reducing *Campylobacter* colonization in broiler chickens. In two separate, replicate trials, day-old-chicks (Cobb500; $n = 10$ birds/treatment) were fed with BR (0, 0.25, 0.5, or 1%) in feed for a period of 14 days ($n = 40$/trial). Birds were challenged with a four-strain mixture of *Campylobacter jejuni* (~10^6 CFU/ml; 250 μl/bird) on day 7 and cecal samples were collected on day 14 for enumerating surviving *Campylobacter* in cecal contents. In addition, the effect of BR on the critical colonization factors of *Campylobacter* (motility, epithelial cell attachment) was studied using phenotypic assay, cell culture, and real-time quantitative PCR. Supplementation of BR in poultry feed for 14 days at 0.5 and 1% reduced *Campylobacter* populations in cecal contents by ~2.5 and 1.7 Log CFU/g, respectively ($P < 0.05$). No significant differences in feed intake and body weight gain were observed between control and treatment birds fed the compound ($P > 0.05$). Follow up mechanistic analysis revealed that sub-inhibitory concentration of BR significantly reduced *Campylobacter* motility, attachment to and invasion of Caco-2 cells. In addition, the expression of *C. jejuni* genes coding for motility (*motA, motB, fliA*) and attachment (*jlpA, ciaB*) was down-regulated as compared to controls ($P < 0.05$). These results suggest that BR could potentially be used as a feed additive to reduce *Campylobacter* colonization in broilers.

Keywords: *Campylobacter jejuni*, β-resorcylic acid, pre-harvest safety, chickens, colonization factors, gene expression, cell culture

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INTRODUCTION

Campylobacter contamination of food products is the leading cause of bacterial foodborne illness worldwide (Crim et al., 2015; Mangen et al., 2016). *Campylobacter*, in particular, *Campylobacter jejuni*, is the second most commonly reported foodborne pathogen in the USA with an annual incidence of 13.45 per 100,000 resulting in approximately 1.3 million infections annually (Crim et al., 2015). Actual cases are probably higher than these estimates due to under-reporting or sick individuals not seeking medical attention (Mead et al., 1999; Samuel et al., 2004). *Campylobacter* causes mild to severe gastroenteritis, fever, vomiting, and diarrhea in patients. In a minority of cases, it leads to more serious Guillain–Barré syndrome, reactive arthritis, or irritable bowel syndrome (Spiller, 2007; Gradel et al., 2009). Epidemiological studies have shown that the major risk factors associated with *Campylobacter* infections are improper handling and consumption of chicken or other food products cross-contaminated with poultry meat or juice during food preparation (Rosenquist et al., 2003; Friedman et al., 2004; Danis et al., 2009). The low infectious dose of *C. jejuni* (∼500 CFU) further raises public health concerns since only a few microorganisms are needed to cause the infection (Black et al., 1988).

Campylobacter sp. colonizes the gastrointestinal tract of chickens by the third to fourth week of age as a commensal organism (Annan-Prah and Janc, 1988; Stern et al., 1988; Humphery et al., 1993; Dhillon et al., 2006). Various studies have reported enteric colonization up to 10^8 CFU/g of cecal contents in birds (Beery et al., 1988; Achen et al., 1998). Product contamination mostly occurs during slaughtering of chickens (Berrang et al., 2000; Herman et al., 2003; Reich et al., 2008; Boysen et al., 2016). Therefore, effective strategies to control *Campylobacter* in poultry flocks at the farm level are needed to reduce product contamination and the incidence of campylobacteriosis in humans (Rosenquist et al., 2003; Arsenault et al., 2007; Reich et al., 2008).

A variety of pre-harvest strategies have been employed to reduce *Campylobacter* in poultry with varied degree of success. These include feeding birds with bacteriophages (Carrillo et al., 2005; Wagenaar et al., 2005), bacteriocins (Stern et al., 2005; Svetoch and Stern, 2010), probiotics (Santini et al., 2010; Arsi et al., 2015; Shrestha et al., 2017), and vaccination (Buckley et al., 2010; Chintoan-Uta et al., 2016). With increasing consumer demand for safe and natural products with minimal preservatives, significant research is being conducted to explore the potential of natural antimicrobials for controlling *C. jejuni* in chickens (Hermans et al., 2011b).

Since ancient times, plant compounds have been used for improving shelf life and microbiological safety of food. The antimicrobial activity of several phytochemicals has been previously reported (Burt, 2004; Holley and Patel, 2005; Upadhyay et al., 2014). β-resorcylic acid (BR; 2, 4 dihydroxybenzoic acid) is a phytophenolic compound that is widely distributed among the angiosperms as a secondary metabolite to protect plants against pathogens (Friedman et al., 2003). It is classified under "Everything Added to Food in the United States" (EAFUS; Cas no. 89-86-1) by the US-FDA (U. S. FDA EAF 3045; U. S. Food and Drug Administration, 2013). Previous research has shown that BR is effective in reducing major foodborne pathogens, including *Salmonella* (Mattson et al., 2011), *Listeria monocytogenes* (Upadhyay et al., 2013), and *Escherichia coli* O157:H7 (Baskaran et al., 2013) in food products. However, its efficacy in reducing *C. jejuni* in chickens has not been determined.

The objective of this study was to investigate the efficacy of in-feed supplementation of BR in reducing *C. jejuni* colonization in broiler chickens. In addition, the effect of BR on the various virulence factors critical for *Campylobacter* colonization in chickens was investigated.

MATERIALS AND METHODS

Campylobacter Strains and Culture Conditions

Four wild strains (S-1, S-3, S-4, S-8) of *C. jejuni* originally isolated from the commercial broilers were used in the study. Each strain was grown separately in 10 ml of *Campylobacter* enrichment broth (CEB, International Diagnostics Group, Bury, Lancashire, UK) for 48 h at 42°C under microaerophilic condition (5% O_2, 10% CO_2, and 85% N_2).

Anti-*Campylobacter* Efficacy of BR in Chicken Cecal Contents

The antibacterial activity of BR against *C. jejuni* in cecal contents was investigated as described before (Kollanoor Johny et al., 2010). Cecal contents from broiler chickens were collected and autoclaved at 121°C for 15 min. The autoclaved cecal contents (10 ml) were inoculated with *C. jejuni* to ∼10^8 CFU/ml. Different concentrations of treatment solution were prepared by dissolving appropriate quantities of BR (Sigma-Aldrich, Co., St. Louis, MO, USA) in CEB to make a final concentration of 0.25, 0.50, and 1% BR. *Campylobacter* enrichment broth was used as a control. Then, 100 μl of cecal content inoculated with the four-strain mixture of *C. jejuni* and 900 μl of respective treatment solutions were added in tubes and incubated at 42°C under microaerophilic condition for 24 h. Duplicate samples were serially diluted (1:10) in Butterfield's phosphate diluent (BPD, 0.625 mM potassium dihydrogen phosphate, pH 7.2) and plated at 0, 8, 24 h on *Campylobacter* line agar (CLA; Line, 2001), followed by incubation at 42°C for 48 h to enumerate the surviving *C. jejuni*.

Bird Housing, *Campylobacter* Challenge, and Enumeration

Two *in vivo* studies were conducted with a total of 80 birds. In each trial, 40 day of hatch broiler chicks (Cobb500) were obtained from a commercial hatchery. All the experiments were approved by the Institutional Animal Care and Use Committee of the University of Arkansas and recommended guidelines were followed for animal handling. Birds were weighed and randomly allocated to one of four treatments groups (0, 0.25, 0.5, 1% BR)

($n = 10$ chicks/treatment/trial). BR was thoroughly mixed in mash feed using a commercial feed mixer and was fed throughout the 14-day study period along with *ad libitum* water. The feed consumption, initial and final body weights of individual birds was recorded during the study. Birds were challenged via oral gavage with a cocktail of four wild strains of *C. jejuni* (6 Log CFU/ml; 250 μl/bird) on day 7. On day 14, cecal contents were collected aseptically, serially diluted and plated on CLA for enumeration of *Campylobacter* (Cole et al., 2006). Confirmation of *Campylobacter* colonies was made with a latex agglutination test (Scimedx, Co., Dover, NJ, USA).

Determination of SIC of BR

The sub-inhibitory concentration (SIC) of BR was determined as described previously (Upadhyay et al., 2012). Briefly, two-fold dilutions of BR (0, 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.003125%) were made in a sterile 24-well polystyrene plate (Costar, Corning, NY, USA) containing CEB followed by inoculation with wild type S-8 strain of *C. jejuni* ($\sim 10^6$ CFU/ml). The plates were incubated at 42°C under microaerophilic condition for 24 h and the growth of *C. jejuni* was enumerated. The highest concentration of BR that did not inhibit the growth of *C. jejuni* as compared to controls was selected as the SIC of the compound.

Motility Assay

The effect of BR on the motility of *C. jejuni* was determined as described previously (Upadhyay et al., 2012). Motility test agar plates (Becton, Dickinson and Company, Sparks, MD, USA) with or without (control) SIC of BR were prepared and stab inoculated in the center with 5 μl culture of *C. jejuni* strain S-8 (6 log CFU). The plates were incubated for 48 h at 42°C under microaerophilic condition and the zone of motility were measured.

Adhesion and Invasion Assays

The effect of SIC of BR on *C. jejuni* adhesion to and invasion of human Caco-2 cells was investigated as described previously (Moroni et al., 2006) with modifications. Human enterocytes were maintained in DMEM (VWR Life Science, Rochester, NY, USA) containing 10% fetal bovine serum (VWR Life Science, Rochester, NY, USA). Caco-2 cells were grown in sterile 24-wells culture plates ($\sim 10^5$ cells per well) to form monolayer at 37°C in a humidified, 5% CO_2 incubator. The Caco-2 monolayer was inoculated with the mid-log phase of *C. jejuni* S-8 (~6 Log CFU/ml; multiplicity of infection 10:1) either in the presence or absence of SIC of BR. For the adhesion assay, infected monolayer (after an hour of incubation at 42°C under microaerophilic condition) was rinsed twice in minimal media and lysed with 0.1% Triton-X 100 (Sigma-Aldrich, Co., St. Louis, MO, USA) for 15 min. The enumeration of adherent *C. jejuni* S-8 was made by serial dilution and plating on CLA. For the invasion assay, infected cells (after an hour of incubation) were rinsed twice in minimal media followed by 2 h of incubation in whole media containing gentamicin (200 μg/ml) (Sigma-Aldrich, Co., St. Louis, MO, USA) to kill extracellular bacteria. After incubation, the cells were processed for enumerating the number of invaded bacteria as described above.

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR

The effect of SIC of BR on the expression of *Campylobacter* chicken colonization genes was studied using real-time quantitative PCR (RT-qPCR) as described previously (Woodall et al., 2005; Upadhyay et al., 2012). *C. jejuni* strain S-8 was cultured with or without SIC of BR in CEB at 42° C under microaerophilic condition and total RNA was extracted at mid-log phase (10 h) using RNA mini kit (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was made using iScript cDNA synthesis kit (Bio-Rad) after DNase treatment (Thermo Fisher Scientific, Carlsbad, CA, USA). All the primers in our study (**Table 1**) were designed from published Gene Bank *C. jejuni* sequences using Primer 3 software (National Center for Biotechnology Information) and obtained from Integrated DNA Technologies. The cDNA was used as the template and the amplified product was detected by SYBR Green reagent (iQ SYBR Green Supermix, Bio-Rad). The primer specificity was tested using NCBI-Primer BLAST, melt curve analysis and *in silico* PCR (Bikandi et al., 2004). Data were normalized to endogenous control (16S rRNA) and comparative analyses of expression of candidate genes were determined using the comparative critical threshold ($\Delta\Delta$Ct) method on Quant Studio 3 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA).

Statistical Analyses

The *Campylobacter* mean CFUs were logarithmically transformed (Log CFU) to maintain the homogeneity of variance (Byrd et al., 2001). For all the *in vitro* experiments, duplicate samples were used and the assay was repeated three times. The data from trial 1 and trial 2 (*in vivo* study) and all *in vitro* experiments were pooled and analyzed using PROC MIXED procedure in SAS software (version 9.4, SAS Institute,

TABLE 1 | Primers used for gene expression analysis using real-time quantitative PCR.

Gene with accession no.	Primer	Sequence (5′–3′)
16S-rRNA (NC_002163.1)	Forward	5′-ATAAGCACCGGCTAACTCCG-3′
(product length 78 bp)	Reverse	5′-TTACGCCCAGTGATTCCGAG-3′
luxS (NC_002163.1)	Forward	5′-AGTGTTGCAAAAGCTTGGGA-3′
(product length 106 bp)	Reverse	5′-GCATTGCACAAGTTCCGCAT-3′
motA (NC_002163.1)	Forward	5′-AGCGGGTATTTCAGGTGCTT-3′
(product length 75 bp)	Reverse	5′-CCCCAAGGAGCAAAAAGTGC-3′
motB (NC_002163.1)	Forward	5′-AATGCCCAGAATGTCCAGCA-3′
(product length 51 bp)	Reverse	5′-AGTCTGCATAAGGCACAGCC-3′
fliA (NC_002163.1)	Forward	5′-AGCTTTCACGCCGTTACGAT-3′
(product length 56 bp)	Reverse	5′-TCTTGCAAAACCCCAGAAGT-3′
ciaB (NC_002163.1)	Forward	5′-TCTCAGCTCAAGTCGTTCCA-3′
(product length 50 bp)	Reverse	5′-GCCCGCCTTAGAACTTACAA-3′
jlpA (NC_002163.1)	Forward	5′-AGCACACAGGGAATCGACAG-3′
(product length 66 bp)	Reverse	5′-TAACGCTTCTGTGGCGTCTT-3′
cadF (NC_002163.1)	Forward	5′-CGCGGGTGTAAAATTCCGTC-3′
(product length 135 bp)	Reverse	5′-TCCTTTTTGCCACCAAAACCA-3′

Inc., Cary, NC, USA). The treatment means were separated by least square means, and a probability of $P < 0.05$ was required for statistical significance.

RESULTS

Anti-*Campylobacter* Efficacy of BR in Chicken Cecal Contents *In vitro*

Figure 1 shows the effect of BR in reducing *Campylobacter* in chicken cecal contents *in vitro*. Among the various BR treatments, only 1% BR treatment significantly reduced *Campylobacter* counts at 0 h by 5.2 Log CFU/ml. At 8 h all the BR treatments significantly reduced *Campylobacter* populations in chicken cecal contents. Both 0.5 and 1% BR treatments reduced the counts below detection limit (1 Log CFU/ml) whereas 0.25% BR significantly reduced the counts by 4.6 Log CFU/ml compared to control. All the doses of BR reduced counts below detection limit after 24 h.

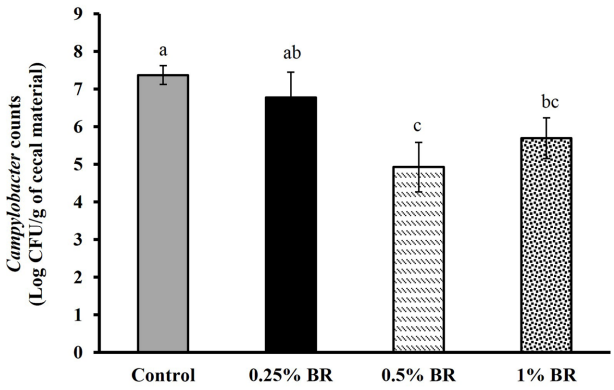

FIGURE 2 | Effect of BR on cecal *Campylobacter* counts in 14 days old broiler chickens. Results are averages of two independent experiments, each containing 10 birds/treatments (mean and SEM). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

Effect of BR on *C. jejuni* Cecal Colonization and Average Body Weight Gain in Broiler Chickens

The effect of BR supplementation on *Campylobacter* cecal colonization in broilers is presented in **Figure 2**. In case of control, an average *Campylobacter* colonization of ∼7.5 Log CFU/g of cecal contents was observed on day 14. Supplementation of BR in feed at 0.5 and 1% level reduced cecal *C. jejuni* counts by ∼2.5 and 1.7 Log CFU/g, respectively as compared to the controls. However, BR supplementation at 0.25% did not significantly reduce *C. jejuni* counts in chickens. There was no significant difference in body weight gains in birds fed with BR compared to control birds at the end of 14 days (**Figure 3**).

Estimation of Sub-inhibitory Concentration (SIC) of BR for Mechanistic Analysis

Based on growth curve analysis, the concentration of BR that did not significantly inhibit the growth of *C. jejuni* was 0.0125% and was selected as the SIC for subsequent mechanistic analysis (data not shown). Since DMSO was used as the diluent for BR, its effect on various colonization factors of *C. jejuni* was also studied.

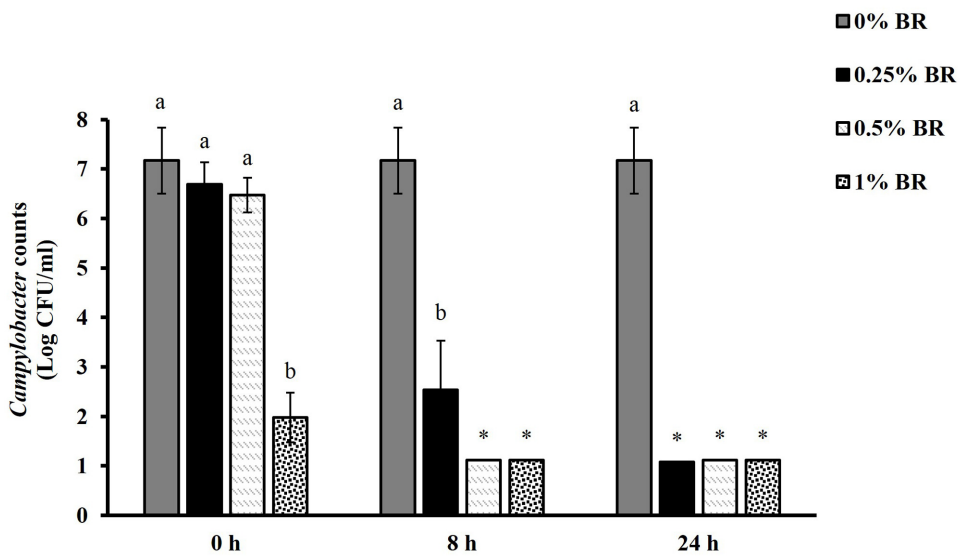

FIGURE 1 | Reduction of *Campylobacter* counts in cecal contents by different concentrations (0, 0.25, 0.50, and 1%) of BR at 0, 8, and 24 h. Results are averages of three independent experiments, each containing duplicate samples (mean and SEM). Bars with different letters represent a significant difference between treatments ($P < 0.05$). *Indicates *Campylobacter* counts below the detection limit (1 Log CFU/ml).

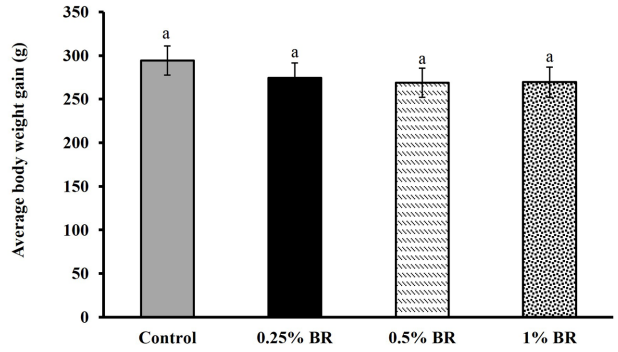

FIGURE 3 | Effect of BR on body weight gain in broiler chickens. Results are averages of two independent experiments, each containing 10 birds/treatments (mean and SEM). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

Effect of SIC of BR on *C. jejuni* Colonization Factors (Motility, Attachment, and Invasion of Caco-2 Cells)

The addition of BR did not change the pH of motility medium or cell culture medium ($P > 0.05$). **Figure 4** shows the effect of 0.0125% BR on *C. jejuni* motility. The SIC of BR reduced the zone of motility of *C. jejuni* to ∼5.9 cm (20% reduction) as compared to control that had a zone of ∼7.3 cm. In addition, BR also reduced *C. jejuni* attachment to and invasion of Caco-2 cells (**Figures 5A,B**). The adhesion of *C. jejuni* to Caco-2 cells was reduced by ∼0.7 Log CFU/ml (16%) as compared to control (**Figure 5A**). Similarly, *C. jejuni* invasion of Caco-2 cells was reduced by ∼0.5 Log CFU/ml (35%) as compared to control (**Figure 5B**). The DMSO treatment did not significantly affect the motility (**Figure 4**), adhesion (**Figure 5A**), or invasion of *C. jejuni* (**Figure 5B**). Taken together, these results show that BR exerts inhibitory effect on major colonization factors of *C. jejuni.*

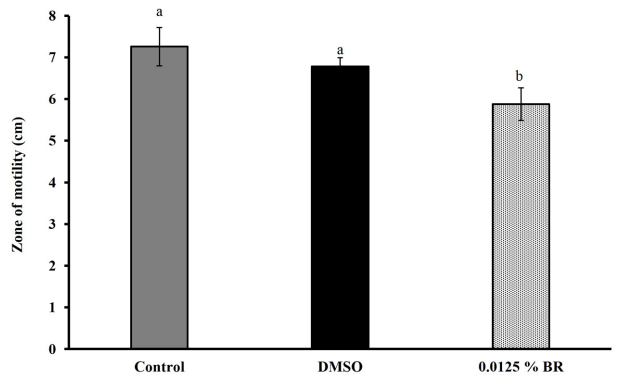

FIGURE 4 | Effect of SIC of BR on the motility of *Campylobacter jejuni*. Results are averages of three independent experiments, each containing duplicate samples (mean and SEM). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

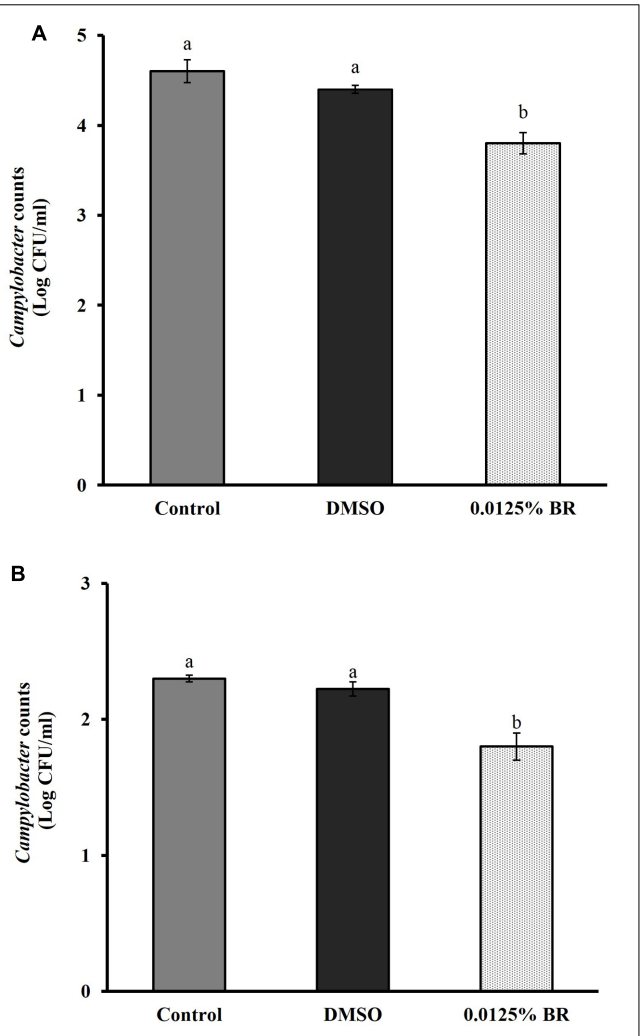

FIGURE 5 | Effect of BR on *C. jejuni* (A) adhesion to and **(B)** invasion of human enterocytes. Results are averages of three independent experiments, each containing duplicate samples (mean and SEM). Bars with different letters represent a significant difference between treatments ($P < 0.05$).

Effect of BR on Expression of *C. jejuni* Poultry Colonization Genes

The effect of SIC of BR on the expression of *Campylobacter* colonization genes is shown in **Figure 6A**. RT-qPCR revealed that BR reduced the transcription of genes critical for motility (*motA*, *motB, fliA*), adhesion and invasion (*jlpA* and *ciaB*) of *Campylobacter* as compared to control. However, other genes coding for quorum sensing (*luxS*) and attachment (*cadF*) were not significantly modulated. The DMSO treatment did not affect the expression of the tested genes ($P > 0.05$) (**Figure 6B**).

DISCUSSION

Despite substantial efforts, *C. jejun*i remains a leading biological contaminant of chicken products (Hermans et al., 2011b).

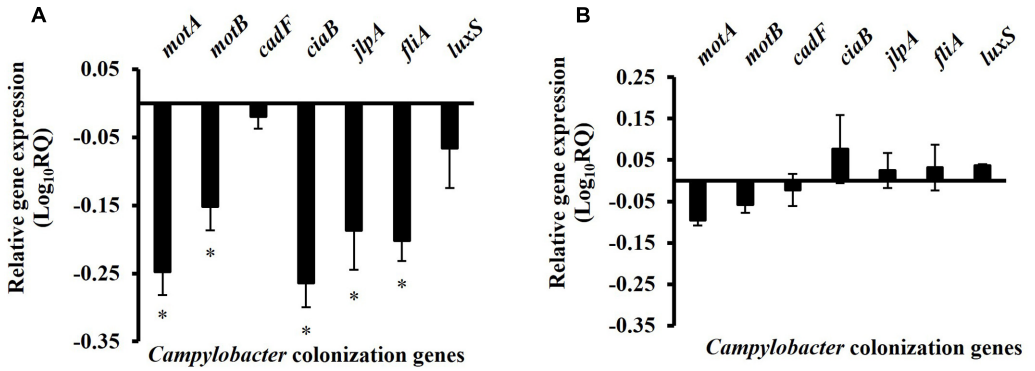

FIGURE 6 | The effect of (A) 0.0125% BR and **(B)** DMSO on the expression of chicken colonization genes of *C. jejuni*. 16S-rRNA served as endogenous control. Results are averages of three independent experiments, each containing duplicate samples (mean and SEM). *Indicates significantly down-regulated genes ($P < 0.05$).

Since *Campylobacter* resides primarily in the cecal crypts of birds, effective pre-harvest control strategy that reduces pathogen colonization in the cecal environment could potentially reduce the risk of fecal shedding and subsequent product contamination (Beery et al., 1988). An antimicrobial treatment that can be administered through feed represents a practical method for controlling pathogen colonization in birds. With increasing consumer demand for antibiotic free chickens, plant derived compounds or phytochemicals represent a large untapped resource that can serve as a safe and effective antibiotic alternative for controlling pathogens in birds.

Since degradation of chemicals in cecal contents is a potential concern, we investigated the anti-*Campylobacter* efficacy of BR in presence of cecal contents as a first step before conducting *in vivo* studies in broilers. We observed that BR is effective at reducing or eliminating *C. jejuni* in presence of cecal contents. The BR treatments reduced *C. jejuni* in a dose-dependent manner with highest tested dose (1%) showing significant reductions immediately (0 h), while the lower doses (0.25 and 0.5%) were effective after 8 h (**Figure 1**). Therefore, we selected these concentrations for testing their anti-*Campylobacter* efficacy in broilers.

Previous research has shown that a 2-Log reduction of *Campylobacter* counts from the poultry would produce up to 95% reduction in the risk of campylobacteriosis in humans (Nauta et al., 2016). In our study, *C. jejuni* colonized the birds effectively (∼7.5 Log CFU/g cecal contents in controls) and the compound consistently reduced *C. jeuni* colonization in both trials, therefore each data were combined (**Figure 2**). Supplementation of BR at 0.5 or 1.0% in feed significantly reduced enteric *Campylobacter* counts by at least 1.5 Log CFU/g when compared with positive control, indicating that BR could potentially reduce risk of subsequent human infections by reducing *Campylobacter* colonization in broilers. Previously, Upadhyaya et al. (2014) reported that supplementation of BR at 1% in feed reduced *Salmonella enteritidis* colonization in cecum, liver and crop by at least 1.5 Log CFU/g in 21 day broiler chickens suggesting that BR has a broad antimicrobial activity that includes major poultry associated foodborne pathogens. Structure-activity studies suggest that the antimicrobial activity of BR is associated with presence of carboxyl and hydroxyl groups on the phenol ring in its structure (Friedman et al., 2003).

Although found to be effective in controlling foodborne pathogen, selection of BR dosage is critical for optimal antimicrobial efficacy in poultry. We observed that feeding BR at level of 1.5% led to slower weight gain in birds (data not shown). Józefiak et al. (2010) reported that supplementation of benzoic acid (dehydroxylated form of BR) at 0.2% depressed the growth of broiler chickens. However, in another study, supplementation of benzoic acid at 0.1% in feed improved the performance in turkey poults (Giannenas et al., 2014). These researchers also observed an increase in lactic acid bacteria and decrease in coliform bacteria in the ceca. There are only a few studies on the absorption, metabolism, and effect of BR on poultry gut. Beta-resorcylic acid has a moderate dissociation constant similar to benzoic acid, and as a weak acid remains in a non-dissociated form in the stomach and intestine (Mattoo, 1959; Milne, 1965). Therefore, it might be acting through similar mechanism(s). It is possible that feeding higher concentration of BR could modulate poultry gut environment leading to reduced appetite and suppressed growth in chickens.

To investigate the potential mechanism of action of BR on *Campylobacter*, we evaluated the effect of BR at its SIC, on various virulence attributes of *C. jejuni. C. jejuni* strain S-8 was randomly selected from the four-strains for mechanistic analysis. Since SICs are not bacteriostatic or bactericidal, the results we observed in our phenotypic assays are not due to killing of *Campylobacter* but potentially due to modulation of its pathophysiology. Motility of *Campylobacter* is one of the essential factors for colonization in poultry gut (Morooka et al., 1985; Hermans et al., 2011a). We found that presence of BR in the medium reduced *Campylobacter* motility as compared

to controls. Similar results of reduced *Campylobacter* motility were observed when *Campylobacter* was exposed to citrus extract (Castillo et al., 2014) and berries (Salaheen et al., 2014). Since a chicken cecal epithelial cell line is not commercially available, we used the well-established human epithelial cells (Caco-2) for conducting attachment and invasion assays. Previously, Byrne et al. (2007) showed that *C. jejuni* attaches and invades both human epithelial cells and primary chicken enterocytes with similar efficiency. Our results from the cell culture revealed that BR reduced *C. jejuni* adhesion and invasion of human enterocytes by 0.7 and 0.5 Log CFU/ml respectively compared to control. This reduction was similar to that observed with other phytochemicals such as berry extracts (Salaheen et al., 2014), and extracts from *Acacia farnesiana*, *Artemisia ludoviciana*, *Opuntia ficus-indica*, and *Cynara scolymus* (Castillo et al., 2014). Since *Campylobacter* adhesion to epithelial cells is an important step for colonization (Jin et al., 2001; Hermans et al., 2011a), a reduction in this virulence attribute could potentially reduce colonization in birds. Bezek et al. (2016) had similar findings with extracts from *Euodia ruticarpa*. In addition, these researchers observed that other virulence attributes, including biofilm formation and quorum sensing were also affected by the phytochemical.

It is previously reported that the SIC of antimicrobials modulates the expression of various virulence proteins and associated genes in bacteria thereby resulting in changes in their pathophysiology and virulence (Goh et al., 2002; Tsui et al., 2004; Qiu et al., 2010; Upadhyay et al., 2012, 2014; Maisuria et al., 2016). To study if similar mechanisms exist in *Campylobacter*, we investigated the effect of BR on various genes of *Campylobacter* that are known to facilitate colonization in poultry. *motA*, *motB*, and *fliA* genes are critical for *Campylobacter* motility (Nachamkin et al., 1993; Wassenaar et al., 1993; Fernando et al., 2007). *motA* and *motB* code for flagella motor protein, while *fliA* codes for flagella biosynthesis protein. Similarly, *cadF* along with the genes *jlpA* and *ciaB* facilitate *C. jejuni* adherence onto the intestinal cells (Hermans et al., 2011a). The transcription level of genes coding for motility (*motA, motB, fliA*) and attachment (*jlpA*, *ciaB*) was downregulated as revealed in RT-qPCR results, thus indicating that the anti-*Campylobacter* colonization effect observed with BR could be mediated via downregulation of critical colonization genes in the pathogen.

CONCLUSION

β-resorcylic acid supplementation in feed reduced *Campylobacter* colonization in birds without affecting their body weight gain or feed conversion ratio. Mechanistic analysis using standard bioassays, cell culture and gene expression analysis showed that the reduction in *Campylobacter* colonization in birds could at least partially be attributed to modulation of critical colonization factors, virulence proteins and associated genes in the pathogen. Thus, BR could potentially be used as a feed supplement to control *C. jejuni* colonization in broiler chickens. Although, the results from this study are encouraging, follow-up studies investigating the efficacy of BR in market age birds and cost-benefit analysis of feed application are warranted.

DISCLAIMER

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

AUTHOR CONTRIBUTIONS

BW and DD designed the study. BW, AU, KA, SS conducted the experiments. BW and AU wrote the manuscript. KV, AD, and DD critically analyzed and revised the manuscript.

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The Minor Flagellin of *Campylobacter jejuni* (FlaB) Confers Defensive Properties against Bacteriophage Infection

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A screen of bacteriophages infecting a panel of *Campylobacter jejuni* PT14 gene knock-out mutants identified a role for the minor flagellin encoded by the *flaB* gene, in the defense of the host against CP8unalikevirus bacteriophage CP_F1 infection. Inactivation of the *flaB* gene resulted in an increase in the susceptibility of PT14 cultures to infection by CP_F1 and an increase in bacteriophage yields. Infection of wild type PT14 with CP_F1 produces turbid plaques in bacterial lawns, from which 78% of the resistant isolates recovered exhibit either attenuation or complete loss of motility. CP_F1 produces clear plaques on the *flaB* mutant with no regrowth in the lysis zones. Complementation of the mutant restored overgrowth and the development of resistance at the expense of motility. Further analyses revealed an increase in bacteriophage adsorption constant of nearly 2-fold and burst-size 3-fold, relative to the wild type. Motility analysis showed no major reduction in swarming motility in the *flaB* mutant. Thus, we propose a new role for FlaB in the defense of campylobacters against bacteriophage infection.

Keywords: *Campylobacter*, bacteriophage, flagellin, flaB, phage escape

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INTRODUCTION

Campylobacter represents a major zoonotic pathogen, as emphasized in recent reports published by the European Food Safety Authority that document year-on-year recorded caseloads of >210.000, which are estimated to belie an actual annual infection rate of 9 million people (EFSA, 2012, 2014, 2015). Human infection by this gram-negative bacterium leads to gastroenteritis (termed campylobacteriosis) with symptoms including severe abdominal pain, fever and diarrhea (Blaser, 1997; Allos, 2001). The infection is generally self-limiting and can be treated with rehydration therapy. However, in rare cases chronic sequelae can develop, for example reactive arthritis or Guillain-Barré syndrome (GBS), autoimmune disorders that can lead to temporary or in severe cases permanent paralysis (Nachamkin, 2002). The fact that campylobacters are highly prevalent in the intestinal tracts of farm livestock, such as poultry and pigs, renders it a major foodborne pathogen, and therefore represents a significant risk to consumer health (Boes et al., 2005; McCrea et al., 2005).

In order to effectively colonize their host, *Campylobacter* cells are equipped with one or two polar located flagella that confer high motility. Recent estimates suggest a torque of 3600 pN/nm for *C. jejuni* flagella, which is more than twice as high as that reported for *Salmonella* cells (Beeby et al., 2016). These high mobility structures together with CadF and FlpA adhesins help withstand

peristaltic forces in the intestines of its colonized host, preventing them from being expelled and to facilitate locomotion in the highly viscous environment of the epithelial cell mucus layer (Monteville et al., 2003; Konkel et al., 2004, 2010). The invasion of intestinal epithelial cells is another key function of the flagella apparatus. A model proposed for *Campylobacter* pathogenesis suggests attachment to and disruption of epithelial cell barriers, before migrating toward the basal ends of cells, where incorporation takes place (O'Loughlin and Konkel, 2014). For this purpose, the flagella act as a type III secretion apparatus releasing Cia proteins (*Campylobacter* invasion antigens), that induce the cell invasion process (Konkel et al., 2001). Through these functions flagella are recognized as crucial for colonization and virulence (Bolton, 2015).

Interestingly, these motility structures have also been employed by the natural antagonists of *Campylobacter*, as they serve as attachment sites for bacteriophage infection. It has been shown that loss of the flagellin structure or motility function will result in resistance to infection from flagellotropic bacteriophages (Coward et al., 2006; Scott et al., 2007; Baldvinsson et al., 2014). Javed et al. (2015) further supported these findings by reporting that glycosylated flagellin serves as target for a phage receptor-binding protein (RBP) from bacteriophage NCTC12673. The main initial binding-target of flagellotropic phages, namely the rotating flagellar filament, is coded for by two tandem genes *flaA* and *flaB*, which show 95% sequence identity (Wassenaar et al., 1991). Selective disruption of the major flagellin gene (*flaA*) or a double deletion mutant lead to loss of the full-length flagellar filament, which results in the loss of motility, reduced colonization efficiency and resistance to flagellotropic bacteriophages (Guerry et al., 1991; Nachamkin et al., 1993; Baldvinsson et al., 2014). Inactivation of *flaB* does not have a major impact on motility as the FlaB content within the flagellar filament is sparse (Alm et al., 1993). Expression of the alternative flagellin genes are governed by different sigma factors. While *flaA* is under control of σ^{28}, *flaB* is σ^{54} regulated (Nuijten et al., 1990). The functional significance or benefits of this separate gene regulation have yet to be unraveled. There are suggestions that *flaB* is involved in an intragenomic recombination mechanism aiming to evade immunological responses of the colonized host, and therefore increasing antigenic diversity (Harrington et al., 1997). Whether this could also have an effect on bacteriophage infection has not been tested, since there are no data available in literature concerning links of the minor flagellin gene product (FlaB) to bacteriophage infection.

The aim of the present study was to identify factors that impact on Eucampyvirinae bacteriophage infection that constitute a virulent subfamily of the *Myoviridae* (Javed et al., 2014), and are candidates for phage therapy of farm animals (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Kittler et al., 2013; Hammerl et al., 2014) and phage biosanitization applications (Atterbury et al., 2003; Goode et al., 2003; Bigwood et al., 2008) to control campylobacters in the human food chain (Connerton et al., 2011). For this purpose, we have constructed isogenic knock-out mutants targeting virulence and animal host colonization factors in the bacteriophage propagation strain *C. jejuni* PT14 (Brathwaite et al., 2013). We report for the first time an effect of the *flaB* gene product in connection to bacteriophage infection. In the absence of functional FlaB we observed an increase in susceptibility to infection by bacteriophage CP_F1 and increased phage propagation after a 24 h incubation period in liquid culture. Further, we observed changes in the adsorption rate and burst size that suggests FlaB has a defensive role against bacteriophage infection.

MATERIALS AND METHODS

Bacteriophage, Bacterial Strains, and Growth Conditions

Bacteriophage CP_F1 was originally isolated from a pig manure sample and was propagated on *C. jejuni* PT14 (Brathwaite et al., 2013). All strains, associated plasmids and oligonucleotide PCR primers used in this study are listed in **Table 1**. The *C. jejuni* strains were routinely grown on blood agar base No. 2 plates (BA plates; Oxoid) supplemented with 5% horse blood (TCS Biosciences) under microaerobic conditions in either a modular atmosphere controlled cabinet (5% CO_2, 5% O_2, 2% H_2, 88% N_2) or in anaerobic jars using gas replacement (7.3% CO_2, 5.6% O_2, 3.6% H_2, 83.5% N_2) at 42°C. *Escherichia coli* strain TOP10 (Invitrogen) was used for cloning and cultured aerobically at 37°C on Luria agar plates or in Luria broth containing appropriate antibiotic as required.

Construction of *C. jejuni* PT14 Mutant and Complemented Strain

Transformation of *C. jejuni*

Site-specific knock-out mutations in *C. jejuni* PT14 were generated by transformation with genomic DNA from established and verified constructs in donor strains (**Table 1**). As most strains of *C. jejuni* exhibit efficient natural transformation, the protocol from Wang and Taylor (1990) was employed. The *C. jejuni* PT14 overnight cultures were collected from BA plates and dissolved in Mueller Hinton (MH) broth (Carl Roth). Cells were adjusted to OD_{600} 0.5 (corresponding to approx. 3×10^9 CFU/ml) and aliquots of 500 µl of adjusted culture were added to 1 ml of MH broth in a 15 ml tube. Following incubation at 42°C under microaerobic conditions for 5 h, 1–5 µg of extracted chromosomal DNA (GenElute Bacterial Genomic DNA Kit, Sigma Aldrich) was added and cells were further incubated overnight. On the following day, cells were concentrated by centrifugation at 13,000 × *g* for 15 min. Pellets were resuspended in 100 µl of MH broth and spread on BA plates containing the selective agent according to the selective marker in use (34 µg/ml chloramphenicol or 50 µg/ml kanamycin from Sigma Aldrich). Selection plates were then incubated for 24–48 h at 42°C under microaerobic conditions. Genomic insertion of the desired mutant genes were verified via PCR amplification and DNA sequencing.

Trans Complementation of the *flaB* Mutant Strain

To achieve a trans-complementation of the disrupted *flaB* gene in strain *C. jejuni* PT14, a suicide vector based on pCfdxA (Gaskin et al., 2007) was constructed. For this purpose, the wild type *flaB* gene and the region 100 bp upstream carrying the native

TABLE 1 | Bacterial strains, plasmids, and primers used in this study.

Strain	Characteristic or genotype	Sources
C. jejuni NCTC12662 PT14	propagating strain for bacteriophages CP_F1, CP220	National Collection of Type Cultures
C. jejuni NCTC12662 PT14 Δ*flaA*	Δ*flaA::Kan*	This study
C. jejuni NCTC12662 PT14 Δ*flaAB*	Δ*flaAB::Kan*	This study
C. jejuni NCTC12662 PT14 *kpsM*	*kpsM::Kan*	This study
C. jejuni NCTC12662 PT14 *flaB*	*flaB::Kan*	This study
C. jejuni NCTC12662 PT14 *flaB, 0046::flaB*	*flaB::Kan, 00230::flaB cat*	This study
C. jejuni NCTC11168H *flaA*	Δ*flaA::Kan*	Jones et al., 2004
C. jejuni NCTC11168 *flaAB*	Δ*flaAB::Kan*	M. Jones Univ of Nottingham
C. jejuni NCTC11168 *flaB*	*flaB::Kan*	M. Jones Univ of Nottingham
C. jejuni NCTC11168 *kpsM*	*kpsM::Kan*	Karlyshev et al., 2000
E. coli TOP10	Plasmid propagation strain	Invitrogen
PLASMIDS	**CHARACTERISTIC**	
pCfdxA	*cj0046*::*cat*	Gaskin et al., 2007
pC_flaB	*cj0046*::*flaB cat*	This study
PRIMERS	**NUCLEOTIDE SEQUENCE**	
flaA_fw	GATTGCACGATATAGCATTTAACAAG	
flaA_rv	TCTAAACTAGGCTTGGATTTGTA	
flaA_int_fw	AGGACTTGGAGCTTTAGCAGATGAGAT	
flaA_int_rv	CGGCAGTATTAGCATCAAGCTGTC	
flaA_i5′_rv	GTAAGATACCTAAAGCATCGTTAC	
flaB_fw	GCCATGGCACAGGCTAATTC	
flaB_rv	GGGTTTATGCACACGAAGCTTTGATAG	
flaB_int_fw	AACGACCATAATTTTCCATCATATTTG	
flaB_int_rv	GGTGAAGTGCAATTTACTCTTAAAAATTAC	
kpsM_fw	AAGGTGTGCAAGCTAAGGCCGAGTT	
kpsT_rv	GATCTCCAACAGCTCCTGCTTCAT	
flaB_NgoMIV_fw	CGGCCGGCACAAATCCAAGCCTAGTTTAGAA*	
flaB_BsmBI_rv	AACGTCTCACATGGACGAAGCTTTGATAGAAAATATCAT*	
cj0046_fw	GCTCTTAGTGGCATTACCACTACC	
cj0046_rv	GCCACACTAGTCGCATCAAGAGAA	

**Restriction sites are underlined.*

promoter were PCR amplified from the genomic DNA of *C. jejuni* PT14 with primers flab_NgoMIV_fw and flab_BsmBI_rv. After restriction digest the fragment was cloned into the vector adjacent to a chloramphenicol resistance cassette (*cat*). This vector further harbored regions of pseudogene *cj0046* from *C. jejuni* NCTC11168 flanking the cassette and the gene of interest which enables recombination with the homologous pseudogene A911_00230 of strain *C. jejuni* PT14. Complementation of the mutant strain *C. jejuni* PT14 *flaB::kan* was performed by electroporation, as natural transformation of *E. coli* derived plasmids was not efficient. Colonies were sub-cultured and analyzed for positive integration by colony PCR using primers cj0046_fw and cj0046_rv that bind in the flanking regions of the A911_00230 pseudogene. Additionally, the amplicon was gel purified and sequenced for additional verification.

Spot Test Assays and Titer Determination

A determination of bacteriophage infection of the *C. jejuni* PT14 wild type and mutant strains was performed by employing the traditional spot test assay. A fresh overnight culture of the test *Campylobacter* strain was collected from a BA plate and emulsified in MH broth supplemented with 10 mM $MgSO_4$ (Thermo Fisher) to a cell density of approximately 10^7 CFU/ml. A volume of 500 μl of emulsified cells was added to 5 ml of molten NZCYM (0.6%, Carl Roth) top agar and poured on top of a NZCYM agar plate. After the top agar had solidified, 5 μl aliquots of the phage suspensions, at the routine test dilution of approximately titer of 10^7 PFU/ml (Frost et al., 1999), were dispensed on top of the soft agar layer. The plate was then incubated at 42°C under microaerobic conditions for 24–48 h after the spots dried into the agar. In a similar way the phage titer was determined by serial 10-fold dilutions of phage suspensions, which were applied as 10 μl droplets in triplicate to the surface of prepared host bacterial lawns and allowed to dry. After incubation the plaques were counted and titers calculated.

Motility Assays

In order to assess swarming motility, fresh overnight cultures were taken from a BA plate and resuspended in MH broth. After adjustment of cell suspensions to OD_{600} 0.1, 2 μl of suspension

was removed with a pipette tip and stabbed into a swarming agar plate (0.4% Mueller Hinton agar). Inoculated plates were then incubated under microaerobic conditions at 42°C for 48 h. Growth zones were measured for each of triplicate samples after 24 and 48 h to assess cell motility.

Analysis of Bacteriophage Infection in Liquid Growth Media

The growth from *C. jejuni* BA plates incubated overnight at 42°C were collected and emulsified in PBS buffer (Merck Millipore) for the adjustment of inoculum concentrations. Flasks containing 50 ml nutrient broth No. 2 (Oxoid) were inoculated with *C. jejuni* cells at starting concentrations of approximately 10^5 or 10^7 CFU/ml. After inoculation, flasks were sealed with cotton stoppers and placed in anaerobic jars (Oxoid). Microaerobic conditions were introduced using the gas replacement method (John et al., 2011). Cultures were then incubated in a shaking incubator at 42°C and 100 rpm. After 2 h incubation, bacteriophage CP_F1 was added at an MOI of 2. Samples were collected at 2 h intervals for determination of viable cell counts and phage titers using the Miles and Misra method. Phage samples were treated with 2 μl chloroform (Thermofisher) and centrifuged at 13,000 × *g* for 5 min. The supernatant was removed and used for titration. The *C. jejuni* PT14 wild type and knock-out strains were tested in triplicate to ensure statistical certainty. Growth controls without phage addition served as references.

Adsorption Assay

Phage adsorption rates were determined as described previously (Siringan et al., 2014) with minor modifications outlined as followed. Suspensions of *C. jejuni* PT14 wild type, *flaB* mutant and trans-complemented *flaB* cells that contained approximately 5×10^7 CFU/ml, were inoculated in nutrient broth No. 2 and incubated at 42°C under microaerobic conditions at 100 rpm for 5 h to obtain cells in the exponential growth state. The actual viable count was determined following serial dilution and incubation as described above. For the experiment, bacteriophage CP_F1 was diluted and added to the suspensions to give a final titer of 10^5 PFU/ml, then briefly mixed and kept static at 42°C under microaerobic conditions. Sampling was performed every 5 min over a period of 30 min. Samples were immediately centrifuged at 13 000 × *g* for 5 min and the supernatants removed. The titer of free bacteriophages in the supernatants was determined and used to calculate numbers of bound bacteriophages. Bacteriophage adsorption constants were determined using the formula $k = \text{-ln}\ (P_t/P_0)/Nt$, where P_t = phage titer at time point t (PFU/ml), P_0 = initial phage titer (PFU/ml), N = bacterial density (CFU/ml) and t = time (min).

Efficiency of Plating of Phage CP_F1 on Mutant Strains

The efficiency of plating (EOP) of bacteriophage CP_F1 infecting the PT14 mutant strains was determined by enumerating bacteriophages as described above. Subsequently the calculations were made by dividing the bacteriophage titer obtained when applied to the lawns prepared from different mutant strains by the titer obtained when applied to *C. jejuni* PT14 wild type lawns.

Bacteriophage Growth Parameter Determination

In order to assess the burst size and latent period for bacteriophage CP_F1 infection of *C. jejuni* PT14 wild type, the *flaB* mutant and trans-complemented derivatives, single step growth curves were monitored over 4 h. The protocol after Carvalho et al. (2010) was employed for this purpose. Triplicate samples of 10 ml nutrient broth No. 2 were inoculated with 10^7 CFU/ml cells and grown under microaerobic conditions at 42°C under constant shaking at 100 rpm into early exponential phase (approx. 10^8 CFU/ml). Bacteriophage CP_F1 was added at an MOI of 0.001. Samples for titer determination were taken every 15 min for 4 h. These were centrifuged at 13,000 × *g* for 5 min and the supernatants used for titration. Three replicates of each individual experiment were performed and mean values used for presentation of titer development. Non-linear regression was used to determine latent period and burst size of the first burst event.

Whole Genome Sequencing

Genomic DNAs from *C. jejuni* PT14 non-motile variants were prepared using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich) following the manufacturer's instructions. DNA sequencing was performed using the Illumina MiSeq platform. The data consisted of 3.1–4.4 million paired-end sequence reads of 250 bp in length. Initial processing of the raw data, mapping of the sequence reads to *C. jejuni* PT14 (GenBank accession number CP003871) and variant detection were performed using CLC Genomics Workbench version 8.0 (Qiagen, Aarhus, Denmark).

RNA Extraction

Total RNAs were extracted from CP_F1 infected and uninfected control cultures of *C. jejuni* PT14. For each treatment, three independent early-log phase cultures (eclipse phase of the infected cultures) growing in nutrient broth No. 2 were harvested 50 min after phage addition and the RNA content extracted using TRIzol Max with Max Bacterial Enhancement Reagent (Invitrogen) according to the manufacturer's protocol. Subsequently ethanol-precipitation and purification using the RNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions were performed including DNase treatment using RNase-free DNase and related reagents (Qiagen). Pure RNA samples were collected in 40 μl of RNase free water and analyzed for quantity/purity (Nanodrop ND1000) and quality (Bioanalyser 2100, Agilent Technologies Inc.). Prokaryote Total RNA Nano series II software, Version 2.3 was used for analysis of the RNA quality. All purified RNA samples showed a RNA Integrity Number (RIN) of 10.0.

Real Time qRT-PCR

Total RNAs were converted to cDNA with random hexamer primers using the Superscript II (Fisher Scientific) reverse transcriptase system according to the manufacturer's protocol.

Specific primers for qRT PCR were designed with lengths of 18–24 nt and can be found in Supplementary Table S1. An optical 48 well microtiter plate (Applied Biosystems) was used with 20 µl reaction volumes consisting of Power SYBR Green PCR master mix (Life Technologies), 50 nM gene specific primers and 100 ng of the cDNA template. A StepOne real time PCR system (Applied Biosystems) was programmed for an initial set up of 30 s at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 58°C. A melting curve was obtained from 50 to 95°C to control specificities of quantitative PCR reaction for each primer pair. Cycle threshold (CT) values were determined employing the StepOne software version 2.0 (Applied Biosystems). The comparative threshold cycle method was used to calculate change (n-fold) where samples were normalized to the internal control product of the 50 S ribosomal subunit protein L1 gene (*rplA*), which showed no change in expression levels between phage infected and uninfected cultures of *C. jejuni* PT14 during acute CP8 infection in previous experiments. Reactions were performed in triplicate. The fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Verification of acute phage infection was confirmed by PCR using synthesized cDNA, employing specific primers targeting the gene for the major capsid protein (mcp) of bacteriophage CP_F1 (homolog of gp23 from phage NCTC12673; Kropinski et al., 2011).

Statistical Treatment of Data

Statistical differences between paired control and treatment groups were assessed by using the Student's *t*-test with significance $p < 0.05$. Differences between experimental groups were analyzed by analysis of variance. Viable count and phage titer data were $\log_{10}$-transformed for analysis.

RESULTS

Disruption of *flaB* Yields Clear Lysis Zones for Bacteriophage CP_F1

By screening bacteriophage infection of a number of mutant variants of *C. jejuni* PT14 we have analyzed the effects of flagella related factors on this process. Spot test assays showed a clear dependence on rotating flagella for bacteriophage CP220 (prototype of the Cp220likevirus genus), as no replication and host lysis was observed for *flaA* or *flaAB* mutants (Supplementary Figure S1). Similarly, the CP8unalikevirus phage CP_F1 showed decreased infection efficiency on non-motile cells, as infection of *flaA* and *flaAB* mutant strains yielded reduced efficiency of plating (EOP) values (*flaA* 0.08 ± 0.04, $n = 3$; *flaAB* 0.12 ± 0.03, $n = 3$) and highly opaque plaques. Additionally we observed CP_F1 to be strictly dependent on capsular polysaccharide (CPS) as no plaque formation occurred on a *kpsM* mutant (**Figure 1**). Interestingly, we found an aberration in infection by bacteriophage CP_F1 of a *flaB* disruption mutant. CP_F1 was isolated from a pig sample and propagated on strain PT14. It is able to infect and propagate on its host, but yields opaque plaques on soft agar bacterial lawns, which reveal regrowth of the host after infection. We observed similar reactions for phage CP220 (Supplementary Figure S1), suggesting that this reaction is not limited to one class of *Campylobacter* phage (Javed et al., 2014). However, propagation on a *flaB* mutant of PT14 produced clear lysis zones that persisted even after prolonged incubation for 24 h (**Figure 1**). Five individual clones, which all carried the desired disruption of *flaB* were tested in spot test assays to exclude effects of secondary mutations (de Vries et al., 2015). All showed identical phenotypes toward phage-induced lysis on soft agar lawns and motility (Supplementary Figure S2).

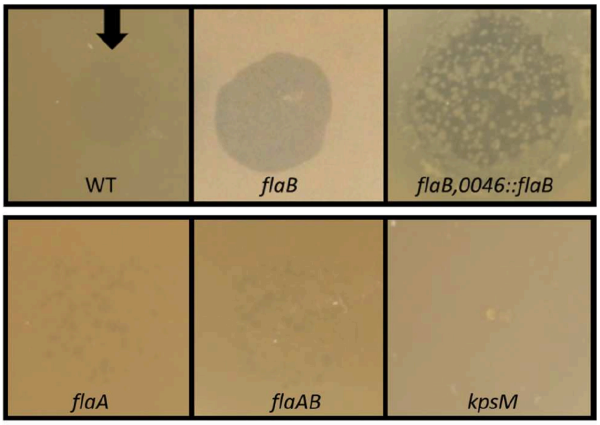

FIGURE 1 | Lysis zones of bacteriophage CP_F1 on bacterial lawns of *C. jejuni* PT14. Opaque lysis areas were observed upon infection of the wild type strain caused by regrowth of a *Campylobacter* sub-population less susceptible to phage infection. Disruption of the *flaB* gene resulted in clear lysis. Complementation of the *flaB* mutant partially restored opaque lysis. Reduced plating efficiency and opaque plaques were observed in *flaA* and *flaAB* mutants. No plaque formation occurred for the *kpsM* mutant.

DNA sequencing of PCR amplicons of all five clones carrying the desired gene disruption, revealed an integration of the resistance marker in the *flaB* gene in the orientation of the *flaB* reading frame, mitigating any polar effects on the adjacent *flaA* gene. We also detected no mutations in the *flaA* gene, as further supported by findings from motility tests. Analysis of the motility of the *flaB* mutant strains revealed no major reduction in swarming motility (**Figure 2**), which is in accordance with previous observations (Wassenaar et al., 1991; de Vries et al., 2015).

To analyze the regrowth of *Campylobacter* cells in lysis zones we collected and sub-cultured surviving cells from the surface of turbid spots. Single colony isolates of these were examined for motility, reaction to phage infection by CP_F1 and DNA sequence analysis of the *flaA/B* region. We found that the majority of clones (78%, $n = 100$) showed either complete loss of motility or attenuation of their swarming ability (Supplementary Table S2), which was accompanied by resistance to reinfection by phage CP_F1. Additionally, eight clones were observed to have shifted into the carrier state (Siringan et al., 2014), as demonstrated by plaque formation inside of the growth zone on the motility agar plates and detectable phage propagation after serial sub-culturing of these clones (Supplementary Figure S3). PCR amplification and DNA sequence analysis of the *flaA* gene of the non-motile isolates revealed no alteration in the gene or

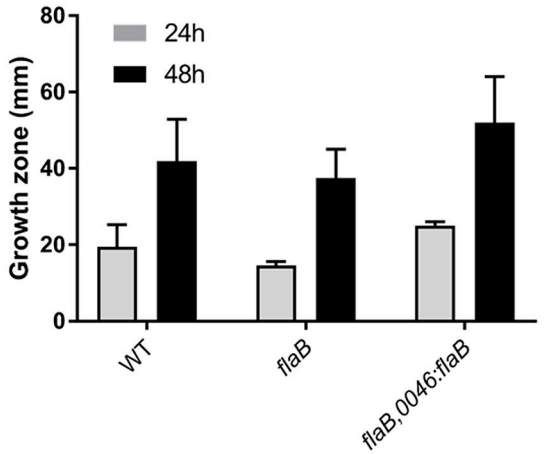

FIGURE 2 | Swarming motility of *C. jejuni* PT14 wild type (WT), *flaB* mutant and the complemented strain on low density agar. The diameters of growth zones after 24 and 48 h of incubation are derived from 3 biological replicates and presented as means with standard deviations.

non-coding sequences. The non-motile isolates were serially sub-cultured and after each passage the motility and phage resistance of the culture were assessed by inoculation of swarming motility agar plates and plaque assay. After two rounds it was observed that cultures arising from a single clone had recovered motility. In one case the process of escape from bacteriophage infection was reversible, which could be attributable to phase variation. Whole genome sequencing of three non-motile clones exhibiting phage resistance revealed all to have suffered a single nucleotide deletion in the N-terminal region of the *rpoN* gene coding for the RNA polymerase factor σ^{54} (A911_03265) at nucleotide position 244–250 in a stretch of 7 adenine residues. Further several phase variable genes were shifted to the off state compared to the wild type genome sequences (**Table 2**). For the *flaB* mutant, the shift to a phage resistant population could not be observed, which we assume is due to an increased susceptibility of the mutant to phage infection that may also be accompanied by an inability to support the mutation(s) leading to phage resistance.

Complementation Leads to Partial Restoration of the Wild Type Phenotype

In order to verify that the observed increase in susceptibility toward phage infection was a result of the disruption of the *flaB* gene, we constructed a plasmid vector for the introduction of an intact copy of *flaB* into the pseudogene A911_00230 of the mutant strain. This construct was used to transform the *flaB* mutant strain and the correct insertion verified by DNA sequencing the target region. Trans-complementation of the disrupted *flaB* gene led to partial restoration of the phenotype with respect to bacteriophage infection. Overgrowth was restored within phage lysis zones but was less turbid than the wild type. The swarming motility of the complemented strain remained comparable to wild type but the survivors of phage infection were compromised in motility (**Figure 2**).

Increased Susceptibility and Burst Size in Liquid Growth Cultures

In order to analyze the changes in phage susceptibility in greater detail, phage replication experiments were performed in 50 ml liquid medium cultures, using either wild type or the *flaB* mutant or the *flaB* trans-complemented strain of *C. jejuni* PT14 over a period of 24 h (**Figure 3**). We tested the effects on phage infection at two different host cell densities, above and below the phage proliferation threshold of $\log_{10}$ 7 CFU/ml, which represents the density of bacteria required for the productive replication of bacteriophage (Cairns et al., 2009). At higher cell densities CP_F1 infection of wild type, *flaB* mutant and *flaB* complement cultures resulted in an initial fall of approximately 1 $\log_{10}$ CFU/ml in the viable count. This event was followed by a recovery in the viable count of wild type cultures (**Figure 3A**). In contrast the *flaB* mutant exhibited a drastic reduction in the growth rate (μ) post the phage-induced crash in viable count (**Figure 3B**). The growth rates post population crash for infected wild type $\mu = 0.67 \pm 0.02$/h and infected *flaB* mutant $\mu = 0.31 \pm 0.05$/h cultures ($p < 0.01$). The *flaB* complement culture also recovered faster than the mutant (**Figure 3C**) exhibiting a significantly greater growth rate of $\mu = 0.44 \pm 0.06$/h ($p < 0.05$). Phage proliferation at high initial cell densities showed significant differences between wild type and *flaB* phage-infected cultures with significantly greater phage yields at 24 h ($p < 0.01$).

Infection of wild type *C. jejuni* PT14 at a bacterial density below the phage proliferation threshold with CP_F1 at an MOI of 2 resulted in minimal differences in the viable bacterial counts between infected and control cultures (**Figure 3D**). In contrast, the viable count for *flaB* mutant cultures remained static for a period of 4 h, post phage infection but subsequently these cultures entered exponential growth (**Figure 3E**). The growth rates of the infected cultures post delay were not significantly different to the uninfected control cultures (control: $\mu = 0.62 \pm 0.10$/h; infected: $\mu = 0.74 \pm 0.06$/h) suggesting under these conditions there was no fitness disadvantage for the emergent phage resistant sub-population. The delay evident in the phage infected *flaB* mutant was not present in trans-complemented cultures, which behaved similar to wild type with similar growth rates to the uninfected control at early stages of growth (**Figure 3F**). Analysis of phage propagation showed no increase in phage numbers during the first 12 h of incubation. However, after 24 h an increase in free virions was detected, implying phage replication. There was a significant difference in the phage titer of approximately 1 $\log_{10}$ PFU/ml ($p < 0.001$) between infected wild type cultures and the infected flagellin B mutant strain after 24 h (Supplementary Figure S4). Further analyses of the phage propagation parameters were examined using a one-step growth experiment. The latent period for CP_F1 was determined as 90 min with two burst events, marked B1 (90–105 min) and B2 (180–210 min) in **Figure 4**, evident over the 240 min period. The latent period for the CP_F1 infection process was in a comparable range to other studies (Cairns et al., 2009; Carvalho et al., 2010). The number of phage particles liberated per cell showed an increase for CP_F1 infecting the *flaB* mutant strain relative to the wild type and *flaB* complement. A burst size of 1.4 ± 0.4 PFU/cell in

TABLE 2 | Observed single nucleotide changes in the genome sequences of escape mutants recovered after CP_F1 infection relative to the reference sequence of *C. jejuni* PT14 (CP003871).

Accession number	Gene product	Changes in coding region	Phase status	Occurance in clones
A911_03265	RNA polymerase factor sigma-54	A deletion pos.244	–	1,2,3
A911_03335	invasion protein CipA	C insertion pos.844_845	phase off (10C)	2,3
A911_05520	1,3-galactosyltransferase	G deletion pos.341	phase off (10G)	1,2,3
A911_06290	Hypothetical protein	G insertion pos.167_168	phase off (10G)	1,2,3
A911_06295	Aminoglycosidase N3′-acetyltransferase	G deletion pos.310	phase off (9G)	2
A911_06340	Hypothetical protein	G insertion / deletion pos.588	phase off (12C/10C)	2 (ins), 3 (del)
A911_06345	Hypothetical protein	G insertion pos.588_589	phase off (10C)	1,2,3
A911_06440	put. methyl transferase	G insertion pos.251_252	phase off (10G)	1,2,3
A911_06490	Motility accessory factor	G insertion pos.168_169	phase off (10G)	1,2,3
A911_06906	SAM dependent methyltransferase	G deletion pos.402	phase off (8C)	1,2,3
A911_06907	put. sugar transferase	G insertion pos.123_124	phase on (8C)	1,2,3
A911_07000	alpha-2,3-sialyltransferase	G insertion pos.1605_1606	phase on (10G)	1,3
A911_08080	Lipoprotein	G deletion pos.497	phase off (9G)	1,2,3

Nine out of thirteen coding regions feature identical nucleotide changes in all three clones.

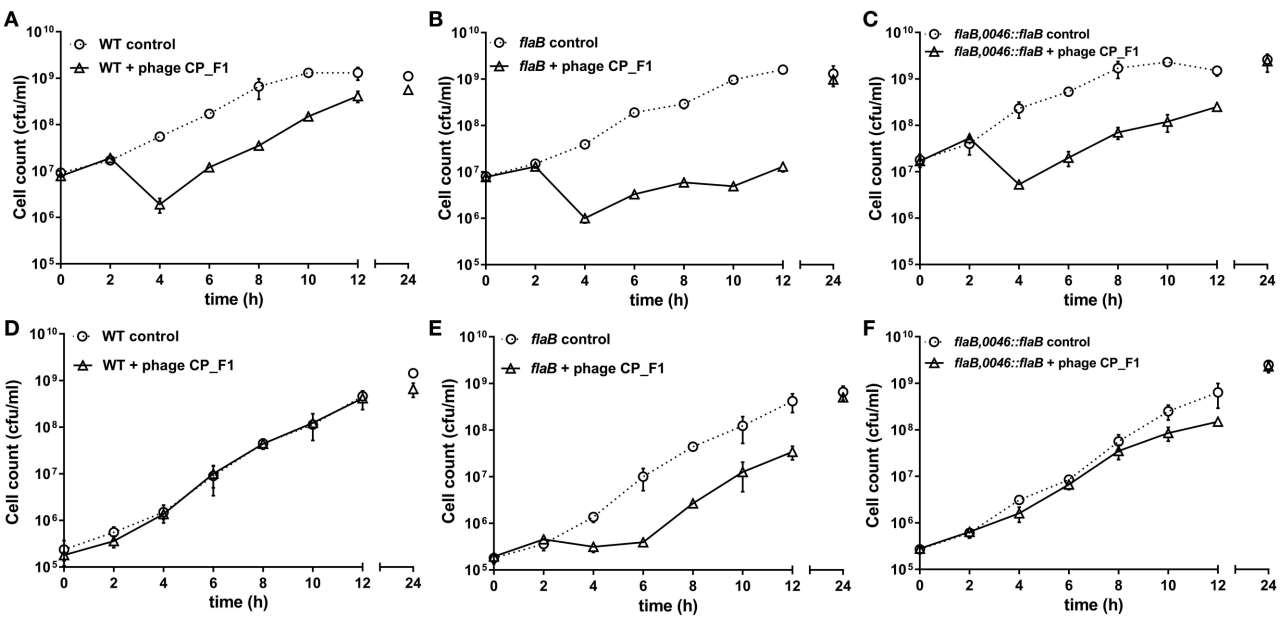

FIGURE 3 | Growth profiles of *C. jejuni* PT14 wild type strain (A,D), flagellin B mutant **(B,E)** and trans-complemented *flaB* cultures **(C,F)** in nutrient broth No. 2. Phage infection was accomplished by addition of bacteriophage CP_F1 to achieve an MOI 2 whilst uninfected cultures were mock infected. Panels **(A–C)** show initial infection above the phage proliferation threshold and panels **(D–F)** below the phage proliferation threshold. Data points indicate mean values for $n = 3$ biological replicates and the error bars show the standard deviation.

the wild type and 4.2 ± 1.2 PFU/cell in the mutant strain were calculated for the first burst event. Differences in the phage yields between the phage infected cultures were statistically significant ($p < 0.05$). Further, the phage yields were magnified after a second burst event. This resulted in a difference of 1 $\log_{10}$ in PFU between mutant culture and the wild type or the *flaB* complement at 225 min after the initial addition of the phage. However, the significant change in the replication process was not accompanied by a major difference in the relative efficiency of plating of phage CP_F1 between infection of the PT14 wild type and the flagellin B mutant strain (1.59 ± 0.38, $n = 3$).

To test if any changes in flagellin gene expression were induced as a response to acute phage infection we performed quantitative RT-PCR analyses. No alterations in the transcript levels of *flaA* or *flaB* in wild type PT14 were observed during the eclipse period of phage infection in which phage DNA replication and capsid assembly occurs prior to host lysis. A time point of 50 min post phage addition was selected for transcriptional

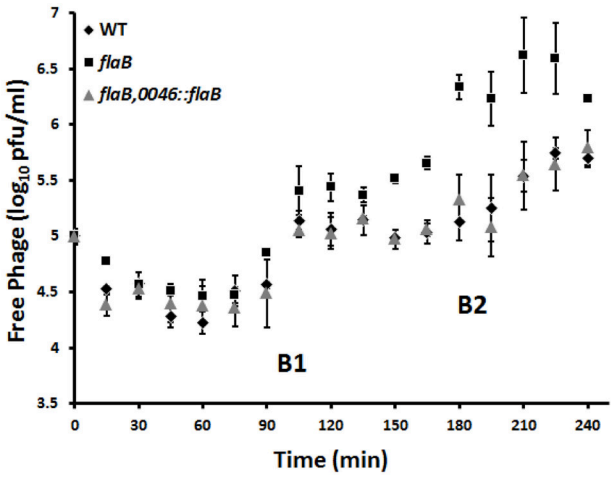

FIGURE 4 | Determination of burst size of *Campylobacter* bacteriophage CP_F1 infecting *C. jejuni* PT14 wild type (WT), flagellin B mutant (*flaB*) and the *flaB* trans-complement (*flaB, 0046::flaB*). Cultures were infected with a MOI of 0.001. Two notable rises in phage titer are marked as burst steps B1 and B2. Calculations of burst size, during the first burst event (90–105 min), resulted in a mean value of 1.4 virions (±0.4) per cell for the wild type strain, 1.8 virions (±0.6) per cell for the *flaB* trans-complement and 4.2 virions (±1.2) for the flagellin B mutant. Data points represent mean values for $n = 3$ independent biological replicates. Error bars represent standard deviations.

analysis, since one step growth experiments revealed a latent period of approximately 90 min for CP_F1 infection. Similarly, no change in the expression of *flaA* was observed in the *flaB* knock-out strain (Supplementary Figures. S5, S6). These data would suggest that the increase in bacteriophage sensitivity or phage yields of *flaB* mutants are not linked to a compensatory increase in *flaA* transcription.

Disruption of *flaB* Affects Bacteriophage Adsorption

As changes in susceptibility toward phage infection and burst size were observed in the mutant strain, we were interested to examine if a change in adsorption might also be an effect of *flaB* inactivation. The calculated adsorption constant of CP_F1 binding to the PT14 wild type was in a similar range to that of the Cp8unalikevirus phages CP30 and CP8 (Siringan et al., 2014). However, there were 2-fold and greater increases in the adsorption constants (k) determined for the *flaB* mutant clones after 30 min incubation (**Table 3**). Differences in the adsorption constant between the wild type and the mutant were statistically significant for five independent clones based on triplicate experiments ($p < 0.01$), verifying that the absence of FlaB has an effect on the adsorption process.

TABLE 3 | Adsorption constants of phage CP_F1 infecting *C. jejuni* PT14 wild type (WT), five independent clones of *flaB* mutants and trans-complemented *flaB* ($n = 3$).

	k-values ($\times 10^{-10}$ ml/ min)	SD	*p*-different to WT
WT	0.54	±0.08	–
flaB c1	2.30	±0.16	$p < 0.01$
flaB c2	1.31	±0.14	$p < 0.01$
flaB c3	3.05	±0.09	$p < 0.01$
flaB c4	1.40	±0.15	$p < 0.01$
flaB c5	0.97	±0.12	$p < 0.01$
flaB,0046::flaB	0.58	±0.06	–

DISCUSSION

Several reports have demonstrated that *Campylobacter* bacteriophages are dependent on certain surface structures for the infection of suitable hosts, for example capsular polysaccharide or a functional flagella (Coward et al., 2006; Scott et al., 2007; Sørensen et al., 2011; Baldvinsson et al., 2014). These are targets for bacteriophage adsorption, which have been recognized as being prone to variation in expression at different stages of bacterial cell growth and are often subject to stochastic phase variation. Campylobacters contain homopolymer GC tracts within dispensable reading frames that can expand or contract to place the reading frame in or out of phase and the gene effectively on or off (Bayliss et al., 2012; Anjum et al., 2016). Variable expression of surface-associated structures may contribute an obstacle to efficient infection by bacteriophages and may affect the efficacy of phage based biocontrol approaches (Nuijten et al., 1995; Park et al., 2000; Karlyshev et al., 2002; Connerton et al., 2011; Holst Sørensen et al., 2012). In search of phage target structures that do not exhibit phase variability we tested the effect of several genes, recognized as essential for *Campylobacter* virulence and colonization, on the infection process of a collection of bacteriophages. The aim was to find novel elements that may be essential for, or have an impact on, bacteriophage infection. During our screening we found a factor that interferes with the infection process of certain bacteriophages. Inactivation of the minor flagellin gene (*flaB*) showed a number of effects on different parameters of bacteriophage infection. Collectively, these effects increased the efficiency of phage infection and impaired the resilience of the affected host population toward infection. For the first time, we show that the presence of a gene product, classified as a structural protein, confers defensive properties against bacteriophage infection. Further, this effect was not limited to one genetically classified genus of *Campylobacter* myovirus phages, as two phages CP_F1 and CP220 from two different genera showed similar reactions during infection of a flagellin B mutant strain. CP_F1 is a CP8una-like phage and CP220 is the archetype of the CP220-like phages (Javed et al., 2014). Furthermore, these two phages differ in requirements for effective infection. We found CP220 to be flagellotropic, while CP_F1 strictly relies on capsular polysaccharide (CPS). However, CP_F1 also showed a preference for motile cells as infection of *flaA* and *flaAB* mutant strains yielded reduced EOP values.

We hypothesize that *flaB* is an essential component of an integral defensive mechanism against bacteriophage infection. In our observations, disruption of this gene prevented cells from

shifting to resistant, non-motile phenotypes during infection of a bacterial lawn on semi-solid media. This phenomenon may be based on the lysis of a large proportion of cells before mutations or gene regulatory responses can develop that lead to a resistant sub-population. In liquid growth cultures, at cell densities below the phage proliferation threshold, bacteriophage CP_F1 was effectively bacteriostatic for *flaB* deficient cultures, whereas the phage did not impair the growth of cultures with an intact *flaB* gene. At cell concentrations that promoted phage proliferation, host bacterial population crashes were observed after phage addition to wild type or mutant cultures. However, the recovery from acute phage infection and the resumption of exponential growth observed for wild type cultures was severely retarded and the growth yields reduced in the *flaB* mutant cultures compared to wild type. These results clearly show that *flaB* deficient strains have a greater susceptibility to phage infection. Inevitably an increase in susceptibility will increase the selective pressure to escape phage infection and the generation of less susceptible sub-populations. Whilst not evident upon infection on semi-solid medium, regrowth was observed post infection of the *flaB* mutants in liquid culture. Spatial differences between liquid and semi-solid media in terms of diffusion, host mobility and distribution might be some of the key factors contributing to the probability of the cells being able to evade infection and develop resistance (Abedon and Yin, 2009). However, it should also be noted that these conditions represent ideal conditions in terms of nutrient availability, suitable oxygen tension and the absence of competitor organisms that would not exist in intestinal or extra-intestinal environments inhabited by campylobacters.

The increase in phage yields observed for the *flaB* mutant are accompanied by a significant increase in the burst size, which suggests that phage maturation is more efficient in the *flaB* mutant. Trans-complementation of the *flaB* mutation results in a reduction in the burst size from 4.2 virions (±1.2) per cell to 1.8 virions (±0.6) per cell, a value similar to that of wild type. The *flaB* mutant also shows an increase in the adsorption constant, which would theoretically make the mutant more susceptible at lower cell densities. **Figure 3E** conclusively demonstrates that the *flaB* mutant culture remains susceptible to phage infection at cell densities below the phage proliferation threshold, such that host growth is arrested as compared to the wild type and *flaB* trans-complement cultures. Lysis times are predicted to become shorter in phage exhibiting high adsorption rates (Shao and Wang, 2008), and it is noteworthy that the first significant increase in free phage titers were observed for the *flaB* mutant in **Figure 4** at 90 min post-infection. From these observations it is evident that any host bacterium that can evolve a countermeasure against this perfect storm would be at a selective advantage when subject to bacteriophage predation. *Campylobacter* bacteriophages have been proposed for the biocontrol of campylobacters in the food chain (Connerton et al., 2011), and from a biotechnological standpoint the introduction of a *flaB* mutant for *Campylobacter* phage amplification strategies offers the prospect of increasing phage yields from growth cultures, which would render large scale phage production more cost effective.

If the FlaB protein acts either directly or indirectly to reduce the burst size, then the *Campylobacter* population would benefit by limiting the release of free virus and the rate of propagation. Such a mechanism could account for the rather low burst size of *Campylobacter* bacteriophages compared to phages infecting other proteobacteria. The peak expression of the *flaB* σ^{54} promoter was reported to occur mid exponential phase (Alm et al., 1993), which would coincide the ideal conditions for efficient phage replication when cells are metabolically replete and at a density beyond the phage proliferation threshold. Under these circumstances the deployment of FlaB as a countermeasure would confer the maximal impact on the bacterial population's ability to evade and adapt to bacteriophage infection.

Campylobacter flagella are highly decorated due to O-linked glycosylation of the flagellin monomer (Guerry, 2007; Ewing et al., 2009). A phage receptor binding protein (RBP) has been reported to specifically interact with flagella decorated with acetamidino-modified pseudaminic acid (Javed et al., 2015). As a consequence, changes in the glycosylation pattern of the flagella may introduce or reduce steric effects on the adsorption process. We have measured 1.8–5.6-fold increases in the adsorption rates of *flaB* mutant clones compared to wild type but how this is manifest is unclear since it has been reported that for *C. coli* VC167 surface exposure of the FlaB protein could not be detected (Guerry et al., 1991).

However, it is also reported that inactivation of *flaB* increases colonization efficiency of *C. jejuni* (Wassenaar et al., 1993). Glycosylation is recognized as an essential colonization factor; therefore it is possible that an altered distribution of glycan molecules may affect a change in colonization efficiency (Howard et al., 2009). There is evidence for diversity in glycan structure and variation in the numbers of residues serving as flagellar glycosylation targets in different *Campylobacter* strains (Thibault et al., 2001; Guerry, 2007), and variations in the occupancy of the glycosylation sites adding to the complexity of surface exposed region of the flagellin proteins (Meinersmann and Hiett, 2000; Ulasi et al., 2015). With respect to flagellin sequence in strain C. *jejuni* PT14, FlaA, and B share 95% identity at the protein level. Differences in amino acid composition can be found predominantly within terminal regions of the protein (26 residues). However, a centrally located region of the protein is predicted to be surface exposed, which contains 4 amino acid differences between FlaA and FlaB proteins of *C. jejuni* PT14, one of which is a serine at position 224 in the FlaB sequence that represents an additional substrate for glycosylation. An analysis of the glycan distributions of FlaA and FlaB may provide further insight as to their potential for interaction with bacteriophage.

Information regarding the function of the minor flagellin is sparse, although several studies have characterized *flaB* as not essential for motility (Guerry et al., 1991; Wassenaar et al., 1993; de Vries et al., 2015). These observations lead to the question as to why a paralogous structural gene has emerged and has been preserved throughout all known *Campylobacter* species. It has been reported that intra-genomic recombination between *flaA* and *flaB* can occur in *Campylobacter* cultures (Wassenaar et al., 1995). Later Meinersmann and Hiett (2000) hypothesized that *flaB* could serve as a driver for antigenic variation, since differences in the amino acid position were found

in residues that function as targets for O-linked glycosylation. A similar conclusion was reached by Harrington et al. (1997), who identified intra-genomic recombination between *flaA* and *flaB* in strain VC670, and proposed that the variation enabled adaptation to eukaryotic host species, since functional flagella are essential for colonization and a key target of the host immune system. We assume that recombination events between the flagellin genes will also assist in the escape of phage infection by varying the flagellin structure and the O-linked glycosylation attachment sites, a response mechanism that is likely to have evolved before the immune systems of the animal hosts of *Campylobacter* species. However, analyses of the *flaA* coding sequences in this study provided no evidence for recombination events between the *flaA* and *flaB* genes in the escape mutants of bacteriophage infection. Further, no alterations in the *flaA* open reading frame or its 5′-untranslated region were detected. Instead whole genome analysis of escape mutants revealed a single adenine deletion in the N-terminal region of the *rpoN* gene coding for the σ^{54} factor. In most polar flagellate species σ^{54} serves as an essential part of flagellar expression. This is also the case for the flagella biogenesis of *Campylobacter*. A knock-out mutation in the *rpoN* gene of strain C. *jejuni* 11168 resulted in complete absence of flagella and flagellin expression (Jagannathan et al., 2001). Motility loss is one strategy by which campylobacters evade phage infection (Scott et al., 2007; Baldvinsson et al., 2014). Moreover, *Campylobacter* bacteriophage carrier state cultures also escape phage lysis by undergoing growth phase dependent motility attenuation as a response to phage association (Siringan et al., 2014). Whereas, *flaA* gene transcription is down-regulated in carrier state cultures, which likely accounts for their impaired mobility, *flaB* transcription is up-regulated, which based on the current data could be an adaption to limit phage infection leading to cell lysis (Brathwaite et al., 2015; Hooton et al., 2016). It is recognized that σ^{54} is part of a group of transcriptional regulators termed class 1 genes that demonstrate hierarchical regulation of class 2 genes, which are fundamental for the formation of the flagellar secretory apparatus and code for the structural components of the basal body of the flagellum (Hendrixson and DiRita, 2003; Lertsethtakarn et al., 2011). The absence of σ^{54} therefore has a profound effect on the formation of functional flagella. We further found several phase variable genes in the off state. Amongst them were several genes coding for sugar transferases, which may have an impact on the cell surface polysaccharides exposed and the phage infection process. High rates of phase variation in *Campylobacter* can facilitate the adaptation toward environmental effects (Gaasbeek et al., 2009; Bayliss et al., 2012). For example, phase variable frame shifts in the key flagellar export regulator FlhA has been reported to cause transcriptional repression of *flaA* and *flaB* to vary motility in *C. coli* (Park et al., 2000). Further, nucleotide deletions in *fliW* and *flgD* have also been found in connection with motility loss. This phenomenon was observed upon analysis of second-site mutations in a *flaB* knock-out mutant strain (de Vries et al., 2015). To exclude that secondary mutation in connection with *flaB* disruption were responsible for the observed increase in bacteriophage sensitivity; we tested five independent clones, which all carried the selective marker in the desired position. All five independent clones showed identical phenotypes with respect to phage induced lysis and motility. Further, trans-complementation partially restored the wild type phenotypes with respect to cell lysis in spot test assays and continued growth at low cell densities in phage infected broth cultures. These results confirm that the observed changes were introduced through the inactivation of the *flaB* gene.

In our efforts to understand how host genes affect bacteriophage propagation in *C. jejuni* we have identified a novel role for the minor flagellin FlaB. Although not essential for motility, the absence of FlaB makes *C. jejuni* more susceptible to bacteriophage infection. Here we propose that the maintenance of the *flaB* gene is not only an evolutionary adaptation to drive antigenic diversity in response to immune pressure but an earlier adaptation to evade infection by flagellotropic phage, and is maintained as a general countermeasure against bacteriophage propagation. Given the ubiquitous presence of virulent bacteriophages in the environment, it is perhaps not surprising that this seemingly redundant gene duplication was fixed and remains a landmark feature of many *C. jejuni* and *C. coli* strains.

AUTHOR CONTRIBUTIONS

LL performed the experiments. LL and IC designed the experiments, analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01908/full#supplementary-material

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Glucose Metabolism via the Entner-Doudoroff Pathway in *Campylobacter*: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates

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Isolates of the zoonotic pathogen *Campylobacter* are generally considered to be unable to metabolize glucose due to lack of key glycolytic enzymes. However, the Entner-Doudoroff (ED) pathway has been identified in *Campylobacter jejuni* subsp. *doylei* and a few *C. coli* isolates. A systematic search for ED pathway genes in a wide range of *Campylobacter* isolates and in the *C. jejuni/coli* PubMLST database revealed that 1.7% of >6,000 genomes encoded a complete ED pathway, including both *C. jejuni* and *C. coli* from diverse clinical, environmental and animal sources. In rich media, glucose significantly enhanced stationary phase survival of a set of ED-positive *C. coli* isolates. Unexpectedly, glucose massively promoted floating biofilm formation in some of these ED-positive isolates. Metabolic profiling by gas chromatography–mass spectrometry revealed distinct responses to glucose in a low biofilm strain (CV1257) compared to a high biofilm strain (B13117), consistent with preferential diversion of hexose-6-phosphate to polysaccharide in B13117. We conclude that while the ED pathway is rare amongst *Campylobacter* isolates causing human disease (the majority of which would be of agricultural origin), some glucose-utilizing isolates exhibit specific fitness advantages, including stationary-phase survival and biofilm production, highlighting key physiological benefits of this pathway in addition to energy conservation.

Keywords: glycolysis, stationary-phase, hexose sugar, polysaccharide, capsule, PubMLST database

INTRODUCTION

The zoonotic pathogen *Campylobacter* is the cause of human campylobacteriosis, the most frequently reported foodborne illness in Europe. The symptoms of campylobacteriosis are gastroenteritis with watery or bloody diarrhea, and the disease is in the majority of cases self-limiting (Butzler, 2004). The most prominent *Campylobacter* species causing disease in humans are *C. jejuni* and *C. coli*, which are most frequently associated with the consumption or handling of

contaminated animal products, especially poultry, but also with animal or environmental contact (Kaakoush et al., 2015). *C. jejuni* is divided into two subspecies: *C. jejuni* subsp. *jejuni,* and *C. jejuni* subsp. *doylei*, which are distinguished by the inability of the latter to reduce nitrate to nitrite (Miller et al., 2007).

Campylobacters are fastidious, microaerophilic, host-adapted organisms with a metabolic capacity highly tuned to their biological niche. For *C. jejuni*, the most widely used carbon sources are primarily the amino acids aspartate, glutamate, serine, and proline (Velayudhan et al., 2004; Guccione et al., 2008; Hofreuter, 2014) as well as certain peptides and organic acids such as lactate, pyruvate and intermediates of the citric acid cycle (Wright et al., 2009; Thomas et al., 2011; Hofreuter, 2014). For many years, the general consensus has been that *Campylobacter* isolates are unable to catabolise various sugars, especially glucose, due to the specific lack of glucokinase (Glk) and phosphofructokinase (PfkA) of the classical Embden-Meyerhof-Parnas (EMP) glycolysis pathway, while the presence of the remaining EMP enzymes (**Figure 1A**) allows the anabolic generation of hexose phosphate via the reverse reactions of gluconeogenesis (Parkhill et al., 2000; Velayudhan and Kelly, 2002). However, catabolism of L-fucose, a hexose sugar, was recently observed in some *C. jejuni* isolates, which overturned this view (Muraoka and Zhang, 2011; Stahl et al., 2011). L-fucose is abundantly present in the mucosal layer of intestinal epithelial cells, originating from fucosylated mucin glycoproteins, and is taken up via the fucose permease FucP (Stahl et al., 2011). The *fucP* gene was found in 30.3% of 710 *C. jejuni* isolates by de Haan et al. (2012) and 57.9% of 266 *C. jejuni* isolates by Zautner et al. (2012), indicating that the ability to utilize L-fucose is quite common but not universal. The genes for L-fucose catabolism are encoded on a genomic island (Stahl et al., 2011) and in *C. coli* isolates, there is evidence that these genes have been introgressed from *C. jejuni* (Sheppard et al., 2013). Significantly, *C. jejuni* mutants lacking the FucP permease displayed a competitive disadvantage in colonization of both chickens and piglets (Stahl et al., 2011). The mechanism of L-fucose catabolism in *Campylobacter* has yet to be fully characterized enzymologically, but seems to proceed by a set of reactions involving non-phosphorylated intermediates, likely forming 1 mol pyruvate and 1 mol lactate per mol L-fucose (Stahl et al., 2011).

Alternative routes to the EMP pathway for the catabolism of glucose include the oxidative pentose phosphate (PP) and the Entner-Doudoroff (ED) pathways. The seemingly universal lack of gluconate-6-phosphate dehydrogenase in campylobacters means the remaining PP enzymes form a purely anabolic pathway. However, the ED pathway genes and a possible glucose transporter gene were first observed in the sequence of *C. jejuni* subsp. *doylei* 269.97 (**Figure 1B**) and proposed as a theoretical way of glucose utilization in this isolate by Miller (2008). Importantly, whereas the EMP pathway produces 2 mol ATP and 2 mol NADH per mol glucose, the ED pathway produces 1 mol each of ATP, NADH, and NADPH per mol glucose (Flamholz et al., 2013). Mechanistically, the ED pathway is somewhat similar to the EMP pathway in that it involves initial activation of the C6 sugar by phosphorylation and a subsequent aldol cleavage to give two C3 intermediates (Conway, 1992), but unlike the EMP pathway the ED aldolase reaction yields one mol of triose phosphate and one mol of pyruvate directly. The key enzymes are 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda) (Conway, 1992; Flamholz et al., 2013). Edd catalyzes the dehydration of gluconate-6P to 2-keto-3-deoxy-gluconate-6P, while Eda catalyzes the aldol cleavage of this compound to pyruvate and glyceraldehyde-3P. In addition, the ED pathway requires a glucokinase (Glk), a glucose-6-phosphate dehydrogenase (Zwf) and a 6-phosphogluconolactonase (Pgl) for conversion of glucose to gluconate-6P, the substrate of Edd (**Figure 1A**).

There are a number of variations of the ED pathway in different groups of bacteria (Conway, 1992). For example, in *Zymomonas mobilis* (a common cause of spoilage of fermented beverages) the pathway is constitutive, effectively linear and is the sole mechanism of converting glucose to pyruvate. In enteric bacteria like *Escherichia coli*, the ED pathway enzymes are inducible and serve primarily in gluconate catabolism, with glucose itself being catabolized preferentially by EMP-mediated glycolysis (Conway, 1992). Gluconate is present in the intestine; interestingly, although campylobacters cannot catabolise gluconate (Pajaniappan et al., 2008; Vorwerk et al., 2015), *C. jejuni* has been shown to use it as an electron donor for respiration via a periplasmic gluconate dehydrogenase (Pajaniappan et al., 2008). A third "cyclic" ED pathway variation is found in *Pseudomonas* spp. that, like campylobacters, lack 6-phosphofructokinase. Here, there is evidence from labeling studies (Nikel et al., 2015) that a portion of the triose-phosphate formed by the Eda aldolase reaction is cycled back to hexose phosphate via the gluconeogenic reactions of the EMP pathway (see **Figure 1A**); this has recently been dubbed the "EDEMP" pathway (Nikel et al., 2015). From genomic studies it seems that the ED pathway (and its variants) is much more common in facultative and obligate aerobes, while anaerobes rely on the EMP pathway (Flamholz et al., 2013). One attractive hypothesis is that both triose-phosphate and pyruvate can be produced with far fewer enzymes by the ED pathway compared to the EMP pathway, which can be viewed as a trade-off with the reduced ATP yield (Flamholz et al., 2013). This may be of particular importance for glucose utilization in small genome, host adapted pathogens.

Recently, in an elegant and detailed study using mutagenesis, ^{13}C-isotopolog analysis and enzyme studies, Vorwerk et al. (2015) discovered that a functional ED pathway exists in certain *C. coli* isolates, which enables this pathogen to utilize glucose as a growth substrate. Moreover, the pathway (like that for L-fucose) was found to be encoded on a genomic island or 'plasticity region,' designated the *glc* locus, that could be transferred between ED-positive and ED-negative isolates by natural transformation, suggesting acquisition of the *glc*-enoded ED pathway could contribute to the expansion of metabolic diversity in campylobacters (Vorwerk et al., 2015). However, knowledge of the actual distribution of the ED pathway genes in isolates from a range of different sources is lacking and it is thus not clear what the role or importance of glucose utilization is in the context of the known genomic diversity of campylobacters.

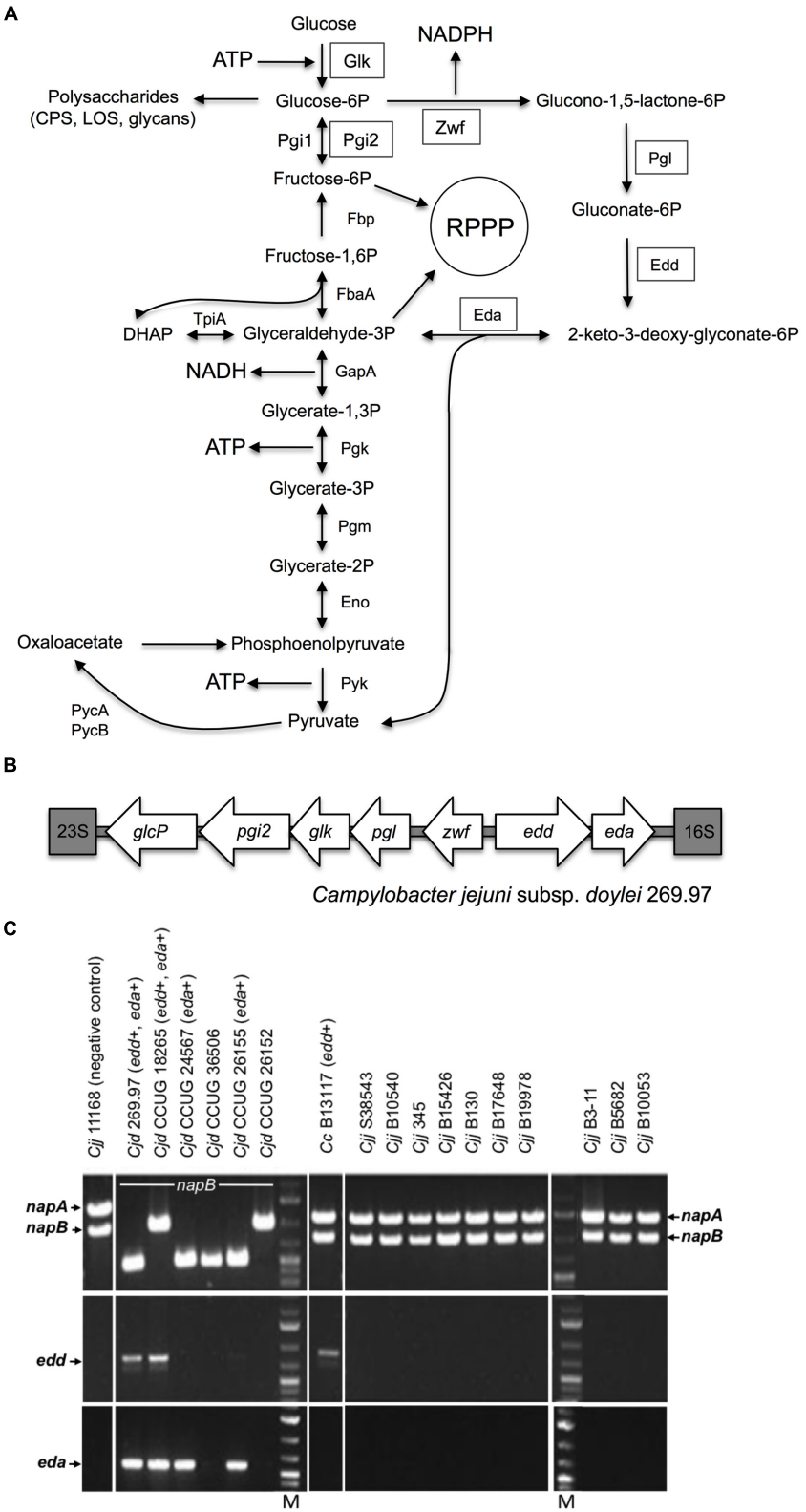

FIGURE 1 | The Entner-Doudoroff pathway in *Campylobacter* isolates. (A) Scheme of the ED pathway to illustrate how the reactions effectively bypass the lack of phosphofructokinase, which prevents glycolysis by the EMP pathway in *Campylobacter*, but can also allow cycling of triose-phosphate back to hexose phosphate. The key enzymes 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda) catalyze the dehydration of

(Continued)

FIGURE 1 | Continued
phosphate back to hexose gluconate-6P to 2-keto-3-deoxy-gluconate-6P and the further aldol cleavage to pyruvate and glyceraldehyde-3P, respectively. In addition, the ED pathway requires a glucokinase (Glk), a glucose-6-phosphate dehydrogenase (Zwf) and a 6-phosphogluconolactonase (Pgl) for conversion of glucose to gluconate-6P, the substrate of Edd. Two phosphoglucose isomerases (Pgi1 and Pgi2) catalyze the same reaction. ED pathway specific enzymes are boxed; Pgi2 is encoded at the *glc* locus (Vorwerk et al., 2015) and may be required to feed fructose-6-phosphate into the reductive pentose phosphate pathway (RPPP). **(B)** Organization of the *glc* locus encoding the ED pathway in *Campylobacter jejuni* subsp. *doylei* 269.97. The locus is situated between the 16S and 23S ribosomal rRNA genes and contains the genes *pgi2*, *glk*, *pgl*, *zwf*, *edd* and *eda* in addition to the glucose transporter *glcP*. **(C)** Screen for *napA, napB, edd,* and *eda* genes in *Campylobacter* isolates. Top panels show PCR fragments of the nitrate reductase genes *napA* (internal fragment) and *napB* (flanking fragment) used to discriminate *C. jejuni* subsp. *doylei* (*Cjd*) from *C. jejuni* subsp. *jejuni* (*Cjj*) and *C. coli (Cc)*. *Cjj* and *Cc* encode both *napA* and *napB* and thus the ability to reduce nitrate. In contrast, *Cjd* has deletions in *napA* and sometimes also *napB*, thus leaving this subsp. unable to reduce nitrate (Miller et al., 2007). None of the *Cjd* isolates showed amplicons with the *napA* internal primers. The *napB* diversity of *Cjd* is illustrated by the variable fragment lengths amplified with the *napB* flanking primers, as described by Miller et al. (2007). Middle panels show amplified ca. 810 bp fragments of the *edd* gene from *Cjd* 269.97, *Cjd* CCUG 18265 and *Cc* B13117. Bottom panels show amplified ca. 600 bp fragments of the *eda* gene from *Cjd* 269.97, *Cjd* CCUG 18265 *Cjd* CCUG 24567, and *Cjd* CCUG 26155. Lanes M contain molecular size markers.

In this study we show by systematic genomic analyses that the ED pathway genes are present in only 1.7% of >6,000 genomes encompassing a diverse range of isolates of both of the major pathogenic species of *Campylobacter* (*C. jejuni* and *C. coli*). However, we demonstrate that the ED pathway provides a significant benefit for *C. coli* in prolonged growth experiments in rich media, where ED-positive isolates display significantly extended stationary-phase survival in the presence of glucose. Interestingly, metabolic profiling revealed that individual ED-positive isolates can respond very differently to glucose, correlating with glucose-stimulated formation of a pellicle or floating biofilm in some isolates. We conclude that although the ability to utilize glucose is generally uncommon in *Campylobacter*, it is found in a more diverse range of isolates than previously suspected and we provide evidence that the ED pathway, in addition to a purely catabolic role, also confers distinct physiological advantages in survival and biofilm formation in some isolates.

MATERIALS AND METHODS

Bacterial Isolates and Growth Conditions

The origins and details of the *Campylobacter* isolates used in this study are given in Supplementary Table S1. Isolates were routinely cultivated on Blood Agar Base No. 2 (Oxoid) supplemented with 5% (v/v) bovine blood and incubated at 37°C under microaerobic conditions (6% v/v CO_2; 6% v/v O_2; 3.4% v/v H_2, and 80.6% v/v N_2). Growth experiments were carried out with and without 100 mM glucose supplementation in Tryptic Soy Broth (TSB) without Dextrose (Becton Dickinson and Co.) or in modified MCLMAN minimal media (Alazzam et al., 2011) without lactate and with 2 mM aspartate. Viable cell counts were determined by plating serial dilutions in phosphate buffered saline on Blood Agar Base No. 2 (Oxoid) and incubating under the conditions described above.

PCR Screen for ED Pathway Genes

Campylobacter isolates (Supplementary Table S1) were screened for the ED pathway genes by PCR using oligonucleotides for *edd* (5′-ATAAATTGGGATGATTTTG and 5′-TCTAAACCCTGCAAAAAGCTC giving a ca 810 bp fragment) and *eda* (5′-GGAGAAAAAATGCAAACAA and 5′-TCTAAACCCTGCAAAAAGCTC giving a ca 600 bp fragment). Oligonucleotides for *napA* and *napB* (periplasmic nitrate reductase) were used in multiplex PCR reactions to discriminate *C. jejuni* subsp. *doylei* from *C. jejuni* subsp. *jejuni* and *C. coli* using the *nap* mpx2 primer set as described by Miller et al. (2007), which consists of internal *napA* primers and *napB* flanking primers. Chromosomal DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen) and the DreamTaq Green DNA polymerase (Thermo Fisher) was applied for the PCR amplifications.

Whole Genome Sequencing and Assembly

Genomic DNA was extracted from bacterial cultures of CCUG 18265, CCUG 24567, CCUG 26155, B13117, and CV1257 using the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK). Whole-genome sequencing (WGS) was carried out at the Wellcome Trust Sanger Institute, UK. Illumina multiplex libraries were generated by acoustic shearing, after which up to 96 libraries were pooled in equimolar ratios and sequenced on a single flow cell lane on the Illumina HiSeq platform, producing 100 bp paired-end reads, as previously described (Cody et al., 2013). The short-read data were submitted to an automated pipeline (Jolley and Maiden, 2010; Bratcher et al., 2014), which integrates VELVET version 1.2.01 (Zerbino and Birney, 2008) and VELVETOPTIMISER version 2.2.0 (Zerbino, 2010) for *de novo* assembly. Draft genomes were then uploaded to the *C. jejuni*/*coli* PubMLST database (Supplementary Table S2).

Genome Annotation and *In silico* Identification of ED-Positive Isolates

Loci corresponding to *glcP* (CAMP2017), *pgi2* (CAMP2018), *glk* (CAMP2019), *pgl* (CAMP2020), *zwf* (CAMP2021), *edd* (CAMP2022), and *eda* (CAMP2023) were added to the *C. jejuni*/*coli* PubMLST database (PubMLST locus names shown in parentheses), using sequences from *C. jejuni* subsp. *doylei* 269.97 (GenBank accession number NC_009707) to seed the database. The *glc* loci, in addition to MLST, rMLST, and *C. jejuni*/*coli* core genome scheme loci, were annotated in the study genomes using the Bacterial Isolate Genome Sequence Database (BIGSDB) 'autotagger' functionality implemented in PubMLST (Jolley and Maiden, 2010). The autotagger uses BLAST to search a genome for sequences similar to loci defined in the

database. For sequences with ≥98% identity to existing alleles, the autotagger defined the position of the locus and assigned a unique allele number in order of discovery (Jolley and Maiden, 2010). Sequences with <98% identity to existing alleles were curated manually. The same approach was used to annotate components of the *glc* locus in publicly available genomes present in the *C. jejuni*/*coli* PubMLST database. A summary of the allelic data for MLST and *glc* loci from all ED-positive isolates was generated using the BIGSDB data export plugin. ED types were generated as for MLST sequence types: unique combinations of alleles across the *glc* loci, taken in gene order from *glcP* to *eda*, were assigned arbitrary numbers in order of discovery.

Genomic Analyses

Genomic analyses were carried out using the hierarchical gene-by-gene approach implemented in BIGSDB, which allows users to compare isolates at varying numbers of loci, depending on the level of resolution required (Jolley and Maiden, 2010; Maiden et al., 2013). Species assignments of all ED-positive isolates, and *C. coli* clade membership, were confirmed using rMLST (Jolley et al., 2012; Jansen van Rensburg et al., 2016). A maximum likelihood tree based on concatenated nucleotide sequences of the *glc* genes was reconstructed in MEGA version 6.06 (Tamura et al., 2013) using the General Time Reversible model with gamma-distributed rates with 500 bootstrap replicates. The resulting phylogenetic tree was annotated online using the Interactive Tree of Life version 3 (Letunic and Bork, 2016). Relationships among ED-positive isolates were established based on wgMLST analyses carried out using the GENOME COMPARATOR module implemented in the *C. jejuni*/*coli* PubMLST database (Jolley and Maiden, 2010). Following the exclusion of genomes in which ≥1% of the 1,343 *C. jejuni*/*coli* core genome scheme loci were incomplete (i.e., at the ends of contigs), relationships among *C. jejuni* and *C. coli* isolates were evaluated using wgMLST. Isolates were compared to the reference genomes of *C. jejuni* NCTC11168 (GenBank accession number AL111168) (Parkhill et al., 2000; Gundogdu et al., 2007) or *C. coli* 15-537360 (CP006702) (Pearson et al., 2013), using the default GENOME COMPARATOR settings. Distance matrices generated by GENOME COMPARATOR were visualized as networks using the NeighborNet algorithm (Bryant and Moulton, 2004) in SplitsTree version 4.13.1 (Huson and Bryant, 2006). Further analyses were carried out to compare the isolates B13117 and CV1257. The genome sequences of these isolates were annotated using Prokka version 1.0 (Seemann, 2014). The number of shared and unique coding sequences was estimated with Roary version 3.6.0 (Page et al., 2015) using the default settings. The capsule gene regions of these isolates were compared using ACT (Carver et al., 2005).

Phenotype Microarray Assays

BIOLOG™ phenotype microarrays were set up according to the manufacturer's description for *C. jejuni*. Briefly, individual isolates were cultivated overnight on Blood Agar Base No. 2 supplemented with 5% v/v bovine blood at 37°C in a microaerobic atmosphere. Cells were harvested from plates with 1.2x IF-0a solution (Biolog, Inc.), gently resuspended and adjusted to an optical density (600 nm) of 0.8. For each strain, 8 ml cell suspension was mixed with 12 ml 1.2x IF-0a (Biolog, Inc.), 0.24 ml Dye mix D (Biolog, Inc.), 6 mg ml^{-1} BSA, 1.26 mg ml^{-1} $NaHCO_3$, and 1.76 ml water. This mixture (0.1 ml) was added to the wells of PM1 phenotype microarray plates (Biolog), and the initial absorbance at 590 nm was read in an ELISA plate reader. Plates were incubated at 37°C in a microaerobic atmosphere and the absorbance read again following 24 and 48 h of incubation.

Biomass Dry-Weight Determination

Following 7 days of incubation at 37°C in a microaerobic atmosphere, *C. coli* cultures in glucose-free TSB with and without supplementation with 100 mM glucose were homogenized carefully by pipetting. Biomass was harvested from 5 ml culture by centrifugation and the pellets incubated overnight at 50°C for evaporation of water content. The dry weight of biomass was determined as the mean of four replicates.

Metabolite Analysis by Gas Chromatography–Mass Spectrometry

Campylobacter coli B13117 and CV1257 were cultivated at 37°C under microaerobic conditions in TSB with and without supplementation with 100 mM glucose. Three independent cultures for each strain and condition were grown; following 24 h of incubation, cells were harvested from 5 ml culture by centrifugation for 1 min at 8,000 × *g* at 0°C. The supernatants were filter sterilized (0.2 μm pore size) and stored at −20°C until analysis. A procedure based on the methyl chloroformate derivatisation protocol described by Smart et al. (2010) was used for the analysis of mixtures containing known metabolites with and without complexation. All samples were analyzed in a randomized order. The system was controlled by ChemStation (Agilent technologies). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported into Matlab R2014b (Mathworks, Inc., Natick, MA, USA) and processed using PARAFAC2 (Bro et al., 1999; Kiers et al., 1999) to obtain relative concentrations for each peak. PARAFAC2 was applied using in-house algorithms.

RESULTS

Carriage of the ED Pathway Genes Does Not Correlate with Ability to Grow in Blood

The key ED pathway genes were originally identified in the genome sequence of the human blood isolate *C. jejuni* subsp. *doylei* 269.97 (Miller, 2008). Since *C. jejuni* subsp. *doylei* is overrepresented in human cases of *Campylobacter* bacteremia (Lastovica, 2006; Parker et al., 2007) and given the millimolar concentrations of glucose in blood, we initially wanted to test the hypothesis that the ED pathway might be specifically associated with the growth or survival of *Campylobacter* found in blood. Therefore, a range of *Campylobacter* isolates was screened for the ED pathway using PCR amplification of the key genes (**Figure 1C**). This screen included five clinical isolates of *C. jejuni*

subsp. *doylei* (CCUG 18265, CCUG 24567, CCUG 26152, CCUG 26155, CCUG 36506), a *C. coli* bacteremia isolate (B13117), an in-house *C. coli* isolate of unknown origin (CV1257) and 10 *C. jejuni* subsp. *jejuni* bacteremia isolates (S38543, B10540, B345, B15426, B130, B17648, B19978, B3-11, B5682, B10053) (see Supplementary Table S1 for details). The screen revealed the presence of 6-phosphogluconate dehydratase (*edd*) and/or aldolase (*eda*) genes, encoding the key ED pathway enzymes, in three out of five clinical *C. jejuni* subsp. *doylei* isolates (CCUG24567, CCUG18265, CCUG26155), both *C. coli* isolates (B13117, CV1257), but not in any of the examined *C. jejuni* subsp. *jejuni* bacteremia isolates (**Figure 1C** and results not shown). These findings therefore do not support an association between blood culture isolates and the key genes of the *glc* locus. Analysis of WGS data for CCUG 24567, CCUG 18265, CCUG 26155, B13117, and CV1257 (Supplementary Table S2) confirmed that all isolates carried the complete *glc* locus.

In silico Identification of ED-Positive *Campylobacter* Isolates

The availability of assembled WGS data in the *C. jejuni*/*coli* PubMLST database facilitated large-scale *in silico* searches for additional ED-positive isolates. We found that 113 out of 6,184 isolates with genomic data contained one or more components of the *glc* locus. The *glc* locus did not assemble into a single contig in six isolates, all of which corresponded to draft genomes. Breaks occurred within the genes of interest, likely due to misassembly or lack of coverage; these isolates were excluded from further analyses. The complete *glc* locus was present on a single contig in the remaining 107 isolates (1.7% of the total) (Supplementary Table S2), which were collected in the UK ($n = 76$), Finland ($n = 30$), and the USA ($n = 1$).

Based on ribosomal multilocus sequence typing (rMLST) (Jolley et al., 2012), 70 and 37 isolates corresponded to *C. jejuni* and *C. coli*, respectively. At the population level, *C. coli* isolates segregate into three groups known as clades 1, 2, and 3 (Sheppard et al., 2008; Sheppard et al., 2010); the ED-positive *C. coli* isolates were all assigned to clade 1, except OXC7653, which belonged to clade 3. With respect to sources of isolation, *C. jejuni* isolates were predominantly from rats (51.4%) and wild birds (42.9%). In contrast, human disease samples accounted for 43.2% of *C. coli* isolates, while the remainder were from a diverse range of sources, including animals, food, and the environment (**Table 1**).

TABLE 1 | Sources of ED-positive isolates from the *Campylobacter jejuni*/*coli* PubMLST database.

Source	*n* (%)	
	C. jejuni	*C. coli*
Chicken	0 (0)	1 (2.7)
Environmental waters	0 (0)	4 (10.8)
Farm environment	2 (2.9)	1 (2.7)
Human disease	0 (0)	16 (43.2)
Other food	0 (0)	2 (5.4)
Pig	0 (0)	5 (13.5)
Rat	36 (51.4)	3 (8.1)
Soil	0 (0)	3 (8.1)
Unknown	2 (2.9)	2 (5.4)
Wild bird	30 (42.9)	0 (0)

TABLE 2 | Allelic diversity of components of the *glc* locus among 113 ED-positive *Campylobacter* isolates.

Gene	Number of alleles	Length of seed sequence	Range of allele lengths
glcP	20	1212	1200–1215
pgi2	22	1644	1644–1688
glk	19	999	999–1014
pgl	15	681	681
zwf	13	1401	1398–1401
edd	20	1803	1803
eda	12	624	624

Allelic Diversity of the *glc* Locus

The seven genes comprising the *glc* locus were annotated as described in Experimental Procedures, using the autotagger functionality implemented in PubMLST (Jolley and Maiden, 2010). Unique alleles identified for each gene were assigned arbitrary numbers in order of discovery. Between 12 (*eda*) and 22 (*pgi2*) alleles per *glc* locus gene were present among *C. jejuni* subsp. *doylei* 269.97, the five ED-positive isolates sequenced for this study, and those from the *C. jejuni*/*coli* PubMLST database (**Table 2**). Gene lengths ranged from 624 bp (*eda*) to 1803 bp (*edd*), and allele lengths were variable for all loci except *edd*, *eda*, and *pgl* (**Table 2**). Unique combinations of *glc* locus alleles, taken in gene order from *glcP* to *eda*, were summarized as ED types, which were assigned arbitrary numbers in order of discovery. Overall, 46 ED types were identified, all of which were species-specific: 36 were present in *C. jejuni* and 10 in *C. coli*. *C. jejuni* isolates were evenly distributed across ED types, with the exception of 18 closely related rat isolates that had been isolated from four different farms between 2011 and 2012 and which all carried ED type 2 (ED allelic profile 3-4-4-5-4-5-5). Interestingly, these findings with *C. jejuni* contrasted with a more limited allelic diversity observed for *C. coli* isolates, amongst which ED type 1 (3-5-7-9-5-9-9) was predominant, accounting for 27/37 (73%) of PubMLST isolates and CV1257. Phylogenetic analysis of concatenated *glc* gene sequences indicated that ED types segregated into three groups, with *C. jejuni* ED types occurring in all three (**Figure 2**). There was little diversity among *C. jejuni* sequences from groups I and III, which primarily corresponded to isolates obtained from wild birds or rats, respectively. Sequences belonging to group II were more diverse and included the known *C. jejuni* subsp. *doylei* isolates. With the exception of clade 3 isolate OXC7653, *C. coli* sequences were only found in group III and were highly homogeneous (**Figure 2**).

Genomic Analyses of ED-Positive Isolates

Relationships among ED-positive isolates were examined using whole-genome multilocus sequence typing (wgMLST). Seven isolates were excluded from these analyses as 1–16.5% of

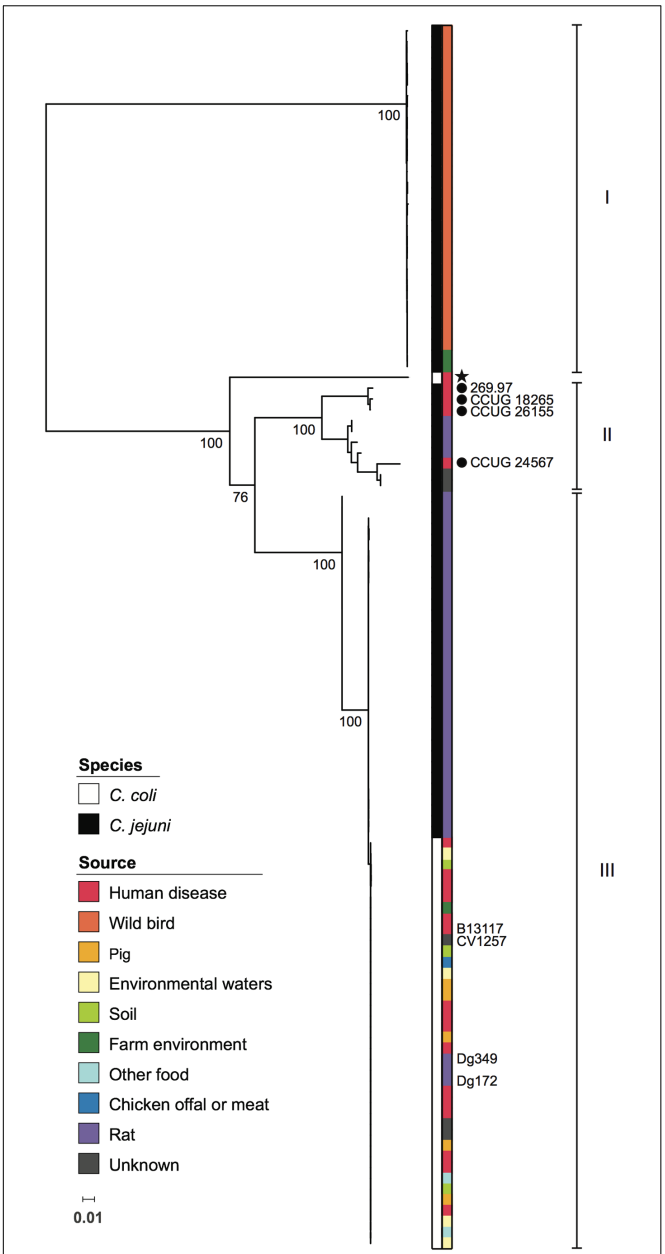

FIGURE 2 | Phylogenetic relationships among *Campylobacter glc* loci. Maximum likelihood tree based on concatenated nucleotide sequences of genes comprising the *glc* locus, encoding the Entner-Doudoroff pathway, from 113 *Campylobacter* isolates. Colored strips adjacent to the phylogeny indicate species and source as shown in the inset legends. Known *C. jejuni* subsp. *doylei* isolates are indicated with filled circles. All *C. coli* isolates were assigned to *C. coli* clade 1, with the exception of a single clade 3 isolate, which is marked with a star. Isolates included in experiments carried out in this study are labeled. Roman numerals indicate groups of *glc* sequences referred to in the text. For major nodes, bootstrap values generated from 500 replicates are shown as percentages. The scale bar represents the number of nucleotide substitutions per site.

loci belonging to the *C. jejuni*/*coli* core genome scheme were incomplete (i.e., at ends of contigs). With respect to *C. jejuni*, wild bird, rat, and known *C. jejuni* subsp. *doylei* isolates largely occupied distinct parts of the network that correlated with membership of the three ED nucleotide sequence groups (**Figures 2** and **3**). Members of these groups were genetically diverse and were separated by an average of 1,294 loci, with the exception of small clusters of closely related isolates, particularly among those obtained from farm-associated rats (**Figure 3A**). Isolates belonging to these clusters were typically separated by fewer than 100 loci and the majority carried ED type 2. Although *C. coli* isolates were also genetically diverse, they were separated by shorter distances, averaging 973 loci (**Figure 3B**). While the majority carried ED type 1, these isolates did not group by source or ED type.

Glucose Supports Growth of *Campylobacter* Isolates Harboring the ED Pathway

The presence of the ED pathway genes within the subset of *Campylobacter* isolates studied here indicates that these isolates should be able to utilize glucose. To confirm this, a Biolog^{TM} phenotypic microarray was initially used to examine the potential metabolism of various carbohydrates by the ED-positive isolates *C. coli* B13117, *C. coli* CV1257 and *C. jejuni* subsp. *doylei* 269.97 in comparison to the widely used lab strain *C. jejuni* NCTC11168, which does not carry the ED pathway. With the Biolog^{TM} system, substrate uptake and metabolism is detected as stimulation of bacterial respiration and quantitated colorimetrically via tetrazolium dye reduction (Bochner et al., 2001). This revealed a correlation between the presence of the ED pathway and metabolism of glucose, as glucose was seen to stimulate respiration of the ED-positive *C. jejuni* subsp. *doylei* 269.97, *C. coli* B13117 and *C. coli* CV1257, while no signal was observed for *C. jejuni* NCTC11168. In contrast, L-fucose was seen to stimulate respiration of all four isolates regardless of the presence of the ED pathway (Supplementary Figure S1).

To investigate if glucose is able to support growth of *C. coli* isolates carrying the ED pathway, the growth of four ED-positive *C. coli* isolates representing three different sequence types (B13117, CV1257, Dg172, and Dg349) was examined in comparison to an ED-negative strain (OXC6725; PubMLST id 18282). Of these isolates, CV1257, Dg172, and Dg349 all had ED type 1 (the same as in the isolates used by Vorwerk et al., 2015), while B13117 carried ED type 45, which only differed by a single synonymous substitution in *pgi2* (471: T- > C). The isolates were grown in minimal media (MCLMAN) without and with glucose supplementation as the main carbon source; a low concentration of L-aspartate (2 mM) was included as a nitrogen source as this was found to greatly aid growth. Following overnight incubation at 37°C under microaerobic conditions, the viable cell count of each of the ED-positive isolates had increased significantly with glucose (by ∼3 logs), while no such increase in cell density was seen for either the ED-negative strain or any of the cultures without glucose supplementation (**Figure 4**). These data show a clear correlation between possession of the ED pathway in these *C. coli* isolates and their growth on glucose.

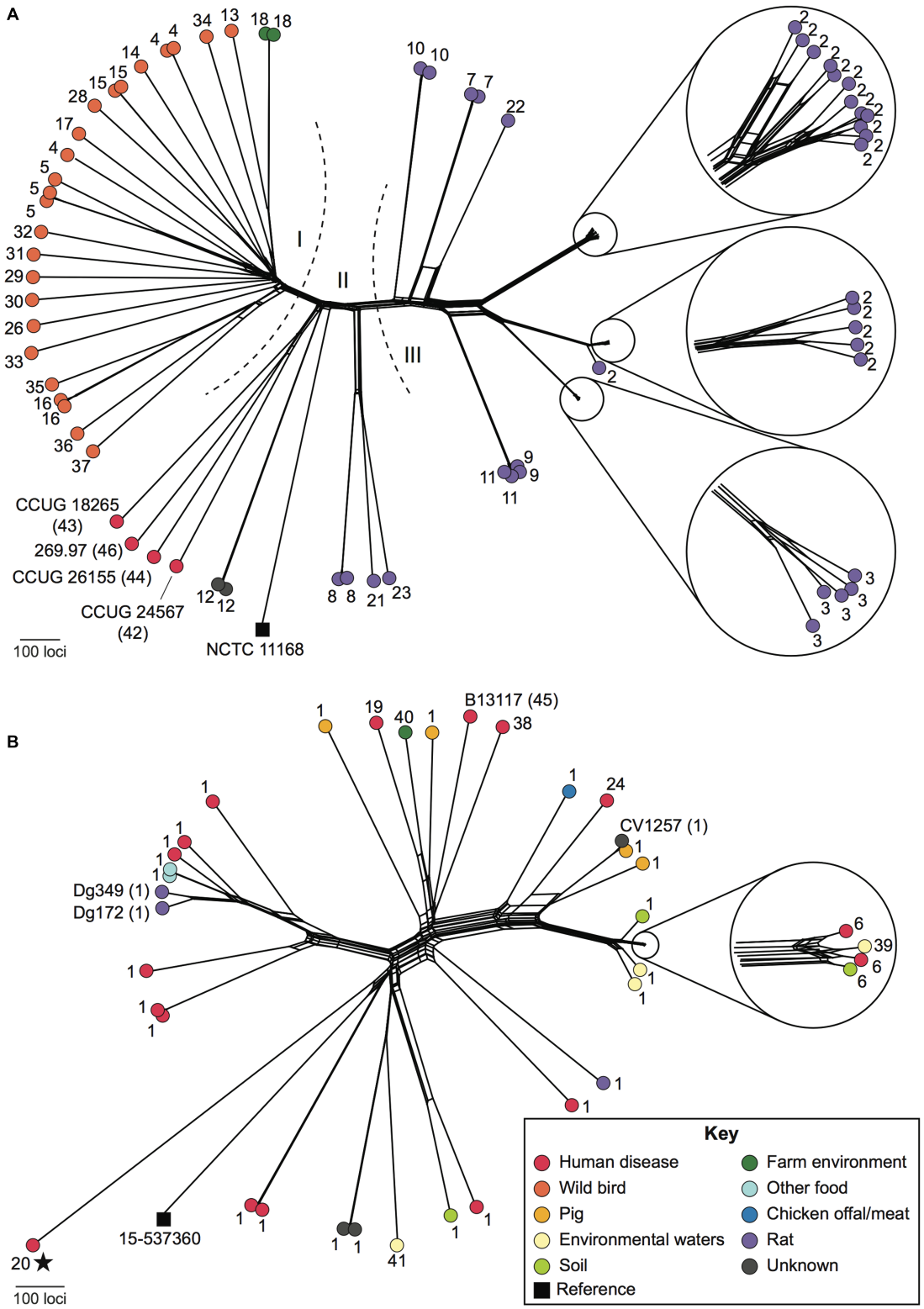

FIGURE 3 | Relationships among ED-positive *Campylobacter* isolates. NeighborNet graphs were generated based on whole-genome multilocus sequence typing (wgMLST) comparisons of 68 *C. jejuni* **(A)** and 38 *C. coli* **(B)** isolates, using NCTC11168 and 15-537360 (black squares), respectively, as reference genomes. ED allele types are shown as numbers adjacent to each isolate. Isolates included in experiments carried out in this study are labeled in full with the ED type in parentheses. Dashed lines and Roman numerals indicate groups of isolates with related ED types referred to in the text. The color of the filled circles indicates the source of each isolate as shown in the key (inset). All *C. coli* were assigned to *C. coli* clade 1 except for a single clade 3 isolate, which is marked with a star.

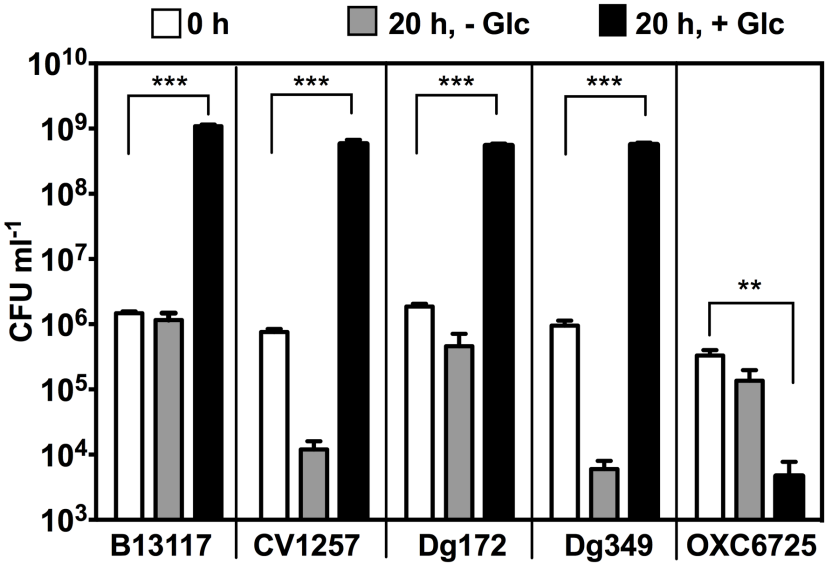

FIGURE 4 | Glucose stimulates growth of ED-positive but not ED-negative *C. coli* isolates. Four ED-positive *C. coli* isolates (B13117, CV1257, Dg172, and Dg349) and an ED-negative control *C. coli* strain (OXC6725) were inoculated into modified MCLMAN minimal media either without or with glucose supplementation. Cultures were incubated for 20 h at 37°C under microaerobic conditions and viable cell numbers measured by plate counts. White bars, colony forming units (CFU) ml^{-1} of inoculum at time 0. Light gray bars, final CFU ml^{-1} in modified MCLMAN without glucose. Black bars, final CFU ml^{-1} in modified MCLMAN supplemented with 100 mM glucose. The bars show the mean and the error bars show the standard deviations of three independent cultures. Statistical significance is indicated by (**$p < 0.01$) or (***$p < 0.001$) as determined by Student's *t*-test.

Extended Stationary Phase Survival and Strain-Specific Biofilm Production with Glucose

In order to examine the physiological impact of glucose metabolism by *C. coli*, the growth of ED-positive isolates was further examined in the rich medium tryptic soy broth (TSB) without and with glucose supplementation. Here, a range of carbon sources (mainly amino-acids and peptides), are available in addition to the added glucose. At 37°C in a microaerobic atmosphere, the exponential growth rates of B13117 and CV1257 were seen to be unaffected by the availability of glucose (Supplementary Figure S2). After 12 h of incubation, however, the viability of the un-supplemented cultures declined significantly. In contrast, viable cell numbers were sustained or slightly increased in the glucose-supplemented cultures (Supplementary Figure S2), hence, suggesting glucose might support extended viability and stationary survival of these ED-positive isolates, at least up to 24 h.

Extending the growth experiments with prolonged incubation time over several days and including four ED-positive *C. coli* isolates (B13117, CV1257, Dg172, and Dg349) and an ED-negative control *C. coli* strain (OXC7218; PubMLST id 22709), revealed that glucose did indeed support significant extended survival of the ED-positive isolates (**Figure 5**). When cultivated with glucose at 37°C in a microaerobic atmosphere, the ED-positive isolates B13117, CV1257, Dg172, and Dg349 displayed at least 10 times higher viable cell numbers after 4 and 7 days as compared to cultures without glucose, while no such effect of glucose could be seen with the ED-negative strain OXC7218 (**Figure 5**). These data show a major impact of glucose on the late stationary phase survival of ED-positive *C. coli* isolates under otherwise nutrient rich conditions.

In the extended incubation experiments, it was observed that glucose stimulated biofilm formation by some ED-positive isolates of *C. coli*. Substantial floating biofilm (flocs or a pellicle), i.e., biofilm unattached to a surface, was observed in the glucose-supplemented late stationary cultures of B13117 and Dg349, and this aggregation was in fact so viscous that it was hardly dispersible by simple pipetting (**Figure 6A**). However, extensive biofilm formation was not observed in either the glucose-free cultures of B13117 and Dg349 or any of the late stationary cultures of the two other ED-positive isolates CV1257 and Dg172 in the presence of glucose. The ED-negative strain OXC7218 displayed a low level of aggregation regardless of the glucose availability (**Figure 6A**). Note that the biofilm produced by *C. coli* B13117 and Dg349 is floating, i.e., unattached to a solid surface, and therefore it was not possible to quantify using traditional dye staining assays for staining of attached biofilms. Consequently, the contribution of the biofilm to the total cell biomass was quantified using the dry weight of culture aliquots following 7 days of incubation with and without glucose (**Figure 6B**). As expected, this showed a significant increase in the biomass of all the ED-positive isolates upon glucose supplementation, while the biomass of the ED-negative strain was unaltered regardless of glucose availability. However, the biomass of B13117 and Dg349 grown with glucose was clearly much greater than that of CV1257 and Dg172 with glucose (**Figure 6B**, arrows), which is in

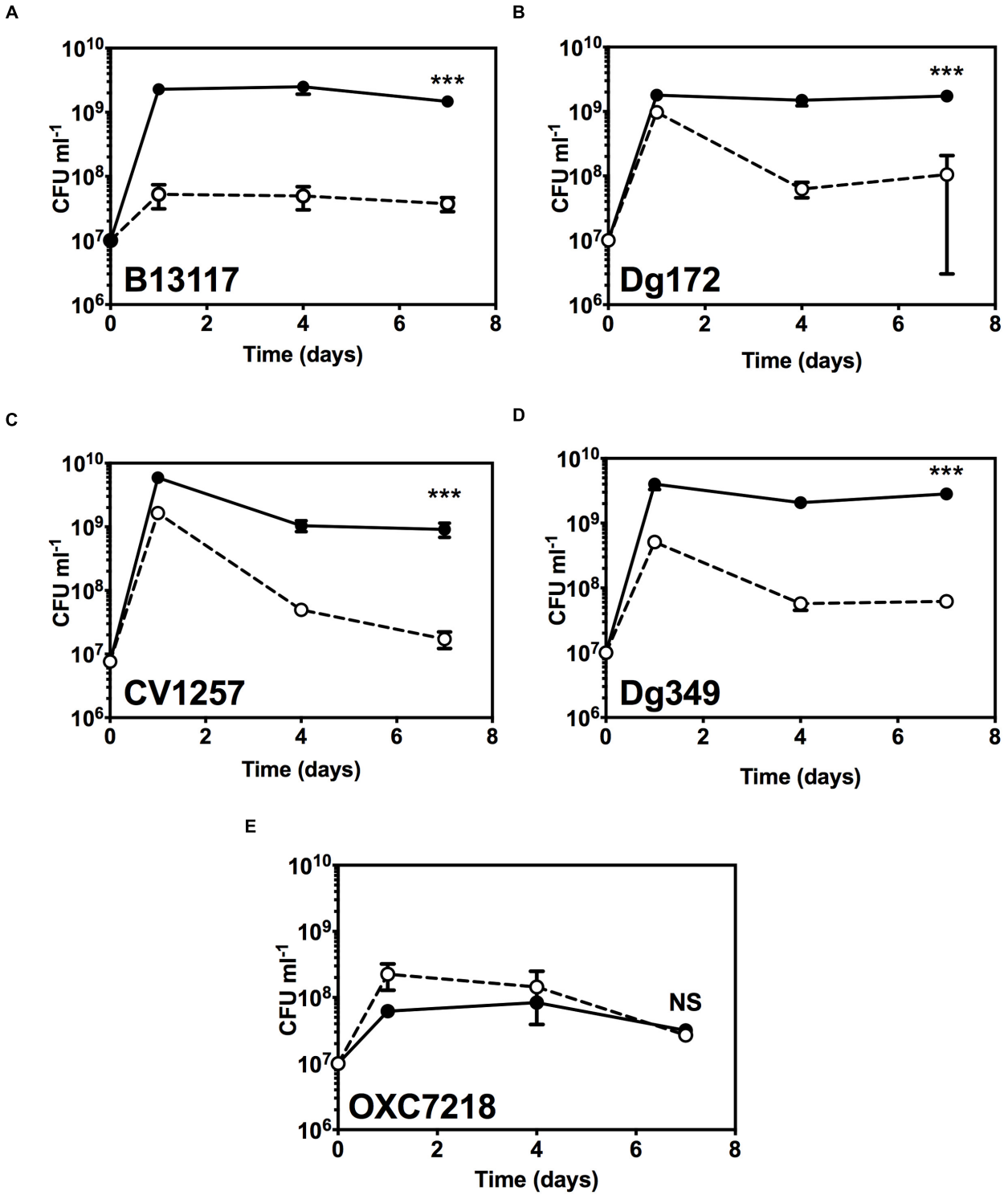

FIGURE 5 | Extended stationary phase survival with glucose. Viable cell numbers of ED-positive *C. coli* isolates (B13117, CV1257, Dg172, and Dg349) and an ED-negative control *C. coli* strain (OXC7218) were determined in TSB with or without supplementation with 100 mM glucose. Cultures were incubated for 7 days at 37°C in microaerobic conditions and the viability determined by enumerating CFU at various time intervals. Results are mean and standard deviations of three independent cultures. The error bars are too small to be seen in some cases. **(A)** *C. coli* B13117; **(B)** *C. coli* CV1257; **(C)** *C. coli* Dg172; **(D)** *C. coli* Dg349; **(E)** *C. coli* OXC7218 (ED-negative control). Open circles and dashed line, cell numbers without glucose; filled circles and solid line, cell numbers with glucose. The statistical significance of the difference between the control without glucose and with added glucose at 7 days was tested by Student's *t*-test, as indicated by (***$p < 0.001$) or NS (not significant).

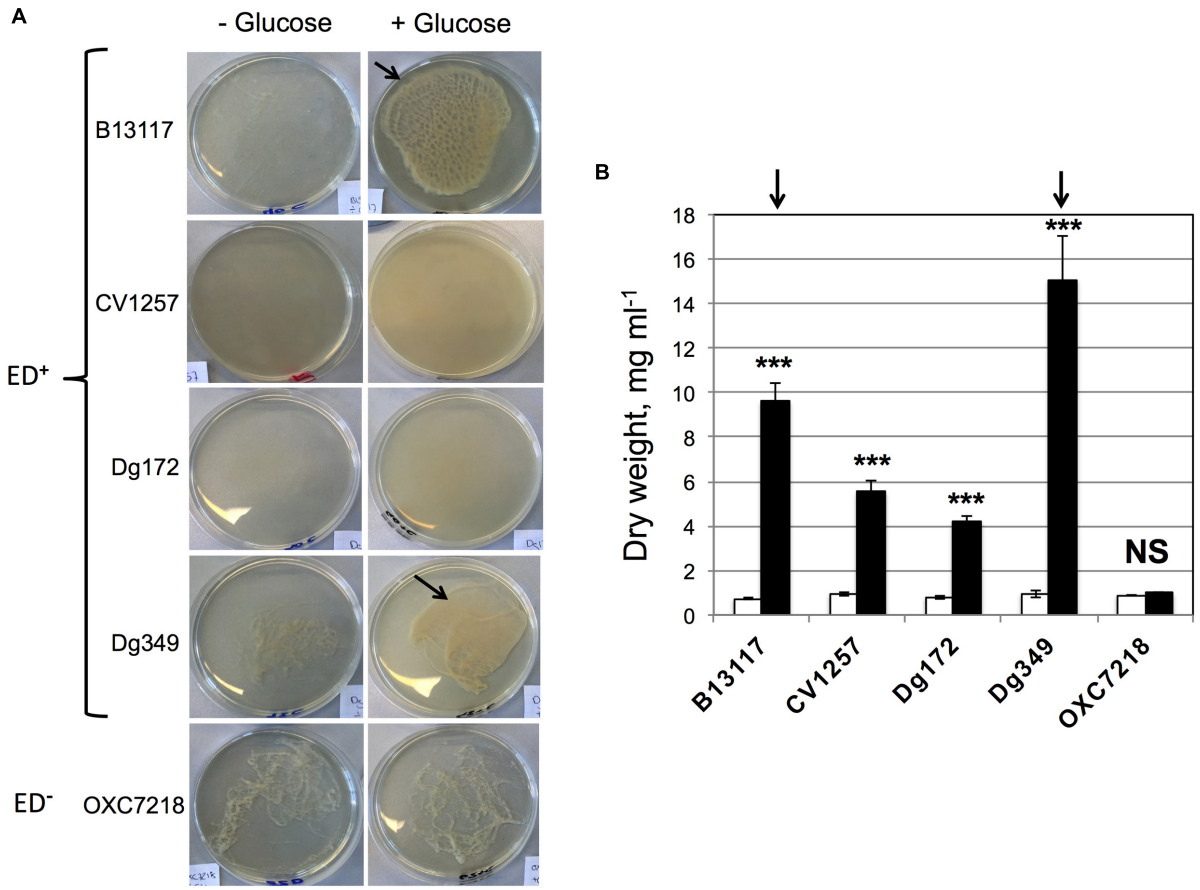

FIGURE 6 | Glucose stimulates production of floating biofilm that increases cell biomass. (A) The isolates of *C. coli* shown were cultivated for 4 days at 37°C under microaerobic conditions in glucose-free TSB with and without supplementation with 100 mM glucose. The glucose-supplemented cultures of B13117 (ED+) and Dg349 (ED+) formed extensive floating biofilm (flocs; *arrowed*). CV1257 (ED+) and Dg172 (ED+) displayed denser growth with glucose but no biofilm formation, while a low level of aggregation was seen in the cultures of ED-negative OXC7218 regardless of glucose-supplementation. Pictures are representative cultures from four independent growths. **(B)** The four ED-positive *C. coli* isolates (B13117, CV1257, Dg172, and Dg349) and the ED-negative *C. coli* strain OXC7218 were cultivated for 7 days at 37°C under microaerobic conditions in glucose-free TSB with or without glucose supplementation. The bars show dry weight biomass of 5 ml aliquots from cultures without glucose (white bars) and with glucose supplementation (black bars). The mean and standard deviations of four independent cultures for each condition are shown. The statistical significance of the difference between the dry weight without glucose and with added glucose was evaluated by Student's *t*-test, as indicated by (***$p < 0.001$) or NS (not significant). The arrows refer to the biofilm producing strains B13117 and Dg349 as in **(A)**, which clearly produce more biomass with glucose compared to the other non-biofilm producing strains tested.

agreement with the visually observed high biofilm production by these isolates (**Figure 6A**, arrows).

Exometabolic Profiling of ED-Positive Isolates Grown with and without Glucose

Campylobacter coli B13117 and CV1257 were subjected to an exometabolomics "footprint" analysis to compare the extracellular metabolic profiles of these ED-positive isolates while growing in TSB with and without glucose. Changes in the media composition after growth, reflecting the metabolic activity of the bacteria (i.e., production and/or consumption of metabolites and changes in growth substrates) were evaluated using a validated method based on gas chromatography–mass spectrometry (GC–MS) analysis of methyl chloroformate derivatised samples (Smart et al., 2010) which primarily identifies amino-acids and organic acids. In the principal component analysis (PCA) shown in **Figure 7A**, it is clear that the footprints of B13117 and CV1257 are highly similar when the isolates are cultivated in TSB without glucose, as these samples group closely together in the same region of the PCA plot (**Figure 6A**). However, upon glucose supplementation, the samples from the two isolates group in opposite quadrants of the plot (**Figure 6A**), indicating a substantial but very dissimilar response to glucose. A heat map, representing the relative concentrations of the compounds detected by GC–MS, is shown in **Figure 7B**. The largely similar extracellular metabolic profiles of B13117 and CV1257, when cultivated in TSB without glucose, are clear from the overall pattern seen in the heat map profile. After growth with glucose a clear difference between the two isolates in their responses in terms of metabolite concentrations was observed (**Figure 7B**). Using known reference standards, some

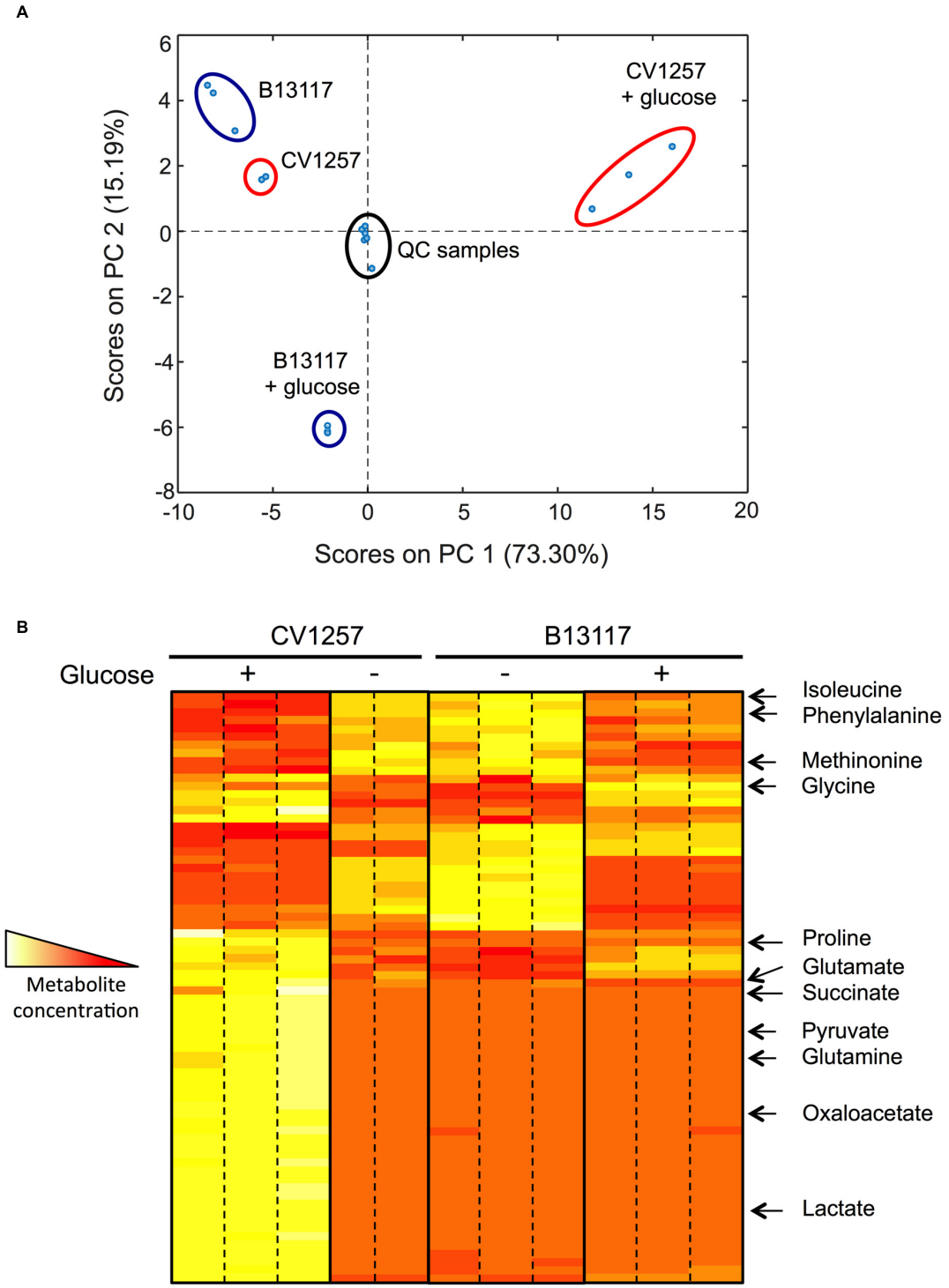

FIGURE 7 | Glucose dependent changes in extracellular metabolic profiles of B13117 and CV1257 isolates. (A) Principal components analysis (PCA) plots of extracellular metabolites determined by gas chromatography–mass spectrometry (GC–MS). The PCA score plot is based on a data set consisting of three independent replicates of *C. coli* B13117 and CV1257 cultivated in TSB with and without glucose for 24 h at 37°C in a microaerobic atmosphere. However, only two replicates of CV1257 without glucose were used due to contamination. For quality control, mixed pooled samples (labeled QC samples) were run alongside the individual separate samples. **(B)** Heat map representation of the most abundant extracellular metabolites of *C. coli* B13117 and CV1257 samples used in **(A)**. When cultivated without glucose the two isolates display very similar metabolite profiles (two middle panels). Upon glucose supplementation both isolates display a distinct shift in metabolic profile (two outer panels), but the two isolates respond very differently to glucose metabolism, which is seen by the highly different overall profile patterns with glucose. Compounds that could be identified by comparison to standards are shown; most compounds that could be detected were not identified. The colors represent the relative concentrations from high (white) to low (red).

of these extracellular compounds could be identified. From the results, it seems that glucose stimulates catabolism more strongly in CV1257, since the extracellular concentration of several metabolites both from and closely related to the citric-acid cycle (e.g., succinate, pyruvate, oxaloacetate, lactate) was found to increase when this strain was cultivated with glucose as compared to a glucose-free medium, while the amino-acids isoleucine, phenylalanine, and methionine decreased (**Figure 7B**). The metabolic profile of B13117 also displayed a clear shift upon cultivation with glucose, with certain amino-acids from the growth medium again decreasing. However, far fewer glucose-dependent extracellular metabolite alterations of B13117 were identified with the GC–MS analysis, and in particular there was little change in citric-acid cycle related organic acids (**Figure 7B**). Given the strong tendency of the B13117 strain, but not CV1257, to produce biofilm upon cultivation with glucose (**Figure 6**), the distinct metabolic profile compared to CV1257 is consistent with glucose carbon being preferentially directed into polysaccharide formation in the former isolate.

Genomic Comparison of B13117 and CV1257

In addition to metabolic differences in the B13117 and CV1257 isolates, we also searched for possible differences in gene content that might be related to polysaccharide formation, in particular in the capsule biosynthesis loci. A total of 1,996 unique coding sequences were identified between B13117 and CV1257, 1,618 of which were present in both isolates. Among the remaining coding sequences, 204 and 174 were specific to B13117 and CV1257, respectively. Differences in the capsule biosynthesis loci accounted for 35 (9.3%) of the isolate-specific genes, with the region being approximately 5.6 kb longer in B13117 (Supplementary Figure S3). Although the flanking genes shared >90% sequence identity, the intervening sequences were distinct (Supplementary Figure S3). Some of the additional genes in this region in B13117 have predicted functions in polysaccharide biosynthesis (Supplementary Table S3). With respect to the remaining isolate-specific genes, 174 corresponded to hypothetical proteins.

DISCUSSION

Glucose utilization by *Campylobacter* strains has only recently been discovered, and it remains of uncertain physiological, pathogenic and environmental significance. Taken together, the results of this study illustrate that while the ED pathway is indeed present in diverse isolates in the *Campylobacter* genus, overall it is uncommon, as judged by the presence of the complete *glc* locus in only 1.7% of over 6,000 genomes analyzed. Importantly, we did not find a correlation between possession of the key ED pathway genes and the ability to cause bloodstream infections, which might have been expected given the abundance of glucose in this niche. However, from our genome sequence analyses, it is clear that the ED pathway is present in both *C. jejuni* and *C. coli* isolates and we believe that this is the first report of this pathway in *C. jejuni* subsp. *jejuni*. The previous study of Vorwerk et al. (2015) employed a limited number of human disease and pig *C. coli* isolates, while we have shown that the *glc* locus is clearly conserved among *C. coli* from more diverse sources. The majority of genomes in the *C. jejuni*/*coli* PubMLST database were from human disease cases, and, as humans typically become infected due to consumption of contaminated animal products (Kaakoush et al., 2015), the isolates analyzed effectively comprised an agricultural sample. This raises questions about the advantages to *C. coli* of glucose utilization in agricultural hosts. It is known that the *glc* locus forms a plasticity region that can be transferred by natural transformation between isolates (Vorwerk et al., 2015), so it is likely that the region has been gained or lost repeatedly in different isolates possibly in response to the differing availability of glucose within distinct hosts or niches.

The genes comprising the *glc* locus were most variable among genetically diverse *C. jejuni* isolates that were primarily not from agricultural sources, but were from rats and wild birds and also corresponded to *C. jejuni* subsp. *doylei*, the natural reservoir of which remains unknown. Identification of clusters of closely related *C. jejuni* from rats that shared identical ED types was likely due to sampling: isolates with ED type 2 were recovered primarily from two different farms over 18 months, while four of the five isolates with ED type 3 were recovered from a third farm over the same period (MacIntyre, in preparation). In contrast, although *C. coli* isolates were also genetically diverse at the wgMLST level, the *glc* locus was less variable in these isolates, 73% of which corresponded to ED type 1. This difference in *glc* diversity between *C. jejuni* and *C. coli* may be related to when the region was gained or lost with respect to the timing of divergence of *Campylobacter* species/subspecies, or to host differences, or to sparse sampling of *C. coli* from non-agricultural sources. Whether this has any physiological consequences in terms of overall pathway activity or specific enzyme activity will require further investigation. There were also slight differences in the gene contents of the *glc* locus, with wild bird isolates carrying an additional ∼2700 bp downstream of *glcP*. Further work is needed to understand the significance of this with respect to the function of the pathway as a whole.

We have shown for the first time that individual glucose metabolizing isolates can show distinct physiological responses to the availability of this substrate, which suggests that glucose can act as more than just an energy source for campylobacters. What are the additional specific physiological advantages of the possession of the ED pathway by a subset of *Campylobacter* isolates? The production of NADPH by glucose-6-phosphate dehydrogenase may be particularly significant, as this is not only required for anabolic enzyme reactions, but also supplies reductant for oxidative stress protection, mainly via the thioredoxin system (Lu and Holmgren, 2014). In the oxygen-sensitive microaerophilic campylobacters, this system (NADPH + thioredoxin reductase + thioredoxin) is crucial, as it supplies electrons to the peroxiredoxins AhpC, Tpx and Bcp, each of which has been shown to protect against oxidative stress by removing damaging peroxides (Baillon et al., 1999; Atack et al., 2008). Thioredoxin also reduces the cytoplasmic methionine sulfoxide reductases MsrA and MsrB, which repair oxidized

proteins (Atack and Kelly, 2008). Thus, ED-positive isolates may be better able to cope with oxidative stress than ED-negative isolates; this has been shown to be the case experimentally in *Pseudomonas putida* (Chavarria et al., 2013) and in a range of marine bacteria (Klingner et al., 2015). Secondly, the absence of Pfk but the presence of the remaining EMP pathway enzymes in campylobacters means that the ED reactions are not "linear." Instead, the metabolism of glucose in ED-positive isolates could occur via the "cyclic" fusion of the ED and EMP reactions, as has been shown in *Pseudomonas* spp., which interestingly also lack Pfk (Nikel et al., 2015). As suggested for pseudomonads, the physiological rationale for this might be related to an enhanced hexose phosphate supply; this would be particularly important for LOS, capsule and polysaccharide formation in campylobacters.

In the Biolog^{TM} assays and liquid batch culture growth experiments in minimal media, we confirmed that glucose utilization and glucose-stimulated growth, respectively, occurred in several ED-positive but not ED-negative isolates. This was as expected and is in agreement with the findings of Vorwerk et al. (2015). However, we found that addition of glucose to the ED-positive isolates in rich, complex media, afforded a significant stationary phase survival benefit, in terms of the maintenance of viability over several days compared to the absence of glucose. This effect is likely a product of the multiple functions of the ED pathway in energy conservation, NADPH production and hexose phosphate generation, which may allow better environmental resilience. Moreover, in further physiological studies on glucose utilization in rich complex media, we found some unexpected differences between the ED-positive isolates with regard to biofilm formation. It was clear from the dry weight measurements and visual appearance of the cultures that glucose utilization led to a massive increase in a pellicle or floating biofilm in isolates like B13117 and Dg349 but not CV1257 and Dg172, which suggested a fundamental difference in the way in which glucose is being metabolized in these isolates. This conclusion was supported by the metabolic profiling analysis we carried out using GC–MS of broth culture supernatants, where we could distinguish a distinct metabolic footprint when comparing the high biofilm forming B13117 strain with the low biofilm forming CV1257. In the latter strain only, several citric-acid cycle organic acids and also lactate were increased in the culture supernatants in a glucose dependent manner. Our conclusion is therefore that the ED pathway in CV1257 primarily feeds glucose into the primary metabolism of this strain. In contrast, B13117 may primarily utilize the ED encoded glucose kinase (Glk) and possibly the EMP gluconeogenic reactions, for conversion of glucose to glucose-6-phosphate and/or fructose-6-phosphate which could be used for the production of surface structures such as capsule or free polysaccharide. This would be consistent with the observed massive increase in biofilm production by this strain when cultivated with glucose.

Although, future analysis of gene expression patterns and *in vivo* activities of ED and EMP pathway enzymes will be required to explain the molecular basis for the different metabolic responses to glucose in B13117 and CV1257, we found differences in gene content in these isolates that might also contribute to the differences in biofilm formation, with 378 isolate-specific coding sequences identified. We speculate that some of these might be involved in polysaccharide synthesis specifically related to biofilm formation. For example, the capsule biosynthesis locus is ∼5.6 kB longer in B13117, and, although the flanking regions are conserved, the intervening sequences are distinct (Supplementary Figure S3). For example, there are several distinct *rbf* genes in B13117: glucose-1-phosphate cytidylyltransferase (*rfbF*), CDP-glucose 4,6-dehydratase (*rfbG*), and CDP-abequose synthase (*rbfJ*), that encode enzymes of polysaccharide synthesis, which might contribute to the observed biofilm formation (Supplementary Table S3). The role of these and other polysaccharide biosynthesis genes, biochemical analysis of the biofilm polymer together with further detailed analysis of gene function and regulation and enzyme activities will elucidate the different phenotypes of these isolates seen in the presence of glucose. It would also be informative to determine how common the glucose stimulated survival and biofilm phenotypes are by screening a much larger number of isolates than was possible in this study.

Finally, there is overwhelming evidence showing that biofilm production is of prime importance in many aspects of the biology of *Campylobacter* isolates, particularly protection against environmental stress (e.g., Svensson et al., 2009; Haddock et al., 2010; Bae and Jeon, 2013; Pascoe et al., 2015) and that it is affected by multiple external factors such as medium composition, osmolarity and oxygen availability (Reeser et al., 2007; Reuter et al., 2010). Host signals also play a role; a recent study showed that *C. jejuni* 11168 can produce a glucan biofilm composed of α-dextran as a specific response to the presence of host pancreatic amylase (Jowiya et al., 2015). In contrast to the clear stimulatory effect of glucose on biofilm formation we found here, the only other sugar known to be metabolized by campylobacters, L-fucose, was very recently shown to *reduce* biofilm formation in the *C. jejuni* NCTC 11168 strain (Dwivedi et al., 2016). The reduction was dependent on L-fucose transport and metabolism and the authors speculated that L-fucose might be an intestinal signal to maintain cells in a planktonic state. Taking our data together with the findings of Dwivedi et al. (2016) it is now clear that these two related hexose sugars are not only metabolized very differently, but they also play very different roles in modulating the crucial biofilm response of campylobacters.

AUTHOR CONTRIBUTIONS

CV, JR, LJ, MD, designed and executed experiments and analyzed the data. MJ performed the sequencing and bioinformatics analyses. CV and DK wrote the manuscript. MJ, MM, SM, HI, LJ, MD, and DK critically evaluated and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01877/full#supplementary-material

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Characterisation of Aerotolerant Forms of a Robust Chicken Colonizing *Campylobacter coli*

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Campylobacter contaminated poultry meat is a major source of human foodborne illness. *Campylobacter coli* strain OR12 is a robust colonizer of chickens that was previously shown to outcompete and displace other *Campylobacter* strains from the chicken's gastrointestinal tract. This strain is capable of aerobic growth on blood agar. Serial aerobic passage increased this aerotolerance as assessed by quantitative assays for growth and survival on solid media. Aerotolerance was also associated with increased peroxide stress resistance. Aerobic passage did not alter cellular morphology or motility or hinder the microaerobic growth rate. Colonization of broiler chickens by aerotolerant *C. coli* OR12 was significantly lower than the wild-type strain at 3 days after challenge but not by 7 days, suggesting adaptation had occurred. Bacteria recovered from chickens had retained their aerotolerance, indicating this trait is stable. Whole genome sequencing enabled comparison with the wild-type sequence. Twenty-three point mutations were present, none of which were in genes known to affect oxidative stress resistance. Insertions or deletions caused frame shifts in several genes including, phosphoglycerate kinase and the b subunit of pyruvate carboxylase that suggest modification of central and carbohydrate metabolism in response to aerobic growth. Other genes affected include those encoding putative carbonic anhydrase, motility accessory factor, filamentous haemagglutinin, and aminoacyl dipeptidase proteins. Aerotolerance has the potential to affect environmental success and survival. Increased environmental survival outside of the host intestinal tract may allow opportunities for transmission between hosts. Resistance to oxidative stress may equate to increased virulence by virtue of reduced susceptibility to oxidative free radicals produced by host immune responses. Finally, resistance to ambient atmospheric oxygen may allow increased survival on chicken skin, and therefore constitutes an increased risk to public health.

Keywords: *Campylobacter coli*, aerotolerant *Campylobacter*, chicken intestinal colonization, oxidative stress, *Campylobacter* survival, food safety

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INTRODUCTION

Campylobacter spp. have been the most commonly reported gastrointestinal pathogens in the EU since 2005, with 229,213 confirmed cases in 2015, representing 65.5 per 100,000 of population (EFSA, 2016). England and Wales reported 65,032 laboratory confirmed cases of campylobacteriosis in 2012 (PHE, 2013). This is a significant underestimate due to under-diagnosis and under-reporting. A recent study estimated that in 2009 there were 280,400 cases

of campylobacteriosis in the UK, accounting for 38,860 general practitioner consultations and 562 hospital admissions (O'Brien et al., 2016). The economic costs associated with *Campylobacter* infections over the same period was estimated to be £50 million (Tam and O'Brien, 2016). The majority of these cases are associated with *Campylobacter jejuni* infection but it is estimated that *C. coli* represents 7% of the cases in the UK (Gillespie et al., 2002).

The primary feature of disease is diarrhea, which can be accompanied by abdominal pain, fever, dysentery, vomiting, or prostration. Most cases are self-limiting with duration of illness varying from days to weeks. Illness can be more severe or life threatening in the young, old and immunocompromised (Blaser and Engberg, 2008). Of the 229,213 reported campylobacteriosis cases in the EU in 2015, 19,302 required hospitalization (31.2%), and there were 59 deaths (0.03%) (EFSA, 2016). In addition to the acute gastrointestinal disease, *Campylobacter* infection can be associated with a number of severe post-infectious complications. The most significant of these are believed to be Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome, and inflammatory bowel disease (WHO, 2013). Many other sequelae are described including: pancreatitis, septic abortion, cholecystitis, nephritis, peritonitis, and myocarditis (Blaser and Engberg, 2008).

Broiler meat is considered to be the main source of human campylobacteriosis (Wilson et al., 2008; Sheppard et al., 2009), with undercooking or cross contamination likely to be the main routes of infection (EFSA, 2016). Consumption of undercooked or contaminated poultry meat is the largest single risk factor for sporadic infection, and other sources are described such as milk, water, other meats, and contact with pet or farm animals (Jacobs-Reitsma et al., 2008). *Campylobacter* colonization is widespread throughout poultry flocks. A recent European Food Safety Authority baseline survey found 75.3% of UK broiler flocks to be positive for *Campylobacter* (EFSA, 2010). In 2014, 76% of 497 chicken neck skin samples collected from UK slaughterhouses tested positive for *Campylobacter* (EFSA, 2015). Caecal or crop contents of broilers at slaughter can contain up to 10^9 or 10^5 CFU/g, respectively, presenting a huge potential for the contamination of meat during evisceration (van Gerwe et al., 2010).

Resistance to oxidative stress is essential for *C. jejuni* colonization of the gastrointestinal tract (Hermans et al., 2011). Common sources of oxidative free radicals include the organisms' own respiratory metabolism, the ambient atmosphere in the extra-intestinal environment, and reactive oxygen species (ROS) generated by phagocytic or cytotoxic cells involved in the host immune response (Atack and Kelly, 2009). ROS damage DNA, proteins and lipids, limiting growth or killing the bacteria (Flint et al., 2016a). *C. jejuni* lacks several of the key oxidative stress response regulators identified in *Escherichia coli*, such as SoxRS, OxyR or Crp, and whereas *E. coli* possesses three separate superoxide dismutase and two catalase enzymes, *C. jejuni* encodes only one of each (Parkhill et al., 2000; Imlay, 2013; Flint et al., 2016b).

The three canonical enzymes involved in oxidative stress resistance in *C. jejuni* are superoxide dismutase, catalase, and alkyl hydroperoxide reductase. A superoxide dismutase (*sodB*) deficient mutant of *C. coli* had diminished environmental survival and decreased ability to colonize day old chicks (Purdy et al., 1999). A catalase (*katA*) deletion mutant *C. jejuni* demonstrated attenuated colonization fitness in a neonatal piglet model (Flint et al., 2012); however, a *katA* deficient *C. coli* had the same chick colonization characteristics as its parental strain (Purdy et al., 1999). Deletion of alkyl hydroperoxide reductase (*ahpC*) in *C. jejuni* significantly reduced its ability to colonize day old chicks (Palyada et al., 2009). Other peroxiredoxins involved in resistance to oxidative stress are the thiol peroxidase Tpx and bacterioferritin comigratory protein BCP (Atack et al., 2008). Methionine sulfoxide reductase enzymes MsrA and MsrB protect *C. jejuni* from R- and S-methionine sulfoxides, which are produced from the oxidation of methionine. Deletion of *msrA* and *msrB* from *C. jejuni* resulted in increased sensitivity to ROS assays (Atack and Kelly, 2008). The DNA–binding protein from starved cells (Dps) has also been identified as a colonization factor for *C. jejuni* in a chick model and has been studied as a potential antigen candidate for a subunit anti-*Campylobacter* vaccine (Theoret et al., 2012). Other proteins involved in oxidative stress resistance of *Campylobacter* include cytochrome c peroxidases, quinone reductases, and DNA repair proteins (Atack and Kelly, 2009; Flint et al., 2016b). The response to oxidative stress is coordinated by a complex and overlapping network of regulators. These include the peroxide regulator (PerR) and ferric uptake regulator (Fur) (Palyada et al., 2009), the *Campylobacter* oxidative stress regulator (CosR) (Hwang et al., 2011), and the regulators of response to peroxide (RrpA and RrpB) (Gundogdu et al., 2015).

Campylobacter jejuni and *C. coli* are considered to be obligate microaerophilic bacteria, requiring oxygen concentrations between 2 and 10% for optimal growth (Kaakoush et al., 2007). The primary stress encountered in the extra-intestinal environment may be the ambient 21% oxygen (Atack and Kelly, 2009). Reduced sensitivity to ambient oxygen would confer superior environmental resistance, therefore increasing the likelihood of transmission between potential hosts. Improved survival of particular strains on chicken carcasses would be of obvious public health importance as the risks they pose would be higher than previously anticipated. In addition, if aerotolerance is related to oxidative stress resistance, this may render the organism less sensitive to free radicals produced by the host's immune response (Atack and Kelly, 2009), and therefore more pathogenic.

Campylobacter coli OR12 has been shown to be a highly successful colonizer of broiler chickens on organic and free-range farms (El-Shibiny et al., 2005). This strain is also capable of displacing other *Campylobacter* strains from pre-colonized chickens (El-Shibiny et al., 2007). An unusual characteristic of this strain is aerobic growth. Here we report adaptation of the strain to aerotolerance with the objective of determining whether the ability to withstand oxidative stress will modify the characteristics of *C. coli* OR 12 that make it a successful colonizer of broiler chickens.

MATERIALS AND METHODS

Bacterial Strains

The *Campylobacter* strains used for this study were the chicken isolates *C. coli* OR12 (El-Shibiny et al., 2005), *C. coli* RM2228 (Fouts et al., 2005), *C. jejuni* HPC5 (Loc Carrillo et al., 2005), and the human clinical isolate *C. jejuni* NCTC 11168 (Parkhill et al., 2000).

Aerobic Passage on Blood Agar (BA)

Aerotolerant *C. coli* OR12 (Aer) was serially passaged by streaking on blood agar plates (BA; Oxoid) containing 5% v/v defibrinated horse blood (TCS; Buckingham; UK) and incubating aerobically at 37°C for 2–7 days. Intermediate stocks were archived at regular intervals by collecting confluent aerobic growth from a BA plate into freezer stock solution (15% Glycerol, 85% Nutrient Broth No.2; Oxoid) and freezing at −80°C. Gram stains, using standard techniques, were performed at regular intervals to ensure contamination had not occurred. Four colonies were initially selected from the original growth and passaged independently. With the exception of pulsed-field gel electrophoresis (PFGE), all experiments were conducted using isolate B, as it had qualitatively demonstrated the best growth in the early passages. The original wild type *C. coli* OR12 (WT) was maintained and stored microaerobically on BA.

Quantitative Aerobic Growth or Survival on BA

Blood agar 48 well plates were prepared by dispensing 1 ml of BA to each well (Nunclon Delta; Nunc; Denmark). Inoculum suspensions of approximately 10^7 CFU/ml of microaerobically grown *C. coli* RM2228, *C. coli* OR12 WT, *C. coli* OR12 Aer and aerobically grown *C. coli* OR12 Aer were prepared by suspending confluent growth from BA plates into PBS. Triplicate wells, representing biological replicates, were inoculated with 10 μl of suspension (approximately 10^5 CFU) per strain. Once dried, plates were incubated aerobically at 37°C. After 0, 6, 24, 30, and 48 h of incubation, well contents were suspended by gentle repeated pipetting in maximum recovery diluent (MRD; Oxoid). Suspensions were enumerated on CCDA using a modification of the Miles Misra method (Miles et al., 1938). Briefly, decimal dilutions were performed in MRD, 5 replicate 10 μl droplets from up to six dilutions were plated on modified *Campylobacter* blood-free selective agar (CCDA; Oxoid) with 2% agar. Once dry, plates were inverted, incubated microaerobically (5% CO_2, 5% O_2, 2% H_2, 88% N_2) in a modular atmosphere controlled cabinet at 42°C (Don Whitley Scientific modified atmospheric cabinet, Shipley, UK) and examined after 24 and 48 h. Dilutions giving rise to between 3 and 30 colonies were selected, the sum of the 5 spots was multiplied by 20, then multiplied by the inverse of the dilution factor to give the original CFU/ml.

Qualitative Aerobic Growth on Solid Media (BA and CCDA)

Suspensions of approximately 10^8 CFU/ml of the following strains were prepared: microaerobically grown *C. jejuni* NCTC 11168; *C. jejuni* HPC5; *C. coli* RM2228; *C. coli* OR12 WT; *C. coli* OR12 Aer and aerobically grown *C. coli* OR12 Aer. Each inoculum was decimally diluted five times and one 10 μl droplet of each dilution of each strain was dispensed onto two BA plates and two CCDA plates. Once dry, one of each was incubated microaerobically at 42°C and the other at 37°C aerobically. The microaerobic plates were examined after 48 h and the aerobic plates checked daily for 6 days.

Survival in Aerobic Liquid Medium

Sterile 250 ml flasks containing 50 ml Mueller-Hinton broth (MHB; Oxoid) were inoculated in triplicate to an approximate cell density of 10^6 CFU/ml of microaerobically grown *C. jejuni* HPC5 and *C. coli* OR12 WT and aerobically grown *C. coli* OR12 Aer P25. These were incubated aerobically at 37°C with orbital shaking at 100 rpm. Samples were collected at 0, 2, 4, 6, 24, and 30 h and *Campylobacter* enumerated on CCDA as above.

Growth Rate in Microaerobic Nutrient Broth No. 2

Sterile 100 ml flasks containing 30 ml nutrient broth no.2 (NB2; Oxoid) were inoculated in triplicate with approximately 10^4 CFU/ml of microaerobically grown *C. coli* RM2228 and *C. coli* OR12 WT and aerobically grown *C. coli* OR12 Aer P38. The flasks were incubated microaerobically in anaerobic gas jars using gas replacement (7.3% CO_2, 5.6% O_2, 3.6% H_2, 83% N_2) at 42°C with orbital shaking at 100 rpm (John et al., 2011). Samples were collected and *Campylobacter* enumerated on CCDA at 0, 2, 5, and 8 h.

Motility Assays

Motility was assessed using a motility agar assay and via membrane filtration. For the agar assay, inocula were prepared from freshly grown aerobic cultures of *C. coli* OR12 Aer or microaerobic cultures of *C. coli* OR12 WT. A pipette tip was used to collect a small plug of colony growth, which was then stab-inoculated into the center of a motility agar (Mueller Hinton broth with 0.4% v/v agar) plate, without touching the bottom. Plates were incubated microaerobically at 42°C and examined after 48 h.

For the membrane filtration assays, sterile nitrocellulose membrane filters with 0.45 μm pore size and 47 mm diameter (Millipore) were aseptically placed in the center of BA plates. The centers of the filters were inoculated with 50 μl of *C. coli* suspension taking care not to contaminate the surrounding agar. Once the liquid had passed through the filters, they were aseptically removed with sterile forceps. Plates were inoculated in duplicate, with one incubated microaerobically at 42°C and one aerobically at 37°C.

Peroxide Stress Assay

Resistance to peroxide stress was performed using a method similar to that of Rodrigues et al. (2015). Glass universal containers, filled with 10 ml of MRD containing 0, 0.5 or 1 mM H_2O_2 (Sigma; UK), were inoculated with identical optical densities of approximately 10^8 CFU/ml microaerobically grown

C. coli RM2228, *C. coli* OR12 WT, *C. coli* OR12 Aer P40 and aerobically grown *C. coli* OR12 Aer P40. Containers were incubated microaerobically at 42°C for 1 h with shaking at 100 rpm followed by immediate enumeration of *Campylobacter* on CCDA as above. Three universals were inoculated per strain, representing independent biological replicates.

Transmission Electron Microscopy

Confluent BA cultures of microaerobically grown *C. coli* OR12 WT and aerobically grown *C. coli* OR12 Aer P37 were fixed in electron microscopy fixative (3% glutaraldehyde in 0.1 M cacodylate buffer) prior to centrifugation and resuspension in sterile water. Transmission electron microscopy (TEM) was performed at the Advanced Microscopy Unit, University of Nottingham. Samples were adsorbed onto copper Formvar/Carbon grids (AGS162-3; Agar Scientific) and negatively stained with 3% uranyl acetate. Grids were imaged using a Tecnai G12 biotwin TEM, run at 100Kv, with a SIS megaview camera system and Gatan Microscopy Suite software (Gatan Inc.).

Pulsed-Field Gel Electrophoresis

Cell suspensions of four *C. coli* OR12 and aerotolerant derivatives were incorporated into agarose plugs, lysed and washed as described by Ribot et al. (2001). DNA plugs were cut into 2-mm slices and placed into 100 μl of 1x SuRE/Cut Buffer A (Roche) and equilibrated at 25°C for 15 min. This was replaced with 100 μl of 0.2 U/μl *Sma*I (Roche) in the same buffer and incubated for 2 h at 25°C. The plugs were incorporated in a gel consisting of 1% w/v Agarose (Bio-Rad) in TAE buffer. A 50–1000 kb DNA ladder (Lambda PFGE Ladder; New England BioLabs) was added as a marker. Electrophoresis was performed using a Bio-Rad CHEF-DRII. The gel was stained in 50 μg/ml ethidium bromide in TAE buffer and visualized under UV light using the Gel Doc XR system with the Quantity One basic software, version 4.6.5 (Bio-Rad).

Chicken Colonization Assay

Ross 308 broiler chickens ($n = 30$) were obtained as day old chicks from a commercial hatchery (PD Hook; UK) and reared in biosecure conditions. Chicks were group reared in pens with a bedding of wood shavings and transferred to individual cages with an astroturf floor and environmental enrichment at 18 days of age. A 12-h light-dark cycle was followed for the duration of rearing. Temperatures were as outlined in the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes, with minor adjustments made as guided by bird thermoregulatory behavior. They received commercial broiler diets (starter, grower, and finisher) for the duration. Cloacal swabs were taken at day 18 and tested for *Campylobacter* by direct plating on CCDA and *Salmonella* by enrichment. At 21 days of age, 15 birds (Group 1) were gavaged with an estimated 10^8 CFU of *C. coli* OR12 WT and 15 birds (Group 2) with *C. coli* OR12 Aer P34. After 3 days, seven birds from each group were culled by parenteral barbiturate overdose, with the remaining eight birds culled at 7 days post challenge. Caecal contents were collected and enumerated for *Campylobacter* in triplicate as described above, with CCDA selective supplement (Oxoid) added to the medium. The membrane filtration assay was also performed on the 10^{-1} dilution of each caecal content sample, as described above. A secondary aerotolerance assay was performed on 24 or 48 h growth on the microaerobically incubated BA filtration plates. Growth was suspended in PBS and normalized to an OD_{600} of 0.05, decimally diluted to 10^{-3} and a 10 μl spot of each dilution inoculated to a BA plate which was incubated aerobically at 37°C.

Power calculations were performed using the Spreadsheet for Sample Size Calculation (Brown, 2016). This indicated that seven birds were required per group to detect a 1 $\log_{10}$ CFU/g difference between groups with 95% confidence and a standard deviation of 0.5 $\log_{10}$ CFU/g. One additional bird was added per group to allow for any unexpected mortality.

Ethics Statement

All experimental animal work was performed in accordance with UK and EU law. This study was approved by the Local Ethics Committee of the University of Nottingham and performed under Home Office license.

Statistical Treatment of Data

Viable counts were $\log_{10}$-transformed for analysis. Statistically significant differences ($p < 0.05$) were assessed using parametric (ANOVA) and non-parametric (Mann–Whitney *U*) tests through the statistical packages available within Minitab statistical software (Minitab Inc.).

Whole Genome Sequencing and Analysis

Genomic DNA was extracted from half of a confluent aerobically incubated BA plate of *C. coli* OR12 Aer P43. DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich) according to manufacturer's instructions. Sequencing was performed using the MiSeq platform at Northumbria University. The sequence reads were aligned to the assembled sequence of *C. coli* OR12 (NCBI RefSeq NZ_CP013733.1) and appear in the database as CcOR12aero (NCBI RefSeq NZ_CP019977). DNA sequence variants were detected using the variant detector within CLC Genomics workbench 9.01 (Qiagen Bioinformatics). Candidate high probability nucleotide sequence variants detected were confirmed by careful inspection of the sequence reads, and any predicted changes to the protein encoding sequences confirmed using the Artemis genome browser (Rutherford et al., 2000). The predicted protein sequences were further compared with wild-type *C. coli* OR12 sequences, and with other similar protein sequences using the protein basic local alignment tool, BLASTP mounted on the NCBI website[1]. Nucleotide BLAST[1] was used to identify orthologs of genes with known roles in *Campylobacter* oxidative stress resistance. Protein sequences of gene products in *C. jejuni* NCTC11168 and *C. coli* OR12 were compared using the protein BLAST function.

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

RESULTS

Growth and Survival Characteristics of Aerotolerant *C. coli* OR12

For the initial early passages, growth of aerotolerant *C. coli* OR12 was limited to the heavily inoculated region of the BA plate. After approximately 15 aerobic passages, growth of single colonies became visible within 2–5 days of aerobic incubation at 37°C. Gram staining confirmed typical *Campylobacter* morphologies with variable populations of Gram-negative spirals, filamentous, and coccoid forms. Approximately 10^5 CFU of *C. coli* RM2228, wild type *C. coli* O12 (WT), aerotolerant *C. coli* OR12 (Aer) passage (P) 32, and a microaerobic preparation of *C. coli* OR12 Aer P32 were inoculated onto BA microplates and aerobically incubated at 37°C. No changes in viable count were apparent after 6 h; however, by 24 h the *C. coli* RM2228 and *C. coli* OR12 had declined to below the limit of detection (1.3 $\log_{10}$ CFU). In contrast, both the *C. coli* OR12 Aer P32 and the microaerobically prepared *C. coli* OR12 Aer P32 had grown by more than 1 $\log_{10}$ after 24 h and continued to grow, with viable counts of 7.4 and 8.3 $\log_{10}$ CFU, respectively, by 48 h (**Figure 1**).

Differences between aerobic and microaerobic growth were examined on BA and CCDA plates that had been inoculated with six decimal dilutions of a panel of six *Campylobacter* strains. These included *C. coli* OR12 WT along with aerobically and microaerobically prepared *C. coli* OR12 Aer P38. After 48 h of microaerobic incubation, single colonies were present at the highest dilution (10^{-5}) of all strains on BA and CCDA, correlating with viable count ranges of between 5.7×10^7 and 2.5×10^8 CFU/ml for the initial inocula. Viable counts of all strains were within 1 $\log_{10}$ of each other (**Figure 2**). After 6 days of aerobic incubation, single colonies were present down to the 10^{-4} dilution of both aerobically and microaerobically prepared *C. coli* Aer P38 on BA and CCDA. No aerobic growth was present from any of the other strains examined (**Figure 2**). The growth rates of *C. coli* RM2228, *C. coli* OR12 WT and *C. coli* O12 Aer P38 were assessed by microaerobic incubation at 42°C in nutrient broth no. 2. Viable counts are shown in Supplementary Figure 1. Exponential growth rates for each strain were calculated in the logarithmic growth phase; rates were not significantly different between strains.

Aerobic survival in liquid medium was assessed by inoculating Mueller Hinton broth with an estimated 6 $\log_{10}$ CFU/ml and incubating at 37°C. Viable counts are presented in Supplementary Figure 2. Following 6 h of aerobic incubation, the *C. jejuni* HPC5, *C. coli* OR12 WT and *C. coli* OR12 Aer had declined by 0.17, 0.23, and 0.42 $\log_{10}$ CFU/ml, respectively. By 24 h, both *C. coli* OR12 strains and two of the *C. jejuni* HPC5 flasks had declined to below the limit of detection (1.3 $\log_{10}$ CFU/ml) and one *C. jejuni* HPC5 flask had a viable count of 2.4 $\log_{10}$ CFU/ml. Despite differences in the starting inocula, aerotolerant *C. coli* OR12 did not demonstrate superior survival compared to the wild type and *C. jejuni* HPC5.

Aerotolerant *C. coli* OR12 Remain Motile in Atmospheric Oxygen

Motility assays were performed to determine whether serial aerobic passage resulted in reduced motility. The *C. coli* OR12 WT strain was tested together with *C. coli* OR12 Aer P22 and P50. All isolates demonstrated motility, with diameters of growth of 35, 34 and 33 mm, respectively, therefore aerotolerance was not associated with any loss of motility.

Owing to their highly energetic corkscrew-like motility campylobacters can traverse membrane filters that would exclude

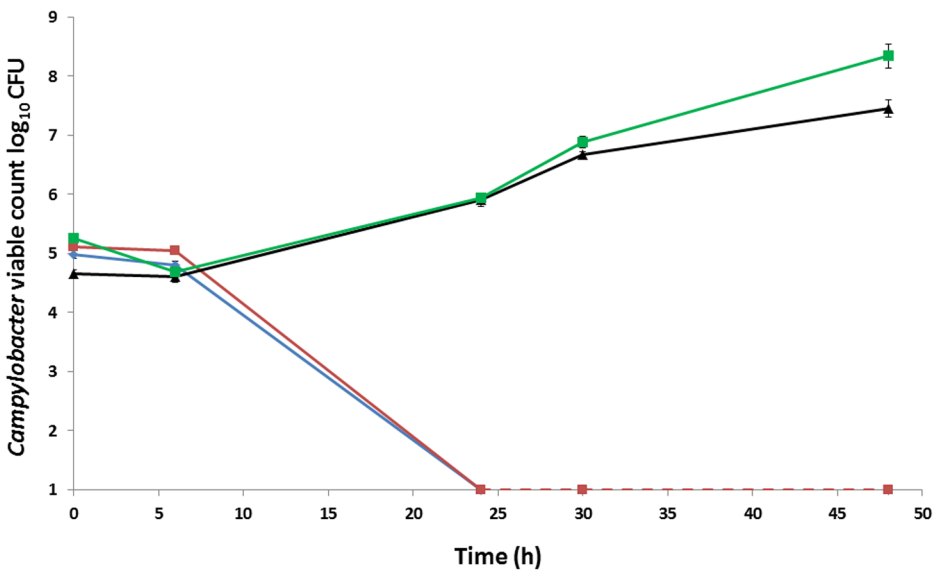

FIGURE 1 | Growth and survival of *Campylobacter coli* strains incubated aerobically on BA at 37°C. *C. coli* RM2228 (blue), *C. coli* OR12 WT (red), aerobically prepared *C. coli* OR12 Aer P32 (black), microaerobically prepared *C. coli* OR12 Aer P32 (green). Error bars represent standard deviations from three biological replicates. Results are representative of three separate experiments. The limit of detection was 1.3 $\log_{10}$ CFU.

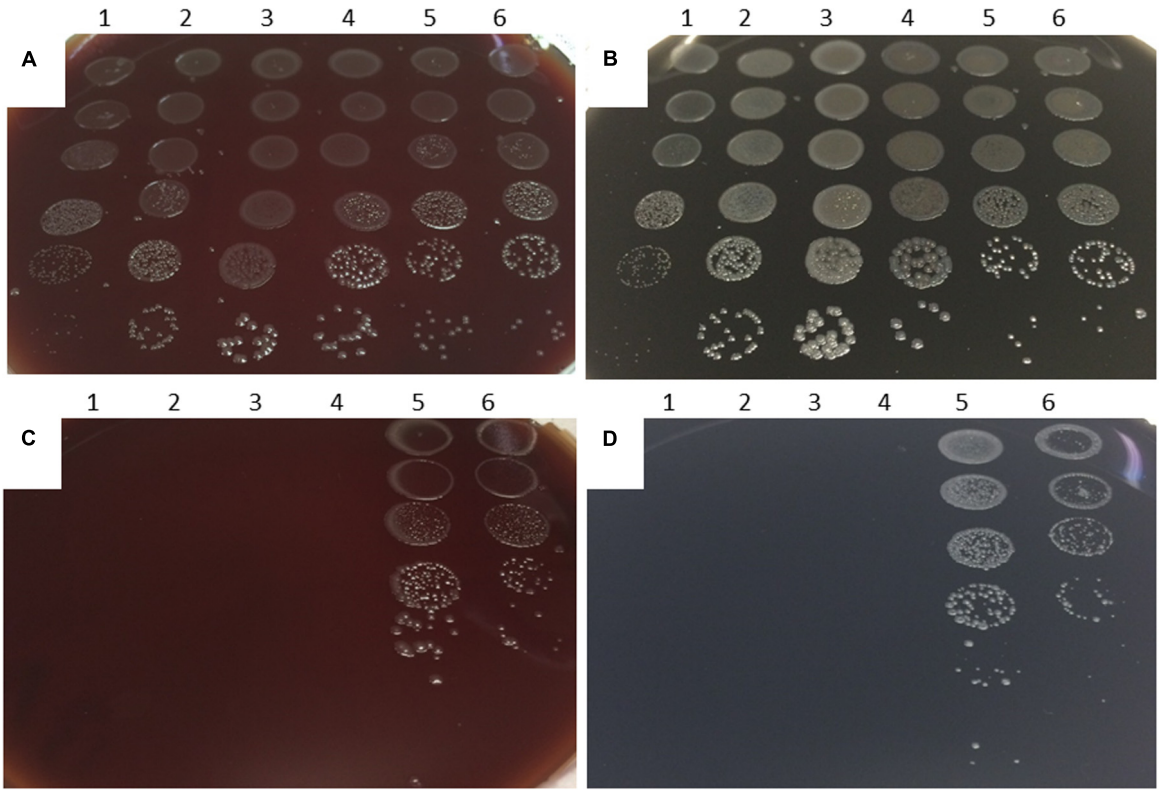

FIGURE 2 | Microaerobic and aerobic growth of *Campylobacter* on BA and CCDA. Decimal dilutions of suspensions of: (1) *C. jejuni* NCTC 11168; (2) *C. jejuni* HPC5; (3) *C. coli* RM2228; (4) *C. coli* OR12 WT; (5) *C. coli* OR12 Aer P38 (aerobic prep); (6) *C. coli* OR12 Aer P38 (microaerobic prep) inoculated onto: Microaerobically incubated BA **(A)** and CCDA **(B)** after 48 h. Aerobically incubated BA **(C)** and CCDA **(D)** after 6 days.

other bacteria, a property that has been used as a basis for their isolation from several animal sources (Moreno et al., 1993). Filtration was therefore used to examine the motility of aerotolerant *C. coli* OR12. Duplicate BA plates with centered nitrocellulose membrane filters were inoculated with 50 μl of suspensions containing 7.6 $\log_{10}$ CFU/ml *C. coli* OR12 WT or 7.6 $\log_{10}$ CFU/ml *C. coli* OR12 Aer P38. One of each was incubated microaerobically at 42°C and one of each aerobically at 37°C. After 24 h, heavy growth was present in the center of the filtered regions of both plates incubated microaerobically. After 4 days, growth was evident on the aerotolerant *C. coli* OR12 incubated aerobically, with confluent colonies by 5 days. No growth was noted on the wild type *C. coli* OR12 incubated aerobically. Under aerobic conditions aerotolerant *C. coli* OR12 remained motile and metabolically active to be able to translocate the membrane filter.

Aerotolerant *C. coli* OR12 Demonstrates Increased Peroxide Stress Resistance

To measure oxidative stress resistance, suspensions containing between 7.3 $\log_{10}$ and 7.8 $\log_{10}$ CFU/ml *C. coli* RM2228, *C. coli* OR12 WT, aerobically prepared *C. coli* OR12 Aer P40 and microaerobically prepared *C. coli* OR12 Aer P40 were exposed to different concentrations of H_2O_2. Marked differences in survival were apparent between aerobically prepared aerotolerant *C. coli* OR12 and the other microaerobically prepared strains (**Figure 3**).

Transmission Electron Microscopy

Transmission electron micrograph (TEM) images are shown in **Figure 4**. Both wild type and aerotolerant *C. coli* OR12 contained populations of typical spiral cells as well as filamentous and coccoid forms.

Colonization of Chickens

Colonization gavage doses were titrated at 1.38×10^8 CFU for group 1 (*C. coli* OR12 WT) and 3.4×10^7 CFU for group 2 (*C. coli* OR12 Aer P34). Caecal *Campylobacter* counts at 3 and 7 days post gavage are shown in **Figure 5**. All the birds that received *C. coli* OR12 WT became colonized, with mean caecal *Campylobacter* counts of 8.71 (±0.2) and 8.57 (±0.3) $\log_{10}$ CFU/g at 3 and 7 days post gavage, respectively. Of the birds challenged with *C. coli* OR12 Aer, only 2/7 had detectably colonized by 3 days and 6/8 by 8 days. Birds with no *Campylobacter* growth were considered ≤2.3 $\log_{10}$ CFU/g, which was the limit of detection. The difference in caecal *Campylobacter* counts between the wild type and aerotolerant strain was significant at 3 days post challenge ($p = 0.006$), but not significant by day 7 ($p = 0.0659$) as determined by the two-tailed Mann–Whitney *U* test. Standard

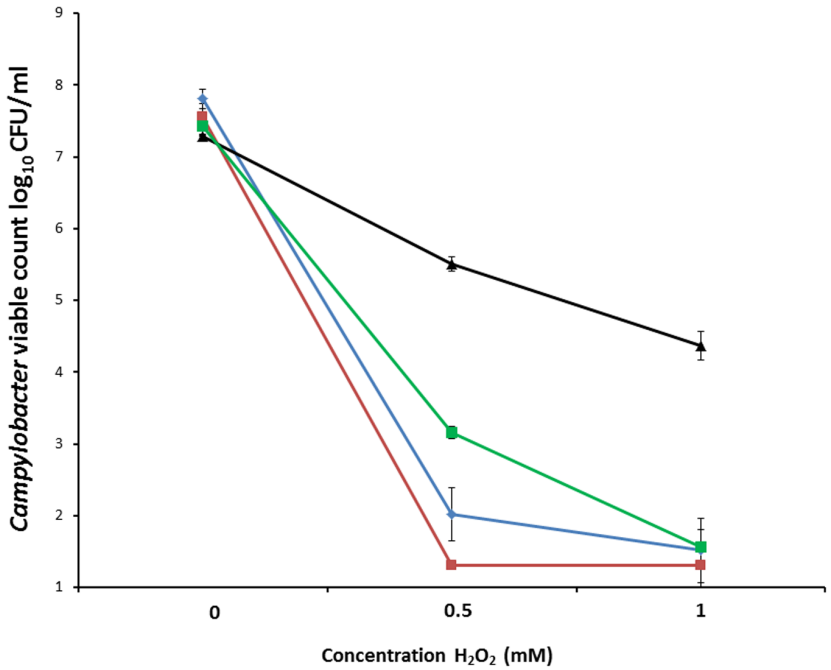

FIGURE 3 | Survival of *C. coli* in differing concentrations of hydrogen peroxide. *C. coli* RM2228 (blue), *C. coli* OR12 WT (red), aerobically prepared *C. coli* OR12 Aer P40 (black), microaerobically prepared *C. coli* OR12 Aer (green). Cultures were incubated for 1 h at 42°C under microaerobic conditions. Error bars represent standard deviations from three biological replicates.

deviations for individual bird *Campylobacter* counts generated by triplicate technical replicates were all below 0.19 $\log_{10}$ CFU/g.

All birds from which *Campylobacter* was detected on CCDA also yielded positive growth on microaerobically incubated membrane filtered BA plates. Colony morphology was consistent with *Campylobacter* (Supplementary Figure 3) and Gram stains provided confirmation. Of the aerobically incubated membrane filtration BA plates, growth was detected from one of the *C. coli* OR12 Aer challenged birds at 3 days post challenge and from 3 birds at 7 days post challenge. No growth was detected on any of the aerobically incubated membrane filtered BA plates from wild type challenged birds.

Secondary aerotolerance assays were performed on the campylobacters isolated by microaerobic membrane filtration. All *C. coli* isolated from birds challenged with *C. coli* OR12 Aer P34 retained their aerotolerance. Supplementary Figure 3A shows the serially diluted and aerobically incubated *C. coli* isolated from the two birds 3 days post challenge with *C. coli* OR12 Aer P34. No such growth was noted from isolates recovered from the seven birds challenged with the wild-type strain.

Whole Genome Sequencing and Analysis

Macrorestriction patterns of wild type *C. coli* OR12 and 4 aerobically passaged isolates determined by PFGE were identical to each other (Supplementary Figure 4) and to the original isolate described by El-Shibiny (2006). The development of aerotolerance is not associated with any significant DNA insertion, deletion or genome rearrangement. Therefore whole genome sequencing was performed to examine the occurrence of any point mutations.

A total of 23 mutations were noted between the wild type and aerotolerant *C. coli* OR12 sequences. There were 11 single nucleotide substitutions, 2 nucleotide insertions and 10 insertions or deletions within homopolymeric GC tracts. The DNA changes within homopolymeric GC tracts are summarized in **Table 1** and all others in **Table 2**. Three of the mutations were silent and one was within a non-coding region. All the predicted protein sequences were analyzed with BLASTP to confirm the protein identity and to determine if the changes induced were present in other similar sequences.

Phosphoglycerate Kinase

Gene *pgk*, which encodes the 400 amino acid protein phosphyglycerate kinase is located between nucleotides 380840–382042. BLASTP analysis of the amino acid sequence identified many *C. coli* and *C. jejuni* sequences with 99–100% coverage and identity. Aerotolerant *C. coli* OR12 has an A insertion at nucleotide position 380,879, which results in a frame shift at residue 19 and early protein termination at 21 amino acids. The entire amino acid sequence of the mutant protein is: MSDIISIKDIDLSKKKSFYKM. BLASTP analysis of this sequence did not find any sequences of similar length.

Biotin Attachment Protein/Pyruvate Carboxylase B

Gene *cfiA* encodes a 597 amino acid product, which was initially annotated as 2-oxoglutarate carboxylase. Subsequent BLASTP

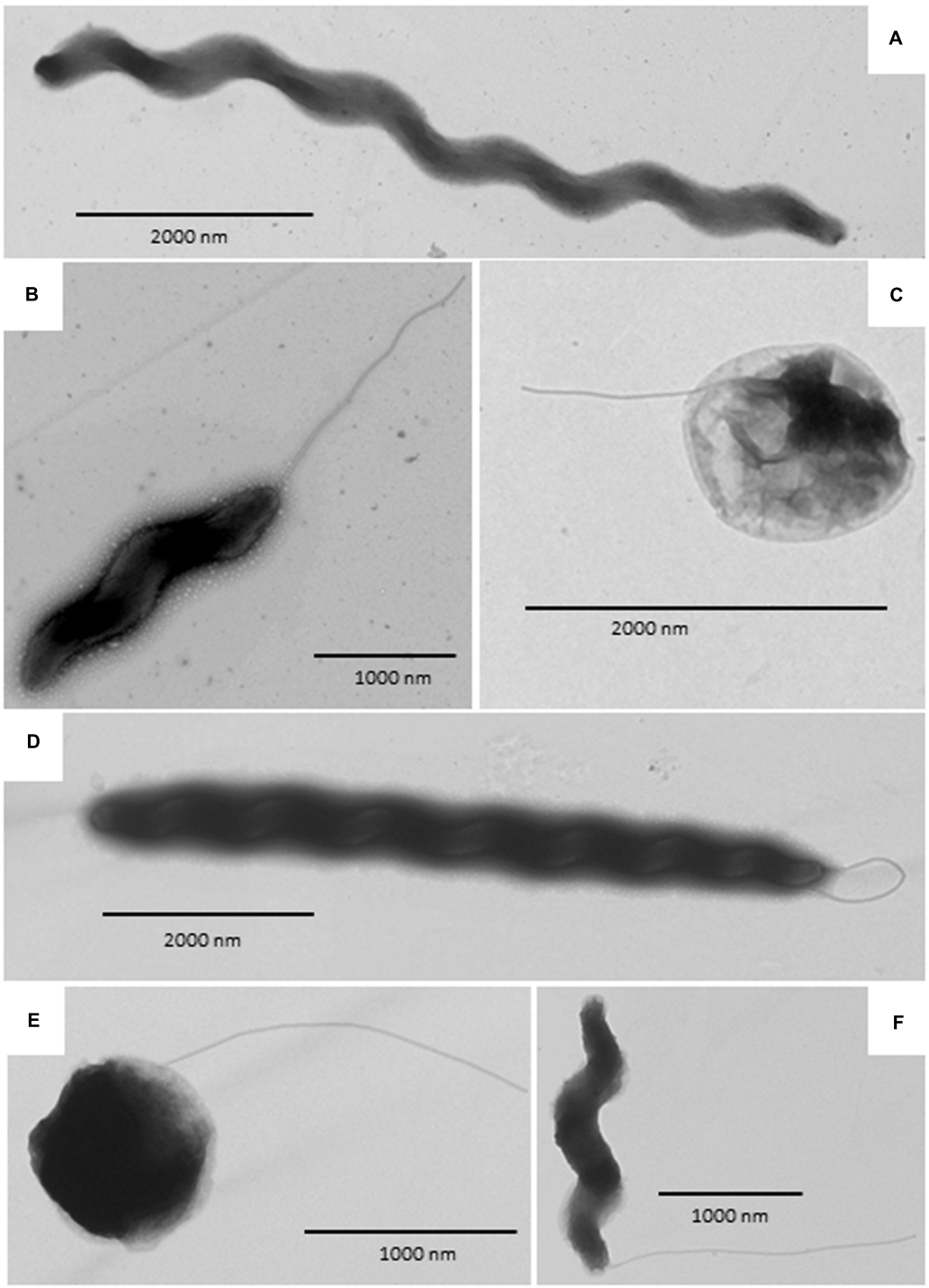

FIGURE 4 | Transmission electron microscopy images of *C. coli* OR12. (A–C)Rrepresent wild type and **(D–F)** aerotolerant *C. coli* OR12. **(A)** Filamentous form; **(B)** Spiral form with flagellum at the poles; **(C)** Coccoid form with flagellum; **(D)**Filamentous form with flagella; **(E)** Coccoid form with flagellum; **(F)** Spiral form with single polar flagellum.

searches identified 99% amino acid sequence identity with many *C. coli* biotin attachment proteins. The sequence also shares identity with several conserved domains, including phosphate binding superfamily, carboxylase, carboxylase interaction sites and a biotinylation site. The sequence of aerotolerant *C. coli* OR12 has a T insertion at position 837,990. This results in a frame shift and premature termination at residue 419. BLASTP analysis of the mutant sequence identified no similar sequences of identical

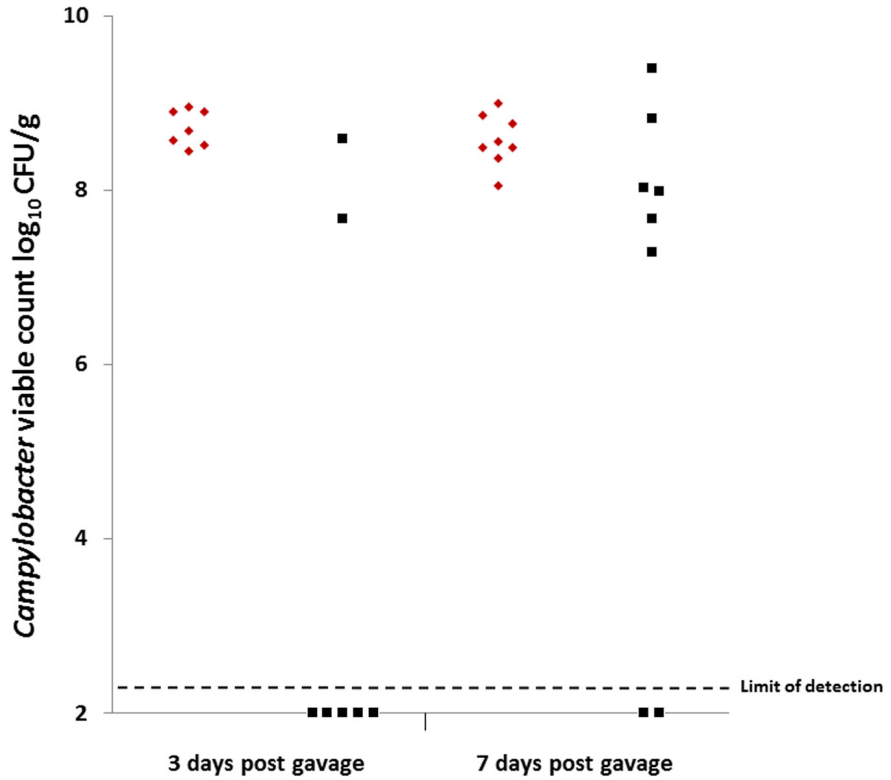

FIGURE 5 | Caecal *Campylobacter* counts from chickens challenged with, *C. coli* OR12 WT and Aer P34 at 3 and 7 days post challenge. *C. coli* WT (red diamonds), *C. coli* OR12 Aer (black squares). Counts between wild type and aerotolerant strains were statistically significant 3 days post challenge ($p = 0.006$) but not at 7 days ($p = 0.0659$) using Mann–Whitney *U* tests.

length, and lacked part of the carboxylase domain as well as the entirety of the biotinylation and carboxylase interaction sites.

PseD/Motility Accessory Factor

Nucleotide position 455,044 lies within a poly-C tract. In wild type *C. coli* OR12 this tract is composed of 10 Cs in 83% of the sequence reads, and 9 Cs in 7% of the sequences. The aerotolerant *C. coli* OR12 sequence has become fixed with 100% 9 Cs at this location. This position is within genes ATE51_00982 and ATE51_00984. In the 10 C configuration, the reading frame ATE51_00982 encodes a 593 amino acid protein that shares high sequence identity with PseD proteins (97 to 100% identity) from

TABLE 1 | Mutations in aerotolerant *Campylobacter coli* OR12 present within homopolymeric tracts.

Position	Consensus nucleotide change	Gene/CDS	Product	Change	AA change
455044	C (–)	ATE51_00892	PseD protein/Motility accessory factor	Premature termination	Y5 FS
455044	C (–)	ATE51_00894	Hypothetical protein/Motility accessory factor	Fusion to ATE51_00892	V60 FS
565851	G (–)	*hxuA*	Heme/hemopexin-binding protein precursor/filamentous hemagglutinin	Premature termination	M19 FS
828036	CC (+)	*pepD*	Aminoacyl-histidine dipeptidase	Premature termination	I186 FS
483767	G (–)	ATE51_00954	Hypothetical protein/carbonic anhydrase (incomplete)	C-terminal change	G196 FS
484990	G (–)	ATE51_00958	Hypothetical protein/carbonic anhydrase (incomplete)	Premature termination	S197 FS
1158035	CC (–)	ATE51_02354	Hypothetical protein/carbonic anhydrase	C-terminal change	G189 FS
64160	CC (–)	ATE51_00096	Iron-binding protein	C-terminal DR deletion	D240 FS
465041	CC (–)	ATE51_00914	Methyltransferase	C-terminal VL→VKL	V88 FS
366720	GG (–)	*yjjG*	Pyrimidine 5′nucleotidase/uridine kinase (incomplete)	C-terminal V→IG	V194 FS
422439	GG (–)	Non-coding	N/A	N/A	N/A

Consensus nucleotide changes represent differences in the aerotolerant C. coli genome sequence by addition (+) or deletion (–) compared to the wild type sequence. FS indicates the change results in a frame shift.

TABLE 2 | Mutations in aerotolerant *C. coli* OR12 not within homopolymeric tracts.

Position	Nucleotide change	Gene/CDS	Product	Change	AA change
380879	A+	*pgk*	Phosphoglycerate kinase	Frame shift and premature termination	V19 frameshift
837990	T+	*cfiA*	Biotin attachment protein/Pyruvate carboxylase B subunit	Frame shift and premature termination	D419 frameshift
116044	C→T	*nhaA1*	Na/H antiporter	Single aa substitution	A166→V
358671	C→T	*epsE*	Glycosyl transferase family A	Single aa substitution	T367→I
615756	A→G	*mnmG*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	Single aa substitution	T368→A
56663	G→A	*TypA/BipA*	GTP- binding protein	Single aa substitution	T210→I
168195	C→T	*sat*	Sulfate adenylyltransferase	Single aa substitution	A290→V
1346838	A→G	ATE51_02794	Hypothetical protein/VgrG type VI secretion protein (incomplete)	Single aa substitution	D19→G
1722359	G→A	*panC*	Pantoate beta alanine ligase	Single aa substitution	E216→K
1948793	C→T	*infB*	Translation initiation factor IF-2	Single aa substitution	R720→H
615323	C→T	*mnmG*	5-carboxymethylaminomethyl modification protein	Silent	None
1205648	T→C	*xerD*	Recombinase	Silent	None
1205660	A→G	*xerD*	Recombinase	Silent	None

many other *C. coli* and *C. jejuni* data base entries. The sequence also has 90% coverage and 71% identity with the sequence of PseD from *C. jejuni* NCTC 11168, which is involved in flagellar modification (McNally et al., 2006). The 9 C configuration, present in the minority of the wild type and all of the aerotolerant *C. coli* OR12 sequences, results in a frame shift at the fifth amino acid residue and protein termination at 21 amino acids. The 10 C configuration of gene ATE51_00984 encodes a 60 amino acid protein. However, the 9 C configuration results in a frame shift after 60 amino acid residues and fusion with ATE51_00982 resulting in a 648 amino acid protein, which has 99% coverage and 72% identity with the PseD from *C. jejuni* NCTC 11168.

Filamentous Haemagglutinin

Gene *hxuA* encodes a 1063 amino acid protein that was annotated as a heme/hemopexin binding precursor. However, BLASTP analysis of the protein sequence identified many *C. coli* filamentous haemagglutinin proteins with over 90% coverage and identity. The amino acid sequence also contains a conserved *N*-terminal haemagglutinin domain, conserved in many proteins with haemagglutination or haemolysin activity. Nucleotide 565,851 lies within a poly-G tract. Of the wild-type *C. coli* OR12 sequences, 96% of contained 10 Gs and 4% contained 9 Gs. Aerotolerant *C. coli* OR12 sequences were composed of 75% containing 9Gs and 25% containing 10 Gs. The 10 G configuration results in encoding of the full 1063 amino acid protein; however, the 9 G configuration results in a frame shift at residue 19 and immediate termination.

PepD

Gene *pepD* is a disrupted pseudogene in the majority of *C. coli* OR12 sequences. Nucleotide 828,036 lies within a poly-C tract. The wild-type *C. coli* OR12 sequences are composed of: 91% containing 8 Cs and 9% containing 9 Gs. Aerotolerant *C. coli* OR12 sequences are composed of: 77% containing 10 Gs and 23% containing 9 Gs. The 8 G configuration, which represents the majority of wild-type sequences, encodes a 221 amino acid protein that shares high sequence identity (83% coverage and 100% identity) with many *C. coli* aminoacyl-histidine dipeptidase proteins that are significantly longer. The 9 C configuration, present in the minority of wild-type and aerotolerant sequences, encodes a 423 amino acid protein, which has high sequence identity (99%) with other *C. coli* aminoacyl-histidine dipeptidase sequences of the same length. The sequence also shares similarity with a conserved zinc peptidase like superfamily domain. The 10 C configuration, present in the minority of aerotolerant sequences, encodes a 194 amino acid protein, which is identical to the 8 G product apart from the final 9 amino acid residues. Of the protein sequences that show high similarity with the 8 G and 10 G products, none ended in the same location or with similar C-terminal amino acid residues.

Carbonic Anhydrases

The first 192 amino acids of this predicted 205 amino acid protein product of gene ATE51_00954 share 67% identity with the first portion of the adjacent ATE51_00952 and nearby ATE51_00958, both of which are annotated as putative carbonic anhydrases. BLASTP analysis indicates 100% identity with several 196 amino acid proteins in *C. coli* and *C. jejuni* also similarly annotated as carbonic anhydrases. Aerotolerant *C. coli* OR12 has a single G homopolymer deletion at position 483,767 which causes a frame shift at the G196 residue, replacing residues GGHMEDTYLYS with GVIWRIPIFTHSKDSSLVCGWRD at the carboxyl terminus. BLASTP analysis of the 217 aa mutant configuration identified 88–90% identity with one *C. jejuni* and one *C. coli* hypothetical protein of 218 aa length, however, the majority of entries demonstrating sequence similarity were for 403–404 aa carbonic anhydrases.

Gene ATE51_00958 encodes a 405 aa protein, annotated as carbonic anhydrase with 97–100% identity with *C. jejuni* and *C. coli* carbonic anhydrase proteins of identical length. Aerotolerant *C. coli* OR12 has a G deletion at nucleotide 484,990 which induces a frame shift at residue 197, altering the aa sequence with a stop at 201 aa. BLASTP analysis of the mutant sequence revealed 97% identity with two *C. coli* hypothetical proteins of identical length, one of which was in *C. coli* RM2228.

Nucleotide position 1,158,035 lies within a homopolymeric tract, with wild-type *C. coli* OR12 sequences consisting of: 90% with 10 Cs; 7% with 9 Cs and 3% with 11 Cs. Aerotolerant *C. coli* OR12 sequences consist of: 85% with 8 Cs and 15% with 9 Cs. The original annotation of ATE51_02354 indicated a hypothetical protein; however, a more recent annotation of the *C. coli* OR12 genome within the Genbank database classified the region as a disrupted carbonic anhydrase pseudogene. The 10 C configuration, representing 90% of wild-type *C. coli* OR12 sequences, results in a predicted 190 aa hypothetical protein, with many highly similar hypothetical proteins in the database. One entry suggests high sequence identity with a partial carbonic anhydrase in *C. coli* (entry KQH53282.1). The 8 C configuration, representing 85% of aerotolerant *C. coli* OR12 sequence reads, results in a frame shift and exchange of the terminal GY residue for VLERFCLEIHMADI. This arrangement is also present in several similar sequences in the database, again mostly annotated as hypothetical proteins. The 9 C configuration, present in 7% of wild type and 15% of aerotolerant *C. coli* OR12 sequence reads results in a frame shift and fusion with the next open reading frame, ATE51_02352, resulting in a 408 amino acid protein. This sequence is also present in the database, with all similar entries annotated as hypothetical proteins. Residues 194–408 have a 99% similarity to a putative carbonic anhydrase domain in *C. jejuni* DFVF1099 (Takamiya et al., 2011). The 11 C configuration (3% of wild-type *C. coli* OR12 sequence reads) results in a frame shift similar to the 8 C configuration, but an additional G residue is retained; only the terminal Y amino acid residue is exchanged for the residues VLERFCLEIHMADI. This configuration is less frequently reported in the database than that of the 8C variant. All variants result in high similarity with the conserved domain DUF2920, a bacterial domain of unknown function. The three putative carbonic anhydrase protein sequences above share significant identity at their N-termini, as demonstrated by the alignment in Supplementary Figure 5.

Oxidative Stress Resistance Genes

Genes involved in *Campylobacter* oxidative stress resistance, as cataloged in a recent review by Flint et al. (2016b), were identified in *C. coli* OR12 and the corresponding protein sequences compared with orthologs from *C. jejuni* NCTC11168. These data are presented in Supplementary Table 1. All the functional genes cataloged by Flint et al. (2016b) could be identified with the exception of those encoding the regulators of response to peroxide A and B (*rrpA* and *rrpB*). No significant sequence similarity to the latter two genes could be identified in the sequences of *C. coli* OR12 or *C. coli* RM1875 or *C. coli* 15-537360.

DISCUSSION

Aerotolerance of *Campylobacter* spp. may represent a survival adaptation, potentially allowing superior environmental persistence and therefore improved opportunities for colonization of new hosts. The ability to withstand atmospheric oxygen is also relevant to survival on chicken meat and is therefore of public health significance. Aerotolerance is related, at least in part, to oxidative stress resistance which has been shown to be a colonization factor for chickens and a virulence factor for human infection (Hermans et al., 2011; Bolton, 2015; Flint et al., 2016a).

Aerotolerance following aerobic passage of *C. coli* OR12 was demonstrated by aerobic growth on both BA and CCDA. Similar viable counts of wild type *C. coli* OR12 and *C. coli* RM2228 were inoculated to BA, but they failed to grow and had declined below limit of detection by 24 h. Aerobic growth of *C. coli* OR12 Aer was noted at 37°C but not at 42°C. Microaerobic passage did not affect the ability of the *C. coli* OR12 Aer to grow aerobically, indicating that the aerotolerant phenotype is stable and likely due to genetic changes rather than temporary physiological adaptation. No difference in swarming motility was observed between the wild type and aerotolerant *C. coli* OR12. Further evidence of motility was provided by a membrane filtration assay, where growth occurred on BA following inoculation through a 0.45 μm nitrocellulose filter. Impaired motility has been reported to significantly reduce the ability of campylobacters to pass through cellulose membranes (Speegle et al., 2009). This suggests that the aerobic growth can be initiated by motile cells and is not solely facilitated by a protective layer of non-viable cells, shielding viable cells from atmospheric oxygen. Aerotolerant *C. coli* OR12 demonstrated superior resistance to H_2O_2 than either wild type *C. coli* OR12 or *C. coli* RM2228. However, *C. coli* OR12 Aer pre-cultured under microaerobic conditions was more sensitive to H_2O_2 than an inoculum from an aerobic culture. Aerobic growth may therefore induce factors which render the cells less sensitive to peroxide stress.

Transmission electron microscopy was performed to determine whether the aerotolerant adaptation was accompanied by any changes in cell morphology, for example cell filamentation was noted by Ghaffar et al. (2015) upon entry to stationary phase in broth cultures. No differences in cell morphology were apparent between the wild type and aerotolerant *C. coli* OR12, with spiral, filamentous and coccid cells present within both populations.

Pulsed-field gel electrophoresis of four *C. coli* OR12 Aer isolates, which had been aerobically passaged in parallel, showed no difference to the parent strain. As well as confirming that the isolates were all *C. coli* OR12 and had not been contaminated, this suggested that the aerotolerance was not associated with any major recombination events, such as the genomic rearrangements observed in *C. jejuni* HPC5 in response to phage predation reported by Scott et al. (2007). The changes associated with aerotolerance were therefore considered more likely to be due to point mutations, and thus, whole genome sequencing was performed to investigate this.

Previous Reports of Aerotolerance

Several authors have reported aerotolerance of *C. jejuni* and *C. coli*, however, the methods adopted to assess this are variable. For example, Jones et al. (1993) report aerobic growth of *C. jejuni* strains on blood agar after 2–3 days of aerobic incubation, but used an initial acclimation period of 18 h under microaerobic conditions. Similarly, Chynoweth et al. (1998) found 29 of 40 *C. jejuni* isolates from various sources could be adapted to grow aerobically on nutrient agar. All isolates were grown microaerobically before streaking on nutrient agar, and though some were initially slow to grow, they became adapted following aerobic subculture. Gaynor et al. (2004) reported the aerobic passage of *C. jejuni* NCTC 11168 on blood agar with antibiotic supplement at 37°C, which resulted in significant attenuation of colonization in a day-old chick model after thirteen passages.

In contrast, Oh et al. (2015) examined aerotolerance of 70 *C. jejuni* isolates obtained from chicken meat samples with respect to survival of approximately 10^9 CFU/ml in aerobic conditions at 42°C in MHB. Of these, 50 were considered aerotolerant based on survival for 12 h and 25 classified as hyper-aerotolerant, with survival for 24 h. The importance of alkyl hydroperoxide reductase was demonstrated by mutation of *ahpC* leading to the loss of hyper aerotolerance (Oh et al., 2015). Purdy et al. (1999) studied the effect of *sodB* mutation on aerotolerance and *in vivo* colonization fitness of *C. coli*. Inactivation of sodB activity did not affect survival of cultures aerobically incubated at 25°C in MHB, however, the colonization of day old chicks was reduced (Purdy et al., 1999). The aerotolerance test used by Purdy may not be equivalent to that described by Oh et al. (2015), as oxidative stress has been demonstrated to have a significantly greater effect at 42°C that at 25°C (Garénaux et al., 2008).

Kaakoush et al. (2007) examined the growth at several cell densities of four *C. jejuni* strains from aerobically and microaerobically incubated brain heart infusion broth. High initial bacterial density cultures grew when incubated either aerobically or microaerobically; however, cultures with less than 10^4 CFU/ml only grew under microaerobic conditions. A combination of lower oxygen saturation in liquid media at 42°C and dense cultures that have a greater oxygen demand will lower the oxygen tension and allow growth. No growth occurred when *C. coli* OR12 Aer or WT were aerobically incubated at 37°C in MHB; indeed similar decline rates were observed for the *C. coli* OR12 derivatives and *C. coli* RM2228.

Rodrigues et al. (2015) recently reported the aerobic growth of *C. jejuni* strain Bf on Karmali agar. For this strain visible growth was reported within 24 h of aerobic incubation at 42°C. Similar to *C. coli* OR12 Aer, growth in liquid medium could not be elicited, and a quantitative assay was used to measure growth on solid medium. Superior resistance to peroxide stress was also noted compared to *C. jejuni* NCTC 11168, which had failed to grow aerobically (Rodrigues et al., 2015). Growth on solid medium creates oxygen gradients, such that facultative anaerobes have been found to be more active on the bottom layer and aerobes more rapidly dividing on the top layer of colonies (Reyrolle and Letellier, 1979). Oxygen gradients in bacterial colonies and solid media have also been measured analytically, with the top 30 μm of a gelatine medium being considered aerobic (Jeanson et al., 2015). Therefore, once initial growth of *Campylobacter* has commenced on a solid medium, bacteria that are not directly exposed to the outside atmosphere may reside in an atmosphere more similar to microaerobic conditions.

Chicken Colonization

Chicken colonization studies confirmed that wild type *C. coli* OR12 is a robust colonizing strain, with reproducible caecal counts between 10^8 and 10^9 CFU/g. However, the aerotolerant strain colonized only 2/7 birds at 3 days and 6/8 birds by 7 days. Birds that were colonized had relatively high counts, with all >7 $\log_{10}$ CFU/g. The binary nature of the outcomes suggests that bottlenecking events and stochastic processes are significant factors in the colonization process. They are also suggestive of adaptation occurring between days 3 and 7, where campylobacters, presumably present at levels below the limit of detection, have become acclimated to the intestinal or caecal environment and proliferate to achieve higher colonization levels. These adaptations may not be identical between birds, as suggested by the wider range of caecal counts compared with the original isolate. This experiment further validates the importance of individual caging of chickens during *Campylobacter* colonization assays as advocated by Conlan et al. (2011). Had the birds been group housed, any chickens in which the aerotolerant *C. coli* OR12 had colonized would likely have shed the modified strain in large numbers in feces, potentially rapidly colonizing the other birds in the group. The reduction in initial colonization fitness could have been missed under these circumstances. A reduction in the challenge dose could potentially allow further resolution between groups and may be of value in future studies. Aerotolerant *C. coli* OR12 recovered from chickens were still capable of aerobic growth demonstrating the trait is stable and compatible with chicken colonization, albeit potentially at a lower initial level. However, it is difficult to distinguish the lower levels of colonization from the overall effect of *in vitro* passage, which is known to reduce the colonization fitness (Stern et al., 1988). Nevertheless these findings correlate with the observations of Jones et al. (1993) in that aerotolerant *C. jejuni* recovered from mice retained aerotolerance. The evident adaptation between days 3 and 7 did not lead to any loss of aerotolerance. The ability of *C. coli* OR12 to adapt to aerobic conditions and re-adapt to chicken colonization may be a driver of diversity within the *Campylobacter* population, where the plastic genome allows rapid adaptation to new or stressful conditions. Given the capacity of campylobacters for inter-strain recombination these changes may then be disseminated amongst cohabiting *Campylobacter* populations. The ability to withstand, and grow, in aerobic conditions could be of major importance not only in environmental persistence, allowing fomite transfer between farms, but could also allow prolonged survival on processed chicken carcasses and therefore be of public health importance. Survival of *C. coli* OR12 on chicken skin following refrigeration and freezing was examined by El-Shibiny et al. (2009). Survival rates were comparable with the other *C. jejuni* and *C. coli* strains tested. Similar experiments with aerotolerant *C. coli* OR12 could provide valuable insight as to

whether improved aerotolerance *in vitro* translates to superior survival on product, which would be of public health significance. This may be further considered in the context of the recently described aerobic *C. jejuni* strain Bf, which was found to be able to adhere to human epithelial cells and form biofilms under aerobic conditions (Bronnec et al., 2016).

This report describes true aerobic growth, without initial support from microaerobic incubation. With the exception of *C. coli* OR12, no growth was noted from any aerobically incubated *Campylobacter* spp. in this study. However, the equality of growth following aerobic or microaerobic incubation, which had been reported by Jones et al. (1993) was not achieved with *C. coli* OR12. Nevertheless the ability to undergo direct growth in atmospheric oxygen, a trait that was retained post either microaerobic growth or *in vivo* passage through chickens, encouraged us to examine the genetic basis for *C. coli* OR12 aerotolerance.

Genomic Analysis

Oxidative stress resistance has been proposed as a key feature of aerotolerance. Almost all the genes whose products have been implicated in oxidative stress resistance could be identified in *C. coli* OR12, none contained any mutations in the aerotolerant *C. coli* OR12. Orthologs of *rrpA* and *rrpB*, encoding MarR-like transcription factors implicated in oxidative stress resistance, could not be identified in the genome sequences of *C. coli* OR12 or RM1875 or 15-537360 (Gundogdu et al., 2015). Bronnec et al. (2016) identified a complete type VI secretion system in *C. jejuni* Bf and suggested that it may contribute to the aerotolerant phenotype. The *C. coli* OR12 genome contains a complete T6SS, although *C. coli* RM2228, which did not demonstrate aerotolerance, also possesses a full T6SS (Bleumink-Pluym et al., 2013). This would suggest that possession of T6SS alone does not confer aerotolerance, although it could be involved when in combination with other features. Changes in 23 coding sequences were observed between the wild type and aerotolerant *C. coli* OR12 genomes. The functional significances of these are discussed in the following paragraphs.

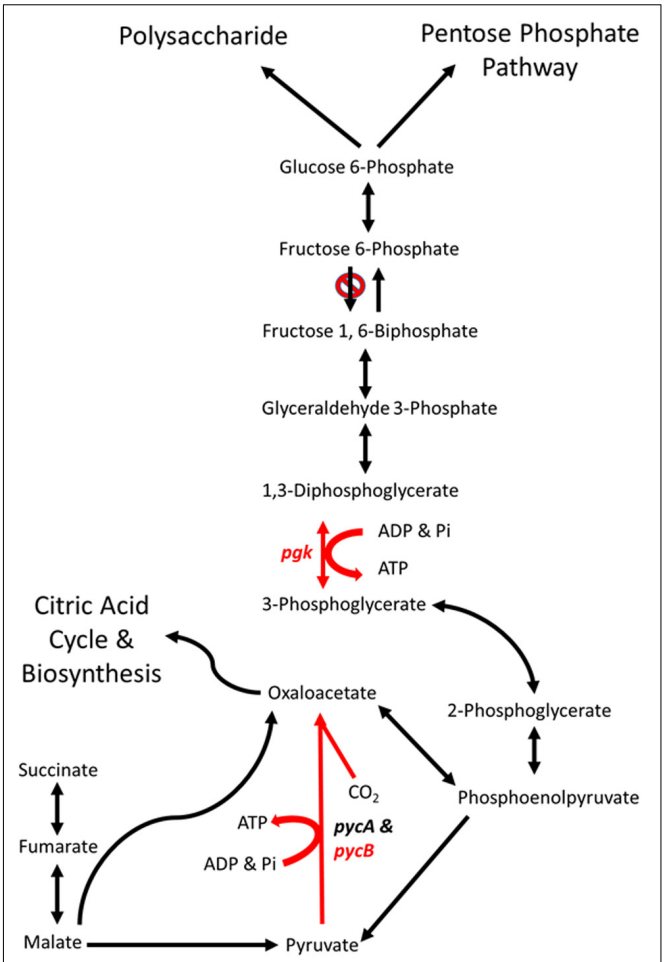

FIGURE 6 | Schematic representations of the major gluconeogenic and anaplerotic pathways of *Campylobacter*. Reaction in red are likely to be affected by the mutations in genes *pgk* (phosphoglycerate kinase) and *pycB* (pyruvate carboxylase B subunit) present in *C. coli* OR12 Aer. Modified from Velayudhan and Kelly (2002).

Phosphoglycerate Kinase

Phosphoglycerate kinase (PGK) is a magnesium dependent kinase involved in glycolysis and gluconeogenesis. In many organisms it performs the first ATP- generating step in glycolysis, however, *C. jejuni* lacks a homolog of 6-phosphofructokinase and therefore cannot metabolize hexose sugars (Velayudhan and Kelly, 2002). Under these circumstances *C. jejuni* PGK is likely to be limited to the gluconeogenic pathway, converting 3-phosphoglycerate to 1,3-diphosphoglycerate (**Figure 6**).

The aerotolerant *C. coli* OR12 sequence has an insertion early in the *pgk* gene that causes a frame shift and termination at 21 amino acids. No other copies of *pgk* are present, so it is likely that no functional PGK protein is produced. This may prevent the gluconeogenic pathway from occurring in aerotolerant *C. coli* OR12, so sugar anabolism must be occurring via different pathways. The *C coli* OR12 strain does possess orthologs to the *serABC* genes present in *C. jejuni* NCTC11168 (ATE51_01764; ATE51_03664; ATE51_03558, BLASTP identities of 94, 79, and 87%, respectively). Therefore, serine biosynthesis from 3-phosphoglycerate should be unaffected (Velayudhan et al., 2004). Circumvention of PGK and reinstatement of gluconeogenesis is theoretically possible via the formation of 2,3-bisphospho-D-glycerate, which would require the enzyme bisphosphoglycerate mutase. A putative phosphoglycerate/bisphosphoglycerate mutase has been annotated for *C. jejuni* NCTC11168, for which an ortholog is present in *C. coli* OR12 (ATE51_01624). The accumulation of 3-phosphoglycerate in the absence of PGK could drive this alternative reaction series. Aerobic growth of *C. coli* OR12 on BA plates prompted this study, and it is worth noting that erythrocytes are estimated to contain 4 to 5 mM 2,3-bisphospho-D-glycerate formed by the Luebering-Rapoport glycolytic shunt, which is used to promote the release of oxygen from deoxygenated hemoglobin at times of need by binding the T-state as an allosteric effector (Benesch and Benesch, 1967). The availability of 2,3-bisphospho-D-glycerate, albeit at declining concentrations, could be utilized by

campylobacters to reduce or remove the requirement of PGK in gluconeogenesis and increase the affinity of any remaining hemoglobin to bind oxygen. However, the adaptation present in the aerotolerant *C. coli* OR 12 also enables aerobic growth on CCDA. Modifications to central metabolism are emerging amongst campylobacters, for example it was recently reported that 1.7% of thermophilic campylobacters have the potential to metabolize glucose via the Entner-Doudoroff pathway (Vegge et al., 2016). This pathway also permits the metabolism of L-fucose, which has been reported to provide a competitive advantage to *C. jejuni* in a piglet colonization model (Stahl et al., 2011).

Biotin Attachment Protein

The *cfiA* gene in the wild-type *C. coli* OR12 sequence encodes a putative biotin attachment protein. The protein sequence shares 85% identity with 100% coverage of the pyruvate carboxylase B subunit of *C. jejuni* NCTC 11168. Therefore, this protein is likely to be the ortholog of PycB, encoded by the gene *cj0933c* in *C. jejuni* NCTC 11168. Pyruvate carboxylase in *C. jejuni* is composed of two subunits, PycA and PycB (Velayudhan and Kelly, 2002). These correspond with the biotin carboxylase and biotin carboxyl carrier subunits of a two-subunit type of carboxylase (Goss et al., 1981). The complete enzyme contains biotin and catalyzes the ATP-dependent carboxylation of pyruvate, yielding oxaloacetate. Velayudhan and Kelly (2002) performed mutation studies of *pycB* and *pycA,* and they noted that mutation of *pycB* resulted in significantly reduced growth in BHI broth, and the inability to use lactate, pyruvate or malate as carbon sources.

The *C. coli* OR12 Aer sequence contains a T insertion at nucleotide position 837,990 compared to the wild-type sequence. This would result in a frame shift and premature termination at 419 amino acids. This is likely to affect the activity of the enzyme as it is missing almost one third of its amino acids, including part of the conserved carboxylase domain as well as the entirety of the putative biotinylation and carboxylase interaction sites. This is surprising as the aerotolerant *C. coli* OR12 has a growth rate very similar to the wild type when grown microaerobically in NB2. Colonies on solid medium from microaerobically incubated aerotolerant *C. coli* OR12 were slightly smaller than the wild type, but this is not similar to the *pycB* mutant which had a threefold reduction in growth compared to the wild type (Velayudhan and Kelly, 2002). Comparisons of carbon metabolism in *C. jejuni* and *C. coli* indicate these species share a core set of carbon sources including amino acids, citric acid cycle intermediates and carboxylic acids (Wagley et al., 2014). However, exceptions were noted such as the inability of *C. coli* strains to metabolize D-malic acid and the inability of *C. jejuni* strains to metabolize propionic acid.

PseD/Motility Accessory Factor

Post-translational glycosylation of flagellar proteins is important for *Campylobacter* virulence. For example, mutation of *pseA* in *C. jejuni* 81-176 resulted in deficiency of flagellar glycosylation with pseudaminic acid and reduced virulence in a ferret model (Guerry et al., 2006). The function of the gene *pseD, cj01333* in *C. jejuni* NCTC 11168, which is a member of the motility accessory family, was examined in the context of flagellar glycosylation in *C. jejuni* 81-167 by McNally et al. (2006). Mutation of *pseD* did not affect cell motility or flagellin glycosylation with pseduaminic acid. However, mutation did prevent glycosylation with the pseudaminic acid acetamidino derivative PseAm, though it did not prevent cellular production of PseAm. Changes within a homopoylmeric C tract of *pseD* in aerotolerant *C. coli* OR12 effectively reinstates a longer reading frame to fuse ATE51_00982 and ATE51_00984 to create a 648 amino acid predicted product that exhibits 99% coverage with the *C. jejuni* NCTC 11168 PseD.

Filamentous Haemagglutinin

The gene *hxuA* encodes a protein which shares high sequence identity with other *C. coli* proteins annotated as filamentous haemagglutinin. The sequence also contains a region with high similarity to several conserved haemagglutination activity domains. Filamentous haemagglutinin proteins are involved in virulence of several bacterial pathogens, including *Bordetella pertussis* and *Avibacterium* (formerly *Haemophilus*) *paragallinarum* (Hobb et al., 2002; Inatsuka et al., 2005). The genome of *Arcobacter butzleri* ATCC 49616 contains the gene *hcpA* which encodes an iron activated filamentous hemagglutinin (Douidah et al., 2012). BLASTP comparison of this protein with *C. coli* OR12 HxuA revealed no significant similarity, nor did comparison with the *C. concisus* HcpA, the HagA protein of *A. paragallinarum*, or the *Bordetella* proteins FhaA and FhaB. *C. fetus* subsp. *fetus* 82-40 encodes a putative filamentous haemagglutinin of the HcpA family. It may be coincidental that this species is associated with bacteraemic infections, however, it is unknown whether this potential virulence factor is functional *in vivo* (Miller, 2008). The protein may have a possible role in iron acquisition or metabolism, which is known to be involved in the oxidative stress response (Atack and Kelly, 2009). The function of the putative filamentous haemagglutinin encoded by *hxuA* in *C. coli* OR12 remains obscure but the reading frame is notable in that it is phase variable, being intact in 96% of the wild-type sequence reads but appears disrupted in aerotolerant *C. coli* OR12.

Carbonic Anhydrases

Carbonic anhydrase enzymes catalyze the reversible hydration of CO_2 to bicarbonate and can be divided into at least six different phylogenetic groups, with varying expressions across kingdoms (Bury-Moné et al., 2008; Al-Haideri et al., 2015). These groups have similar function but differing structures, which are believed to have arisen by convergent evolution (Smith and Ferry, 2000). Strains of *C. jejuni* are reported to encode a β and a γ carbonic anhydrase, the former class are oligomeric, containing between 2 and 8 monomers and the latter are homotrimers (Smith and Ferry, 2000). However, the putative carbonic anhydrase annotated genes affected in the aerotolerant *C. coli* OR12 are not similar to those that have been investigated in campylobacters thus far. Whether these represent

a different class of carbonic anhydrases not yet characterized in *Campylobacter*, or encode a hitherto unknown function requires further investigation.

Eleven other genes contained coding mutations, all of which involved single nucleotide substitutions or changes of up to three amino acids at the C-terminus. None of these involved genes which could be expected to influence aerotolerance, such as those encoding oxidative stress resistance proteins or their regulatory genes. Further investigation of these changes would include protein modeling software to determine whether the changes induce significant structural alterations or cause relocation of the protein.

CONCLUSION

The *C. coli* strain OR12, which is a robust colonizer of chickens, was found to be capable of aerobic growth on blood agar. This aerotolerance became further evident after serial passage and was associated with increased peroxide stress resistance but did not influence cellular morphology or motility. Colonization of chickens by aerotolerant *C. coli* OR12 was lower than the wild-type strain at 3 days after challenge but not by 7 days, suggesting adaptation was occurring. Whole genome sequencing did not reveal any changes in genes known to be related to oxidative stress. Instead, significant mutations were present within genes encoding phosphoglycerate kinase and pyruvate carboxylase, suggesting alterations to cellular metabolism.

AUTHOR CONTRIBUTIONS

PO'K and IC designed and executed experiments, analyzed data, prepared and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00513/full#supplementary-material

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Function and Regulation of the C4-Dicarboxylate Transporters in *Campylobacter jejuni*

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C4-dicarboxylates are important molecules for the human pathogen *C.jejuni*, as they are used as carbon and electron acceptor molecules, as sugars cannot be utilized by this microaerophilic organism. Based on the genome analysis, *C. jejuni* may possess five different C4–dicarboxylate transporters: DctA, DcuA, DcuB, and two homologs of DcuC. Here, we investigated the regulation and function of various C4–dicarboxylate transporters in *C. jejuni*. Transcription of the *dctA* and *dcuC* homologs is constitutive, while *dcuA* and *dcuB* are both directly regulated by the two-component RacR/RacS system in response to limited oxygen availability and the presence of nitrate. The DctA transporter is the only C4-dicarboxylate transporter to allow *C. jejuni* to grow on C4-carbon sources such as aspartate, fumarate, and succinate at high oxygen levels (10% O_2) and is indispensable for the uptake of succinate from the medium under these conditions. Both DcuA and DcuB can sequester aspartate from the medium under low-oxygen conditions (0.3% O_2). However, under these conditions, DcuB is the only transporter to secrete succinate to the environment. Under low-oxygen conditions, nitrate prevents the secretion of succinate to the environment and was able to overrule the phenotype of the C4-transporter mutants, indicating that the activity of the aspartate–fumarate–succinate pathway in *C. jejuni* is strongly reduced by the addition of nitrate in the medium.

Keywords: ***Campylobacter jejuni,*** **C4-dicarboxylates transporters, DctA, Dcu, gene regulation, metabolism, RacRS**

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INTRODUCTION

Bacteria utilize C4-dicarboxylates such as fumarate, succinate, malate, and aspartate when sugars or related compounds are not available (Janausch et al., 2002). C4-dicarboxylates serve as carbon and energy source and are oxidized to CO_2 in the citric acid cycle under aerobic conditions. Under anaerobic conditions fumarate, malate, and aspartate are taken up into the cell. Malate and aspartate are reduced to fumarate, which is used as electron acceptor in the fumarate respiration pathway where it is converted to succinate. Succinate cannot be further metabolized by most bacteria due to the lack of a functional citric acid cycle under these conditions and is excreted.

The microaerophilic Gram-negative bacterium *Campylobacter jejuni* is the most common cause of food-borne bacterial gastroenteritis worldwide. Despite the medical and public health importance of *Campylobacter* infection, it is remarkable that *C. jejuni* is one of the least understood enteropathogens. *C. jejuni* possesses a highly branched electron transport chain, which allows both

aerobic and anaerobic respiration (Kelly, 2008). Most *C. jejuni* strains cannot utilize sugars (Parkhill et al., 2000; Pearson et al., 2007; Stahl et al., 2011) and it seems that selected amino acids and C4-dicarboxylates act as primary energy source (Guccione et al., 2008; Zientz et al., 1999). It remains largely unknown how the transport and regulation of C4-dicarboxylates occurs in *C. jejuni*.

Five C4-dicarboxylate carriers, DctA, DcuAB, DcuC, CitT, and DctPQM, are known to transport C4-dicarboxylates from the periplasm across the inner membrane into bacteria (Janausch et al., 2002). In *Escherichia coli* DctA is a C4-dicarboxylate/H+ or Na+ cation symporter that catalyses the uptake of C4-dicarboxylates during aerobic growth. During anaerobiosis the transcription of the *dctA* gene is strongly repressed by the two-component ArcBA system. Due to the cAMP-CRP complex glucose can also prevent the transcription of the *dctA* gene. DcuAB and DcuC have similar functions as they catalyse the exchange, uptake and efflux of C4-dicarboxylates under anaerobic growth conditions (Zientz et al., 1999). DcuB and DcuC are the main transporters for succinate efflux during anaerobic growth (Zientz et al., 1999). While the *dcuA* gene is expressed constitutively, both DcuB and DcuC are activated by the O_2-dependent regulator FNR. Furthermore, DcuB is repressed by nitrate due to the two-component NarXL regulatory system and activated by the two-component DcuSR system in response to presence of fumarate (Overton et al., 2006). CitT is a citrate:succinate antiporter, which is regulated by the two-component CitAB system in response to citrate (Scheu et al., 2012). Finally, the three proteins DctPQM in *Rhodobacter capsulatus* form a C4-dicarboxylate transporter which is in *Pseudomonas aeruginosa* dependent on the two-component dctSR system (Forward et al., 1997; Valentini et al., 2011).

C. jejuni contains all the enzymes for a complete oxidative TCA cycle, central to a flexible energy metabolism. *Campylobacter* possess only the C4-dicarboxylate carriers, DctA and DcuAB, some strains also contain one or two proteins similar to DcuC (Hofreuter et al., 2006). Like in other bacteria under oxygen-limited conditions, the transcription of the *C. jejuni dcuA* and *dcuB* genes is upregulated and under these conditions the antiporters are able to transport aspartate and fumarate (Woodall et al., 2005; Guccione et al., 2008). In contrast, all the transcription factors known to regulate the C4-dicarboxylate transporters carriers in other bacteria are lacking in *C. jejuni*.

Recently, we have shown that the two-component RacR/RacS system of *C. jejuni* directly represses the operon *aspA-dcuA-cj0089* under oxygen-limited conditions in the presence of nitrate (van der Stel et al., 2015). In this work, we investigated the function and regulation of all C4-dicarboxylate carriers in *C. jejuni*.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *C. jejuni* was routinely cultured under microaerophilic conditions (5% O_2, 10% CO_2, 75% N_2)on Blood Agar Base No. 2 (BA) medium containing 5% horse blood or in Heart Infusion broth (HI; Oxoid). Kanamycin (25 μg ml^{-1}) and/or chloramphenicol (15 μg ml^{-1}) and/or spectinomycin (30 μg ml^{-1}) were added when appropriate. *E. coli* strains were routinely grown at 37°C in Luria–Bertani (LB) broth or on LB agar plates supplemented with ampicillin (50 μg ml^{-1}), kanamycin (30 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}).

Construction of a *dcuA*, *dcuB*, *dcuC*, *dcuC2* or *dctA* Mutant

To disrupt the *dcuA*, *dcuB*, *dcuC*, *dcuC2,* or *dctA* genes, the genes as well as ∼1 kb of the flanking regions were first amplified by PCR using the primer pairs dcuA-F/dcuA-R, dcuB-F/dcuB-R, dcuC-F/dcuC-R, dcuC-2F/dcuC-2R, or dctA-F/dctA-R, respectively. Primers are listed in **Table 2**. The ∼3 kb PCR fragments were ligated into pJET1.2/blunt Cloning Vector resulting into the plasmids pJETdcuA, pJETdcuB, pJETdcuC, pJETdcuC2, and pJETdctA. Inverse PCR was performed on the plasmids pJETdcuA, pJETdcuB, pJETdcuC, and pJETdctA using the primers sets dcuABamHI F/dcuABamHI R, dcuBBamHI F/dcuBBamHI R, dcuCBamHI F/dcuCBamHI R, or dctABamHI F/dctABamHI R, respectively, to delete the *dcuA*, *dcuB*, *dcuC,* and *dctA* genes present in pJET and to introduce a *Bam*HI restriction site. The pJETdcuA and pJETdctA inverse PCR fragment were ligated to a *Bam*HI fragment containing a chloramphenicol resistance gene of pAV35 resulting in the knock-out constructs pJETdcuA::Cm and pJETdctA::Cm. The pJETdcuB and pJETdcuC inverse PCR fragment were ligated to a *Bam*HI fragment containing the kanamycin resistance gene of pMW2 or spectomycin resistance gene of pNBspec resulting into the knock-out constructs pJETdcuB::Km and pJETdcuC::speC, respectively. Plasmid pNBspec is a pAV35 derivate containing the spectomycin resistance gene of pZW2 (Zhou et al., 2012). pNBspec was constructed by amplifying pAV35 with the primers RBSCATrev and CATstop and the spectomycin resistance gene of pZW2 with the primers RBSspec and Specstop. In a second PCR these two PCR fragments were connected and after self-ligating of the PCR product pNBspec was obtained. To disrupt the *dcuC2* gene, plasmid pJETdcuC2 was digested with *XmaJI* and ligated to an *XbaI* fragment containing the pAV35 chloramphenicol resistance gene, resulting in the pJETdcuC2::Cm knock-out construct. To mutate the *dcuA, dcuB, dcuC, dcuC2, and dctA* genes, the knock-out constructs pJETdcuA::Cm, pJETdcuB::km, pJETdcuC::speC, pjetDcuC2::Cm, and pJETdctA::Cm were introduced by natural transformation in *C. jejuni* 81116. Homologous recombinations resulting in double cross-over events were verified by PCR.

RNA Isolation

RNA was extracted from *C. jejuni* wild-type grown in HI with or without 25 mM serine, aspartate, fumarate or succinate under 10 or 0.3% oxygen concentration at logarithmic (10 h) or stationary phase (20 h). RNA was also extracted from the wild-type, the *racR* mutant and the *racR* complemented strain grown in HI with 50 mM of $NaNO_3$ under 0.3% oxygen concentration until

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotype or relevant characteristics	Source or reference
STRAINS		
C. jejuni 81116	wildtype	Palmer et al., 1983
dctA	81116 derivative *dctA*::Cm	This study
dcuA	81116 derivative *dcuA*::Cm	This study
dcuB	81116 derivative *dcuB*::Km	This study
dcuC	81116 derivative *dcuC*::SpeC	This study
dcuC2	81116 derivative *dcuC2*::Cm	This study
dcuAB	81116 derivative *dcuA*::Cm *dcuB*::Km	This study
dcuABC	81116 derivative *dcuA*::Cm *dcuB*::Km, *dcuC*::SpeC	This study
E. coli PC2955	*relA1 Φ80dlacZΔM15 phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44*	NCCB
PLASMIDS		
pJet 1.2 blunt	Ap^R PCR cloning vector, Amp^r	*Fermentas, thermoscientific*
pAV35	Ap^R Cm^R pBluescript II SK containing *Campylobacter coli* Cm^R cassette	van Vliet et al., 1998
pMW2	Ap^R Km^R pBluescript KS containing *C. jejuni* Km^r cassette	Wösten et al., 2010
pZW2	Ap^R $Spec^R$ *E. coli-C. jejuni* shuttle vector	Zhou et al., 2012
pNBspec	Ap^R $Spec^R$ pAV35 containing $Spec^R$ cassette	This study
pJetdctA	Ap^R; 6.2 kb pJet containing *dctA* region	This study
pJetdcuA	Ap^R; 7.5 kb pJet containing *dcuA* region	This study
pJetdcuB	Ap^R; 6.3 kb pJet containing *dcuB* region	This study
pJetdcuC	Ap^R; 6.6 kb pJet containing *dcuC* region	This study
pJetdcuC2		
pJetdcuC2	Ap^R; 8.7 kb pJet containing *dcuC2* region	This study
pJetdctA::Cm	Ap^R Cm^R pJet containing *dctA* gene on a 2009 bp fragment	This study
pJetdcuA::Cm	Ap^R Cm^R; 5.9 kb, *dcuA* replaced by Cm^R	This study
pJetdcuB::Km	Ap^R Km^R; 6.5 kb, *dcuB* replaced by Km^R	This study
pJetdcuC::Spec	Ap^R $Spec^R$; 6.2 kb, *dcuC* replaced by $Spec^R$	This study
pJetdcuC2::Cm	Ap^R Cm^R; 8.5 Kb, *dcuC2* replaced by Cm^R	This study

late logarithmic (log) phase (16 h) using the RNA-Bee™ kit (Tel-Test, Inc.). RNA samples were treated with RNAse-free DNAse I (Fermentas) according to the manufacturer's manual.

Real-Time RT-PCR

Real-time RT-PCR analysis was performed as previously described (Wösten et al., 2004). Primers used in this assay are listed in **Table 2**. The calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct of the *gyrA* gene amplified from the corresponding sample before calculating fold change using the arithmetic formula $2^{-\Delta\Delta Ct}$ (Schmittgen, 2001). Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and are displayed as error bars.

Electrophoretic Mobility Shift Assay (EMSA)

Recombinant His-tag labeled RacR was isolated and EMSA were performed as described before (van der Stel et al., 2015). The promoter regions upstream of the genes *aspA, dcuB, dcuC, dcuC2,* and *dctA* were amplified by PCR using the primer pairs listed in **Table 2** and *C. jejuni* 81116 genomic DNA as template. Radioactive labeled PCR products, ∼25 pmol, were incubated with 0 or 50 pmol of recombinant RacR and 25 pmol RacScyto (van der Stel et al., 2015) for 20 min at RT in binding buffer containing 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 50 μg/ml bovine serum albumin, 10 μg/ml poly-(dI-dC), and 10% glycerol. For competition assays RacR was pre-incubated for 15 min with 10 times excess of unlabelled PCR fragment. Samples were run on 6% non-denaturing Tris-glycine polyacrylamide gels at 4°C. After electrophoresis, gels were dried and autoradiographed.

Growth Experiments

Growth curves were generated under microaerobic conditions (10% O_2, 10% CO_2, 70% N_2, 10% H_2) or under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79% N_2, 10% H_2) at 42°C in a "honeycomb" 10 × 10 well micro-plate using a Bioscreen C MRB (Oy Growth Curves Ab Ltd) computer-controlled incubator placed in the anaerobic chamber (Coy labs, Michigan, United States). *Campylobacter* precultures grown for 6 h in HI were diluted to OD_{600nm} of 0.01 in fresh HI media containing 25 mM fumarate, 25 mM aspartate, 25 mM serine, or 25 mM succinate with or without 25 mM

TABLE 2 | Primers used in this study.

Primer	DNA sequence (5′-3′)
MUTANTS	
dcuA-F	AGTCAAAGTACTAATGATGC
dcuB-R	TCAAGAGCTGCAACAGGACC
dcuA-BamHI F	AGGATCCTTACTTGCAAAATTATCATTATATCC
dcuA-BamHI R	AGGATCCGGCTTTGTATTAGCTCCTATGCTTATT
dcuB-F	TTAGATCCTGTAGGAGTGGG
dcuB-BamHI R	AGGATCCCTCACTAAGGCTTGTTAAAAAGTCC
dcuB-BamHI F	AGGATCCATTTTATTGCTATGGCAGCGGGTTAT
dcuC-F	TAAAATATTGATAATTCTTGTGAGT
dcuC-R	GCCTTTATTATCGGCGAAATTTGCA
dcuC-BamHI F	AGGATCCAAATAGCAGTATCTTTAAATAATAAATA
dcuC-BamHI R	AGGATCCGCTAAAAGTGTAGAACATATCATAAA
dcuC-2F	TAAAATATCACCCATATCTCCAATTTT
dcuC-2R	TATCCACCCCAGCTAGCACCAATTGA
dctA-F	CATAAATTTACCCCTTTTTATTGAAA
dctA-R	CCCTTTTTTATTTTAATTATACACTTA
dctA-BamHI F	TGGATCCTTGCCATCTGGGATAAACAAATTGAT
dctA-BamHI R	TGGATCCAGCAATGGCTAATTCTTTATCTATATA
SPEC CASSETTE	
RBSCATrev	TTATCCTCCGTAAATTCCGATTTGTTG
CATstop	TAAAATCCCAGTTTGTCGCACTG
RBSspec	CGGAATTTACGGAGGATAAATGATGAATAGTTATGAAGTAAC
Specstop	GCGACAAACTGGGATTTTAAGCAAAACCTTTTATTTTTT GTTGAAGG
RT-PCR	
dctARtaq	GTGTAACTGGATCAGGTTTTATAGTGCTT
dctAFtaq	GCTAAGGTTGCATTTGCTTCTGAA
dcuBFtaq	TGGACTTATGCTGTAATGCTTCTTTTA
dcuBRtaq	GCCAAAGGAACAAAAGCTGAA
aspAFtaq81116	TTTGTTAGAGCTTTGGCTAGAGTAAAAA
aspARtaq81116	CGCTTTAATAATCGCATCTTGGA
gyrAFtaq81116	ACGACTTACACGACCGATTTCA
gyrARtaq81116	ATGCTCTTTGCAGTAACCAAAAAA
dcuCFtaq	CACTTGGTGGAGTTAATATCCTTGCT
dcuCRtaq	AAAGAATTCCAAACCATGCAAAA
dcuC2Ftaq	TGCTTGCAGTTTGTGCTTTCTT
dcuC2Rtaq	TTGGGATAAGTGGAGCAAAGCT
dcuAFtaq	GTTTCGGCACTTTTTGTGCTT
dcuARtaq	CCAATTCTAGTCGTTCCTGTATCATC
EMSA	
dcuBR	ATTTGGATTGCAAATTGCCCT
dcuBF	AAAATTCTATCAATCTATCAAACC
dcuCR	TAAGTATATAATAAGCAACGACAATT
dcuCF	TTTCAGTAACCAAACTATACATATT
dctAF	GCGGTTTTCTTTCAGCTAAAGTTTGA
dctAR	GCAAATAATGAGAAATTTTGTAACATT
dcuC2R	GCTGAGTATGGACCAATGGCCTTTG
dcuC2F	TACTCTTTATACTTTAAAACATTTCTT
aspAF	AGCTTGCAAAAATATATTAATTT
aspAR	TAATAAACCTCATCAGAGATTTC

nitrate. The optical density at 600 nm was measured every 15 min. The experiments were repeated at least three times in duplicate.

High-Performance Liquid Chromatography (HPLC)-MS-MS Analysis

During the growth experiments of *C. jejuni* at 10% O_2, culture samples (50 μl) were taken at 4, 8, 16, and 24 h, under oxygen-limited conditions (0.3% O_2) samples were taken at 6, 12, 24, and 36 h. The culture samples were centrifuged at 14,000 rpm for 5 min. The supernatants were diluted in milli-Q water and adjusted to pH 2.4 with formic acid and injected on a Synergi 4u Fusion-RP (150 × 2.0 mm, particle size of 4 μm) analytical column (Phenomenex, Utrecht, NL). Elution was performed isocratically with milli-Q (adjusted to pH 2.4 with formic-acid): acetonitrile (9:1 [v/v]) at a flow rate of 0.3 ml/min, and the column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface, in negative mode with an ionization current of −1 μA and a source temperature of 350°C, into a 2000 QTRAP mass spectrometer (Sciex, Toronto, ON). For maximal sensitivity and for linearity of the response, the mass spectrometer was operated in multiple-reaction monitoring (MRM) mode at unit mass resolution. Peaks were identified by comparison of retention time and mass spectrum with authentic standards. Ion transitions monitored were *m/z* 115.0/71.0 (fumarate), 117.0/73.0 (succinate), 132.1/88.0 (aspartic acid), and 104.0/74.0 (serine) at collision energies of −12, −15, −20, and −18V, respectively. Simultaneously the four molecules were monitored by single-ion monitoring (SIM). Data were analyzed with Analyst software version 1.6.1 (Applied Biosystems).

Statistical Analysis

Prism software (GraphPad, San Diego, CA) was used for statistical analysis. Data was expressed as mean ± *SD*. Results were analyzed by two tailed paired *t*-test, $p < 0.05$ was considered statistically significant.

RESULTS

Genome Locations of the *C. jejuni* C4-Dicarboxylate Transporters

Genome analysis revealed that *C. jejuni* possesses up to five putative C4-dicarboxylate transporters, DctA, DcuA, DcuB, and some strains contain one or two homologs of the DcuC C4-dicarboxylate transporter. *C. jejuni* 81116 possess five C4-dicarboxylate transporters, which are all located at a different locus in the genome (**Figure 1**). The genes *dcuB, dcuC,* and *dctA* are not co-transcribed with other genes located in a single-gene operon, while *dcuA* is located in one operon together with the *aspA* gene and the *dcuC2* gene is located in one operon with C8J_1306 and the *metC* genes.

Transcription Regulation of the *C. jejuni* C4-Dicarboxylate Transporters

In a number of bacterial species, the C4-dicarboxylate transporters are regulated by oxygen, the available C4-dicarbolytes and/or by growth phase. To investigate whether this also applies for the C4-dicarboxylate transporters in *C. jejuni* we performed real-time RT PCR. We first used mRNA isolated from the wild-type 81116 strain grown at 10 or 0.3%

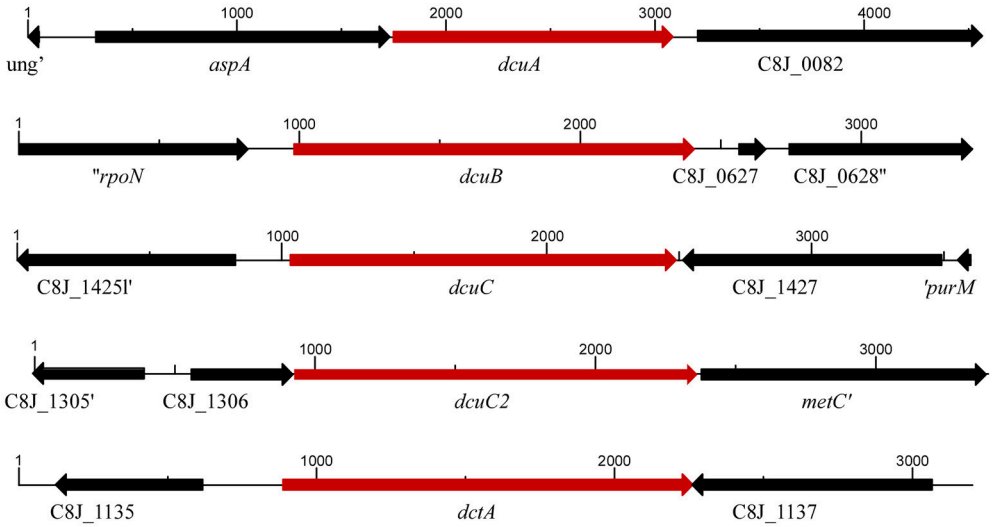

FIGURE 1 | Organization of the C4-dicarboxylate transporters in the genome of *C. jejuni* 81116. The size and orientation of the genes in C4 transporters regions are indicated.

oxygen (**Figure 2A**). Only the transcripts of the *dcuA* and *dcuB* genes showed a minor increase of three- to four-fold under oxygen limited conditions. Addition of serine or the C4-carbon sources, aspartate, succinate or fumarate to the culture medium of the wild-type grown until the logarithmic or stationary phase (data not shown) at 0.3 or 10% oxygen (data not shown) did not influence the transcription of the C4-dicarboxylate transporters (**Figure 2B**). Finally, we tested whether the growth phase (by comparing logarithmic vs. stationary phase) influences the transcription of the C4-dicarboxylate genes (**Figure 2C**). Only the transcription of the *dcuA* gene was regulated by growth phase as a 15-fold higher transcript level was observed at the logarithmic phase compared to the stationary phase. These results indicate that the regulation of C4-dicarboxylate transporters in other bacteria cannot be extrapolated to that of *C. jejuni*.

dcuA and *dcuB* Are Directly Regulated by *racR*

We previously showed that the two-component regulator RacR regulates the *dcuA* transporter gene. As *dcuC* and *dcuC2* were not present on the used microarrays slides we performed real-time RT PCR using RNA isolated of the wild-type and RacR mutant grown under RacR inducing conditions (0.3% O_2 and 25 mM nitrate). Transcription of *dcuC*, *dcuC2,* and *dctA* genes was not affected by mutation of *racR*, however like the proven RacR dependent genes *dcuA* and *aspA,* a decrease of the *dcuB* transcription was observed in the *racR* mutant (**Figure 3A**).

To investigate whether RacR directly regulates the *dcuB* promoter we performed electrophoretic mobility shift assays (EMSA). As previously shown, phosphorylated RacR was able to bind to the ^{32}P-labeled *aspA-dcuA* promoter region, however RacR also binds to the *dcuB* promoter (**Figure 3B**). The band shift disappeared when unlabelled DNA corresponding to the *dcuB* promoter region was added in excess. As expected from the real-time RT PCR results, the *dcuC*, *dcuC2,* and *dctA* promoter regions are not recognized by the RacR protein. These results show that the *dcuA* as well as *dcuB* are directly regulated by the two-component system regulator RacR.

Dcta Is Active at Elevated Oxygen Levels

To phenotypically address the function of the C4-dicarboxylate transporter in *C. jejuni* we disrupted the *dctA*, *dcuA*, *dcuB*, *dcuC,* and *dcuC2* genes by substituting large parts of the genes with an antibiotic resistance cassette. Although the transcription of C4-dicarboxylate transporter in *C. jejuni* is not regulated by oxygen (**Figure 2A**), we investigated whether oxygen has an influence of the activity of these transporters. Hereto growth curves in the presence of 10% O_2 were generated of the wild-type and the *dctA*, *dcuA*, *dcuB*, *dcuC,* and *dcuC2* mutants in HI or with the addition of 25 mM serine, aspartate, succinate, or fumarate. No clear growth differences between the strains were observed when they were growing in HI or HI with 25 mM serine (**Figures 4A,B**). However, all strains reached a higher OD when serine was added to the medium, suggesting that the available serine or carbon is a limited compound in HI. When one of the C4-carbon sources, aspartate, succinate, or fumarate were added to the HI medium, the maximum final OD of all strains except for the *dctA* mutant, was also higher compared to HI alone (**Figures 4C–E**), indicating that carbon availability in HI is a limiting growth factor. The *dctA* mutant reached a similar maximum final OD in HI as in HI with additional aspartate, succinate or fumarate indicating that the *dctA* mutant is unable to utilize or to take up these carbon sources from the medium. Based on these results we conclude that the DctA transporter is the only C4-dicarboxylate transporter needed to allow *C. jejuni* to growth on C4-carbon sources at high oxygen levels.

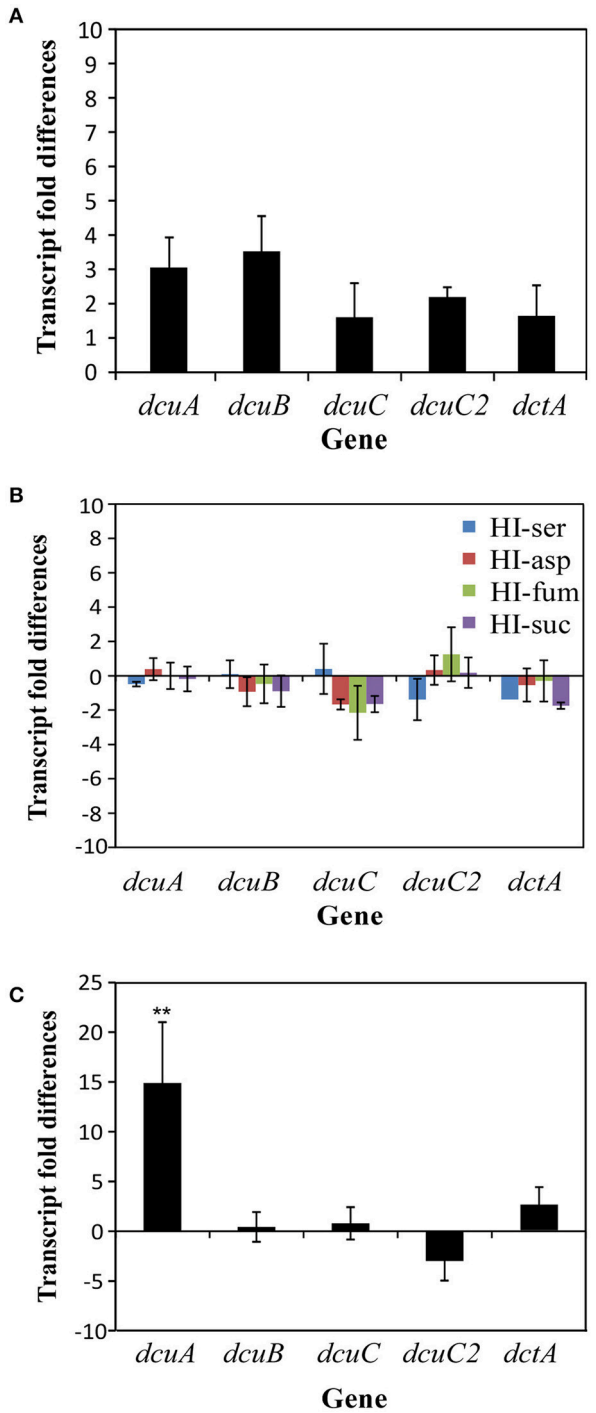

FIGURE 2 | Influence of oxygen, carbon sources, and growth phase on the transcription of the C4-dicarboxylate transporter genes as measured by real-time RT-PCR. (A) Transcript fold difference of the *dcuA*, *dcuB*, *dcuC*, *dcuC2,* and *dctA* genes in logarithmic phase grown wild-type *C. jejuni* (10 h) at 10% O_2 compared to 16 h at 0.3% O_2. **(B)** The effect of adding various carbon sources to the HI culture medium on the transcription of the C4-dicarboxylate genes. Hereto total RNA was isolated from wild-type grown in HI or HI with 25 mM serine, aspartate, fumarate, or succinate for 10 h. **(C)** Influence of the growth phase on the transcription of the C4-dicarboxylate

(Continued)

FIGURE 2 | Continued

genes as estimated for wild-type bacteria by real-time RT-PCR. Total RNA was extracted from logarithmic (10 h), or stationary (20 h) phase cultures. Fold change relative to the transcription levels was calculated using the arithmetic formula ($2^{-\Delta\Delta Ct}$). The *gyrA* gene was used as normalization gene. Data of four independent experiments with two independent preparations of RNA are presented as mean values ± standard deviation, $^{**}P < 0.01$.

Main Function of *dctA* Is the Uptake of Succinate from the Medium at Elevated Oxygen Levels

To investigate why the *dctA* mutant is unable to use aspartate, fumarate, and succinate at 10% O_2, we measured concentrations of serine, aspartate, succinate, and fumarate in the culture supernatants of the wild-type and C4-dicarboxylate transporter mutants at 4, 8, 16, and 24 h (**Figure 5A**). Without the addition of extra C4-dicarboxylate compounds HI medium contains no detectable fumarate, 0.4 mM succinate, 1.0 mM aspartate, and 1.2 mM serine. All added carbon sources, serine, succinate, aspartate, and fumarate were completely utilized by the wild-type bacteria within 24 h growth at 10% O_2, however serine and fumarate were removed earlier from the media than succinate and aspartate. Fumarate was converted to succinate and then secreted to the medium. Once the fumarate was completely used the secreted succinate was taken up again and utilized by *C. jejuni* (**Figure 5A**) Similar results were obtained for the *dcuA*, *dcuB*, *dcuC,* and *dcuC2* mutants (data not shown). A different result was obtained for the *dctA* mutant. The *dctA* mutant was still able to take up serine, to convert fumarate to succinate and, although reduced, to take up aspartate (**Figure 5B**), but was unable to take up succinate from the media and accumulated in the supernatant of media containing excess serine, aspartate, or fumarate. These results indicate that the DctA transporter of *C. jejuni* is involved in the uptake of aspartate and indispensable is for the uptake of succinate from the media at elevated oxygen conditions.

Redundancy of C4-Dicarboxylate Transporter Function under Oxygen-Limited Conditions

To investigate which of the C4-dicarboxylate transporters are active under oxygen-limited conditions, we generated growth curves of the wild-type, the *dctA*, *dcuA*, *dcuB*, *dcuC,* and *dcuC2* mutants as well as a double *dcuA*/*dcuB* and a triple *dcuA*/*dcuB*/*dcuC* mutant in HI or HI with the addition of 25 mM serine, aspartate, succinate, or fumarate (**Figures 6A–E**). The maximum optical densities reached under these oxygen-limited conditions were all lower compared to the growth curves obtained at 10% O_2. No obvious growth differences were observed between the strains when they were grown in HI, HI + serine, or HI + succinate. The maximum OD of all strains was higher in HI + serine compared to HI alone, indicating that serine is utilized by *C. jejuni* under these conditions (**Figure 6B**). In contrast, succinate is not utilized under the oxygen-limited conditions as the growth curves generated in HI + succinate were

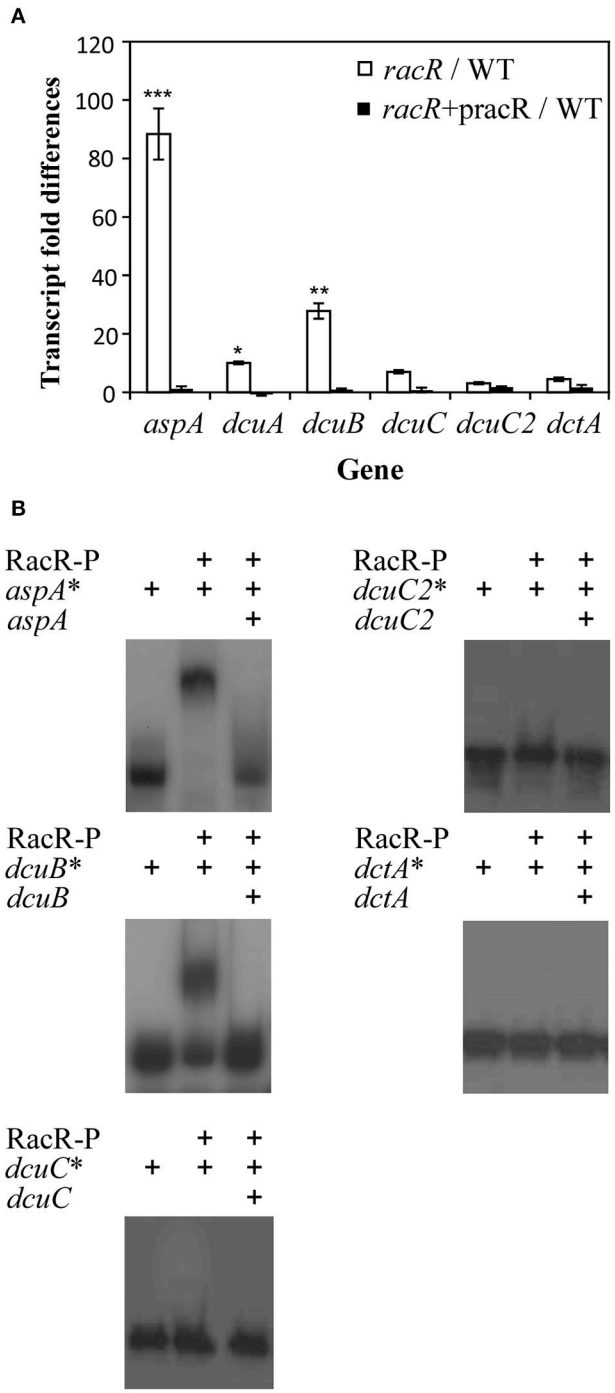

FIGURE 3 | RacR directly regulates the *dcuA* and *dcuB* promoters. (A) Real-time RT-PCR data of the *dcuA*, *dcuB*, *dcuC*, *dcuC2,* and *dctA* genes in wild-type *C. jejuni* compared to the RacR mutant or the complemented RacR mutant strain. Data of four independent experiments with two independent preparations of RNA are presented as mean ds ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(B)** Electrophoretic mobility shift assays with the *aspA, dcuB, dcuC, dcuC2,* and *dctA* promoter regions labeled with [γ-^{32}P]ATP and phosphorylated RacR protein. Radioactive labeled promoter regions are marked with *. RacR was phosphorylated by RacScyto in the presence of ATP. The specificity of the protein-DNA interaction was determined by the addition of a 10-fold excess of unlabelled promoter region DNA.

similar as in HI. Growth defects were observed when fumarate or aspartate was added to the HI medium (**Figures 6C,E**). All single mutants grew less compared to the wild-type in HI + aspartate, especially the *dcuB* mutant. To investigate whether the C4-carboxylate transporter can replace each other function, we also tested double and triple mutants. A more severe growth defect in HI + aspartate compared to the *dcuB* mutant was observed for the *dcuA*/*dcuB* mutant but not for a *dctA*/*dcuA* (data not shown). A similar *dcuA*/*dcuB* growth defect was seen for the *dcuA*/*dcuB*/*dcuC* triple mutant, suggesting that *dcuC* is not involved in the utilization of aspartate. A small reduction in growth yield was observed for the single mutants *dcuC* and *dcuB* in HI + fumarate. The growth defect was more obvious in the triple mutant *dcuA*/*dcuB*/*dcuC,* suggesting that both DcuB and DcuC are involved in the utilization of fumarate.

Secretion of Succinate Is Dependent on a Functional *dcuB* under Limited Oxygen Conditions

To investigate the role of various active C4-transporters under oxygen-limited conditions we measured at time points 6, 12, 24, and 36 h the serine, aspartate, succinate, and fumarate concentration in the culture supernatants of the wild-type and C4-transporters mutants (**Figure 7**). Serine was used within 12 h by the wild-type and within 24 h by the *dcuB* mutant, the slowest grower in HI + serine (**Figure 7A**). When the strains were grown on aspartate they secreted succinate to the medium, except for the strains which were also mutated in the *dcuB* gene (**Figure 7B**). No succinate could be measured in the supernatant of the *dcuB* mutants showing that DcuB under oxygen-limited conditions transports succinate into the environment. Both the *dcuA* and *dcuB* mutants were able to utilize aspartate, however when both genes were mutated no decrease in the aspartate concentration in the medium was observed, indicating that both genes are needed to sequester aspartate from the medium. Growing the strains in excess fumarate revealed that not *dcuC,* but both *dcuA* and *dcuB* might be involved in the uptake of fumarate, as at 12 h the supernatant of these single mutants contain more fumarate than the supernatant of the wild-type (**Figure 7C**). The supernatant of all strains tested grown in excess fumarate contained succinate. However, the supernatant of the *dcuB* mutant strains contained less succinate, indicating that *Campylobacter* possesses a *dcu*-independent fumarate–succinate reductase.

The Availability of Nitrate under Oxygen-Limited Conditions Reduces the Role of the C4-Dicarboxylate Transporters

We showed that RacR directly regulates by inhibition, the *dcuA* and *dcuB* genes under limited oxygen and high nitrate conditions. To address the role of the *dcu* genes under these conditions, we performed growth curves in HI under oxygen-limited conditions with nitrate and aspartate or fumarate (**Figure 8**). The growth rate as well as the maximum optical densities of the wild-type and the mutants were similar under these conditions, suggesting that the C4-dicarboxylate transporters are less active under these conditions.

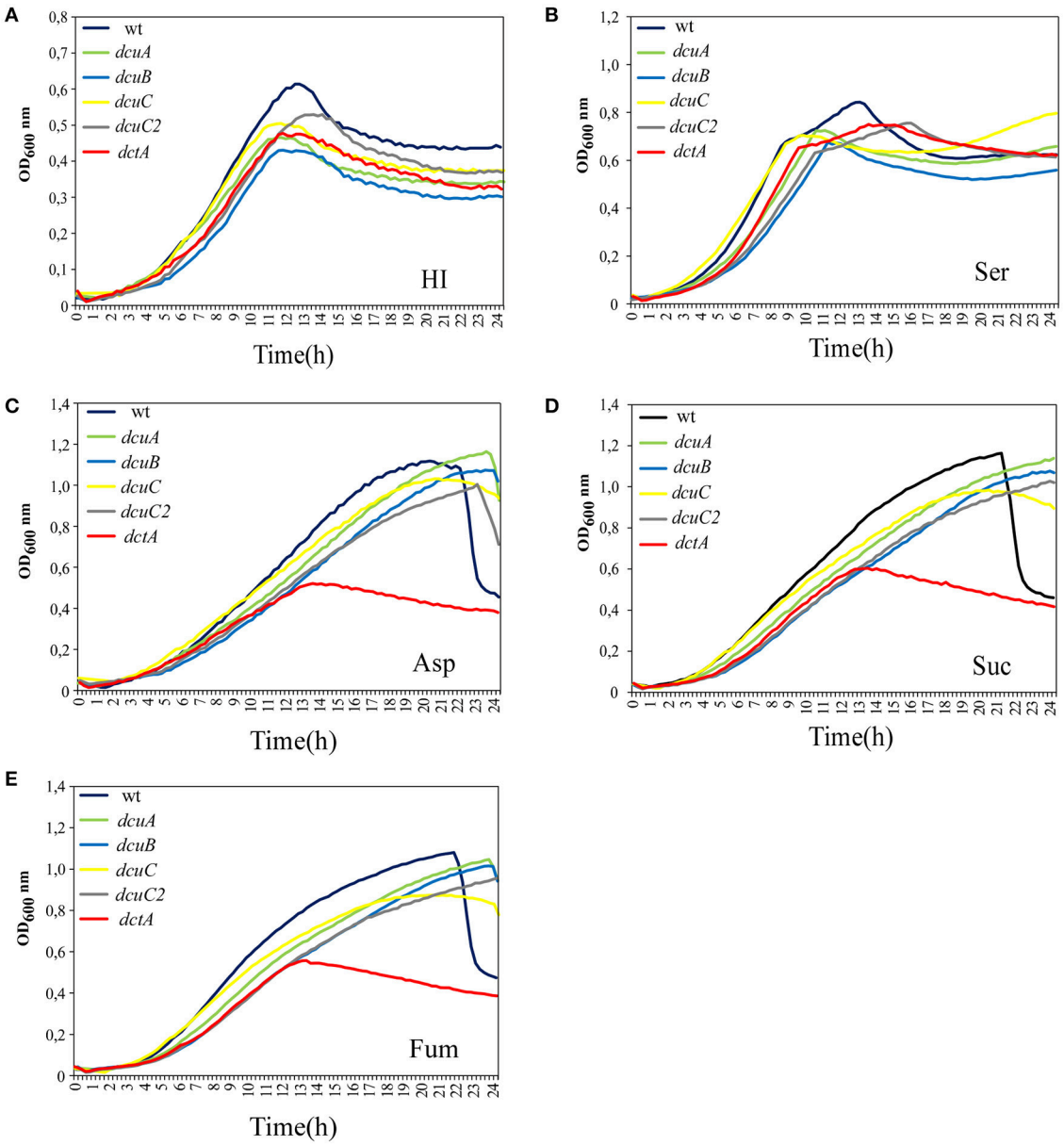

FIGURE 4 | DctA is the only C4-carboxylate transporter necessary at high oxygen conditions (10% O_2). Growth curves of *C. jejuni* wild-type or the *dcuA*, *dcuB*, *dcuC*, *dcuC2,* and *dctA* mutants were generated in HI **(A)**, or HI with the addition of 25 mM serine **(B)**, aspartate **(C)**, succinate **(D)**, or fumarate **(E)** under microaerobic conditions (10% O_2, 10% CO_2, 70% N_2, and 10% H_2) at 42°C. The optical density at 600 nm was measured every 15 min. The experiments were repeated three times in duplicate.

Nitrate Prevents the Secretion of Succinate under Oxygen-Limited Conditions

To further address the role of the C4-dicarboxylate transporters under RacR inducing conditions, we measured the aspartate, fumarate, and succinate concentrations in the supernatant of the wild-type and the C4-dicarboxylate mutants at 6, 12, 24, and 36 h. The C4-carbon content in the supernatants of the strains growing under oxygen-limited conditions with nitrate were much more similar compared to the strains grown under oxygen-limited conditions (**Figure 9**). The aspartate uptake in the wild-type, *dcuA* and *dcuB* mutants under these conditions were similar, however only half of the aspartate in the supernatant of these cultures was utilized (**Figure 9A**). These results are in accordance with the growth curves and show that the C4-transporters are less active under these conditions. No reduction of the aspartate concentration was seen in the supernatant of the *dcuAB* mutant, confirming that both DcuA and DcuB are needed to take up aspartate from the medium. The amount of succinate produced by the wild-type under these conditions was nine-fold lower than under oxygen-limited conditions, similar results were seen for

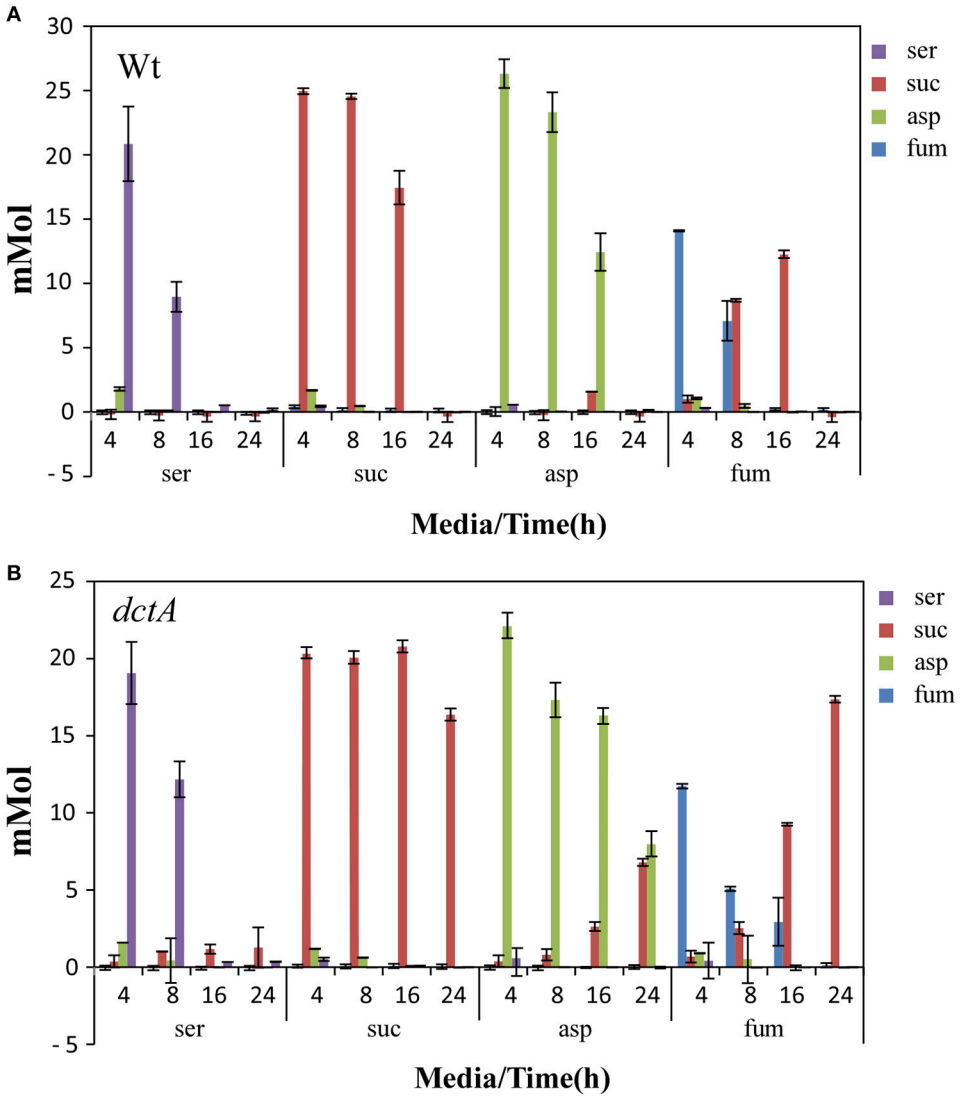

FIGURE 5 | DctA transports succinate into the cell. HPLC-MS-MS analysis of the culture supernatants of wild-type **(A)** and *dctA* mutant **(B)** taken after 4, 8, 16, or 24 h of growth under 10% O_2 with 25 mM serine, aspartate, fumarate, or succinate. Data represent the mean and standard error of three independent experiments.

the *dctA* and *dcuC2* mutants. In all other mutants no succinate could be detected in the supernatant. These results show that the activity like the transcription (**Figure 3A**) of aspartate–fumarate–succinate pathway under oxygen-limited condition is strongly reduced when nitrate is available. In the wild-type the utilization of fumarate in the presence of nitrate was similar as without nitrate, however the amount of secreted succinate was two-fold lower when nitrate was present (**Figure 9B**). Similar results were obtained for all other mutants (data not shown), indicating that *Campylobacter* possesses a *dcu* nitrate independent fumarate–succinate reductase.

DISCUSSION

Dependent on the oxygen concentration, C4-dicarboxylate transporters have been shown to play an important role in the transport of C4-carbon sources such as fumarate, succinate, and aspartate. These carbon sources are even more important for the microaerophilic bacterium *C. jejuni* as most strains cannot ferment nor oxidize carbohydrates. *C. jejuni* cannot grow under aerobic or strictly anaerobic oxygen conditions therefore we have attempted to obtain a more complete understanding of the function and regulation of the C4-dicarboxylate transporters in *C. jejuni*.

Like in other bacteria the C4-dicarboxylate transporter genes of *C. jejuni* are dispersed over the genome (**Figure 1**). As they all are transcribed from different promoters, they might be regulated differently, as is seen in *E. coli,* where several transcription factors are involved in the regulation of the C4-dicarboxylate transporters in response to oxygen, C4-dicarboxylate compounds and growth phase (Janausch et al., 2002). Here we showed by real-time RT-qPCR that the oxygen concentration had only a

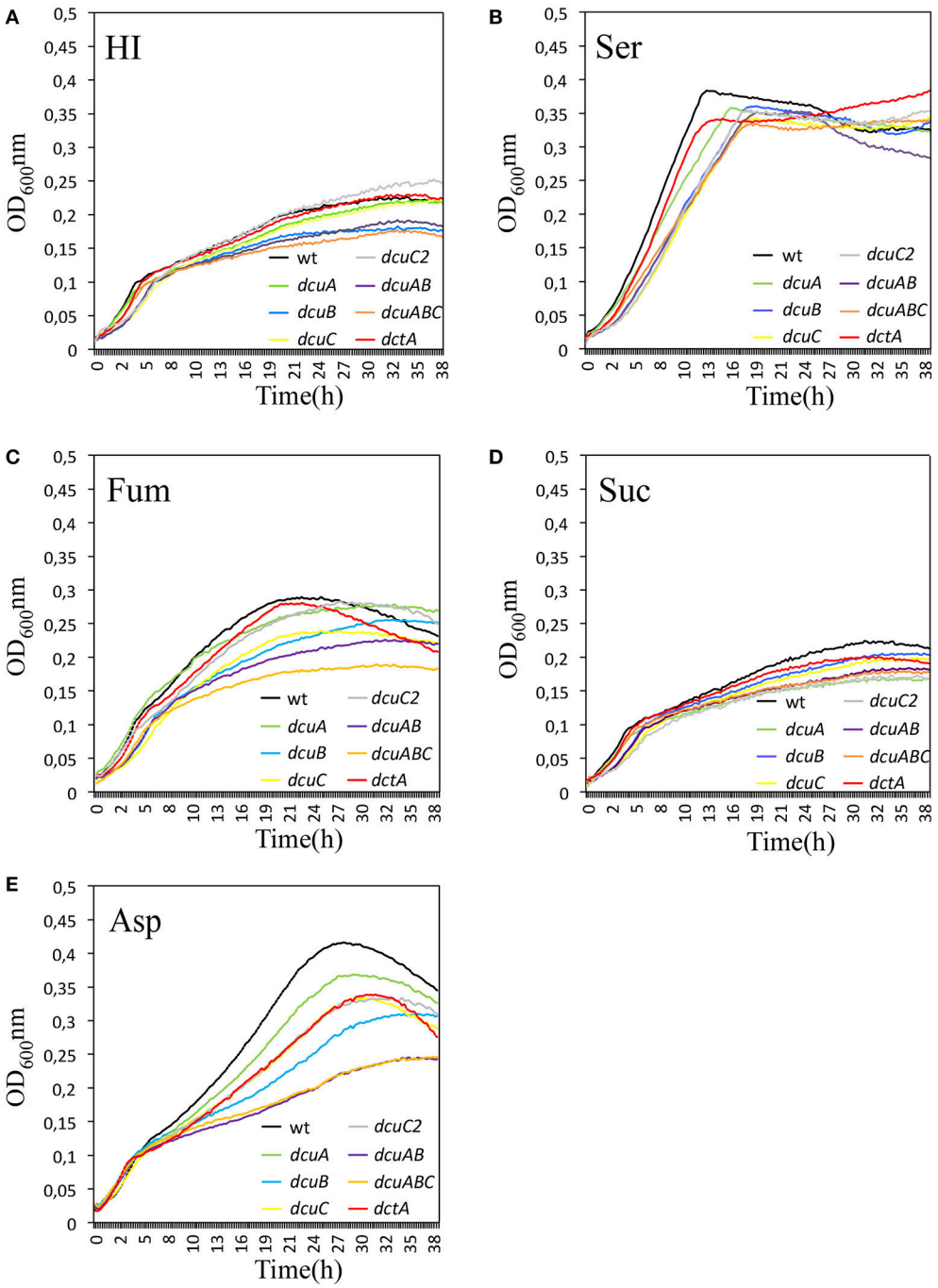

FIGURE 6 | DcuA and DcuB are the main C4-dicarboxylate transporters under oxygen-limited conditions. Growth curves were generated of *C. jejuni* wild-type, the single mutants *dcuA*, *dcuB*, *dcuC*, *dcuC2,* and *dctA,* the double mutant *dcuA/B* and the triple mutant *dcuA/B/C* under oxygen-limited conditions (0.3% O_2). Strains were growing in HI **(A)**, or HI with the addition of 25 mM serine **(B)**, fumarate **(C)**, succinate **(D)**, or aspartate **(E)** under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79% N_2, 10% H_2) at 42°C. The optical density at 600 nm was measured every 15 min. The experiments were repeated three times in duplicate.

minor but not significant effect on the transcription of the *dcuA* and *dcuB* genes in strain 81116, and that the addition of C4-dicarboxylate compounds in the complex media HI had no influence on the transcription of the C4-dicarboxylate transporter genes (**Figure 2**). Similar results were obtained in another *C. jejuni* strain 11168 (Woodall et al., 2005). Growth phase of the culture influenced only the transcription of the *dcuA* gene. Based on these results no transcription factor of *C. jejuni* has obtained a similar function as the *E. coli* transcription factors regulating the C4-dicarboxylates, such as FNR, ArcAB, DcuRS,

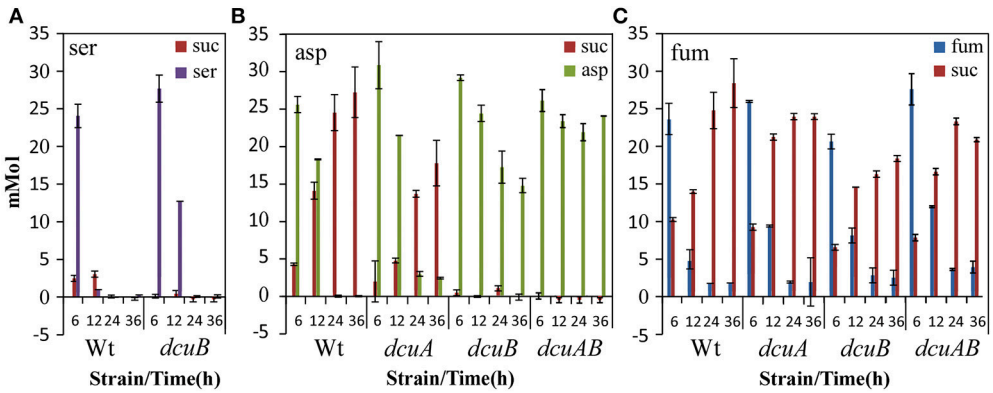

FIGURE 7 | DcuB secretes succinate into the environment under oxygen-limited conditions. HPLC-MS-MS data of the supernatants of wild-type, *dcuA*, *dcuB*, or *dcuAB* mutant cultures taken after 6, 12, 24, or 36 h of growth at 0.3% O_2 with 25 mM serine **(A)**, aspartate **(B)**, or fumarate **(C)**. Data represent the mean and standard error of three independent experiments.

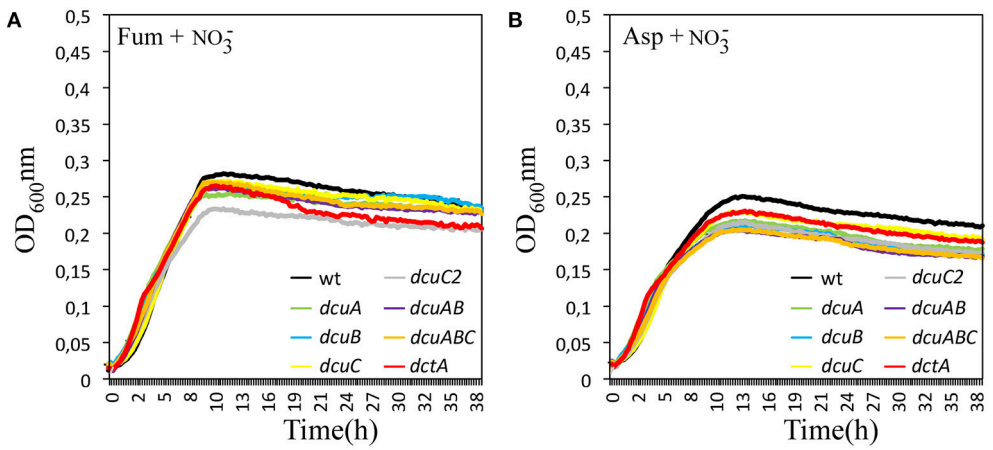

FIGURE 8 | Addition of nitrate under limited oxygen conditions nullifies the C4-dicarboxylate mutant phenotype. Growth curves were generated of *C. jejuni* wild-type, the single mutants *dcuA*, *dcuB*, *dcuC*, *dcuC2,* and *dctA,* the double mutant *dcuA/B* and the triple mutant *dcuA/B/C* in HI with 25 mM nitrate and 25 mM fumarate **(A)** or aspartate **(B)** at 42°C under oxygen-limited conditions. The optical density at 600 nm was measured every 15 min. The experiments were repeated three times in duplicate.

or CRP (Janausch et al., 2002). Apparently, the transcription regulation in response to oxygen, C4-dicarboxylates and growth phase as seen in *E. coli* is not important for the function of the genes in *C. jejuni*.

We have shown that both the *dcuA* and *dcuB* genes are repressed and directly regulated by the RacRS system in response to low oxygen and nitrate. The *dcuA* is in most bacteria constitutively expressed and so far *C. jejuni* is the only organism in which both genes are regulated in a similar manner. The phenotypes observed for the *dcuA* and *dcuB* mutants under oxygen-limited conditions (**Figure 6**) were restored by the addition of nitrate to the medium (**Figure 8**), clearly showing that these genes are not needed under these conditions. Regulation of the C4-dicarboxylate genes by nitrate is not uncommon as the *dcuB* gene in *E. coli* is repressed by the two-component NarXL system (Overton et al., 2006). The use of nitrate as electron acceptor (E_m nitrate/nitrite + 430 mV) is preferred over fumarate (E_m fumarate/succinate + 30 mV) enabling fumarate to be used as carbon source instead of electron acceptor under oxygen-limited conditions. This explains the observed reduced secretion of succinate depicted in **Figure 9**.

The secretion of succinate in *C. jejuni* appeared to be mediated solely by the DcuB C4-transporter (**Figure 7**). In *E. coli*, the DcuB and DcuC C4-transporters, both regulated by FNR, can act synergistically to secrete succinate under anaerobic conditions (Golby et al., 1999). So far, we were unable to address the function of the two DcuC homologs in *C. jejuni* 81116 which are not co-regulated with *dcuB* and are absent in other *C. jejuni* strains (Parkhill et al., 2000; Hofreuter et al., 2006). Both DcuA and DcuB are involved in the uptake of aspartate as well as fumarate, confirming the data of Guccione et al. (2008). By regulating both DcuA and DcuB, the RacRS system completely controls the

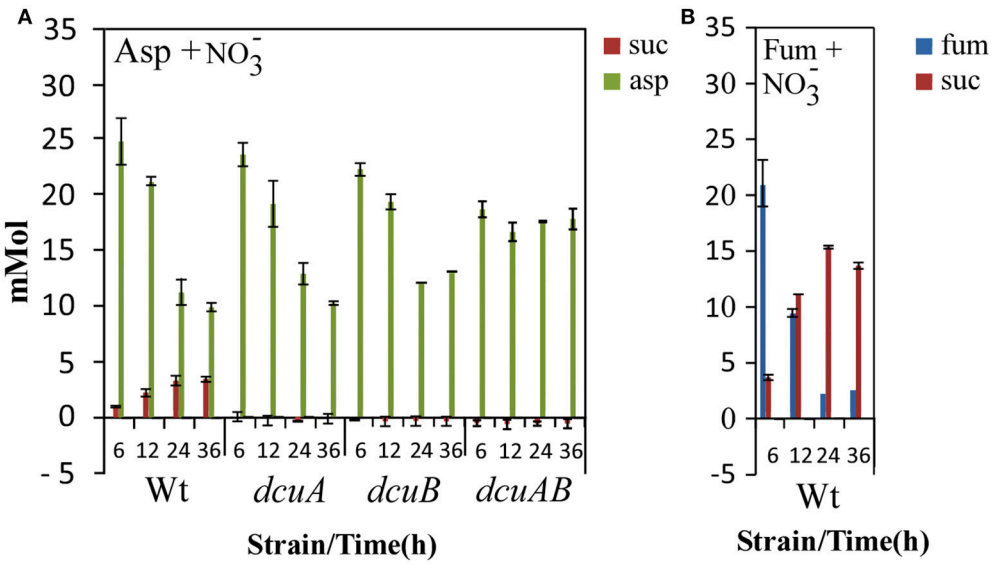

FIGURE 9 | Nitrate prevents the secretion of succinate to the environment. HPLC-MS-MS of the supernatants of wild-type, *dcuA*, *dcuB,* or *dcuAB* mutant cultures taken after 6, 12, 24, or 36 h of growth under 0.3% O_2 with 25 mM nitrate and 25 mM aspartate **(A)** or 25 mM nitrate and 25 mN fumarate **(B)**. Data represent the mean and standard error of three independent experiments.

fumarate respiration in response to limited oxygen availability and the presence of nitrate.

Succinate is taken up by the DctA transporter and used as carbon source by *C. jejuni* when oxygen is not scarce (**Figure 5**). The DctA transporter is also needed to allow *C. jejuni* to use aspartate and fumarate as carbon source, indicating that the *dctA* mutant is unable to use or to take up these carbon sources from the medium. In *E. coli* and *Bacillus subtilis*, DctA also mediates the uptake of succinate as well as fumarate and aspartate under aerobic conditions (Davies et al., 1999; Asai et al., 2000; Janausch et al., 2002). However, no difference was observed between the *C. jejuni* wild-type and *dctA* mutant in the uptake of fumarate, suggesting that *C. jejuni* DctA is not involved in the uptake of fumarate. When fumarate or aspartate are present in the media, a large amount of energy rich succinate accumulated in the media which could not be re-used by the *dctA* mutant explaining the reduced growth of *dctA* mutant under these conditions. Beside the regulation it also appears that the function of C4-dicarboxylate genes differs in *C. jejuni*.

In our work, we highlighted the regulation and function of various C4-dicarboxylate genes in the microaerophilic bacterium *C. jejuni*. The DctA transporter is responsible for the uptake of succinate under high oxygen levels. The *dcuA* and *dcuB* genes are the only C4-dicarboxylate-regulated genes and are dependent on the two-component RacRS system in response to low O_2 and high nitrate concentrations. DcuB is the only C4-dicarboxylate/succinate antiporter in *C. jejuni* which secretes succinate when oxygen levels are low, but is not necessary when nitrate is available.

AUTHOR CONTRIBUTIONS

MW designed experiments, wrote the article, and performed growth experiments. JVP wrote the article, CVDL performed the High-Performance Liquid Chromatography analysis, and LVD performed all other experiments.

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The *Campylobacter jejuni* Oxidative Stress Regulator RrpB Is Associated with a Genomic Hypervariable Region and Altered Oxidative Stress Resistance

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Campylobacter jejuni is the leading cause of bacterial foodborne diarrhoeal disease worldwide. Despite the microaerophilic nature of the bacterium, *C. jejuni* can survive the atmospheric oxygen conditions in the environment. Bacteria that can survive either within a host or in the environment like *C. jejuni* require variable responses to survive the stresses associated with exposure to different levels of reactive oxygen species. The MarR-type transcriptional regulators RrpA and RrpB have recently been shown to play a role in controlling both the *C. jejuni* oxidative and aerobic stress responses. Analysis of 3,746 *C. jejuni* and 486 *C. coli* genome sequences showed that whilst *rrpA* is present in over 99% of *C. jejuni* strains, the presence of *rrpB* is restricted and appears to correlate with specific MLST clonal complexes (predominantly ST-21 and ST-61). *C. coli* strains in contrast lack both *rrpA* and *rrpB*. In *C. jejuni rrpB*$^+$ strains, the *rrpB* gene is located within a variable genomic region containing the IF subtype of the type I Restriction-Modification (*hsd*) system, whilst this variable genomic region in *C. jejuni rrpB*$^-$ strains contains the IAB subtype *hsd* system and not the *rrpB* gene. *C. jejuni rrpB*$^-$ strains exhibit greater resistance to peroxide and aerobic stress than *C. jejuni rrpB*$^+$ strains. Inactivation of *rrpA* resulted in increased sensitivity to peroxide stress in *rrpB*$^+$ strains, but not in *rrpB*$^-$ strains. Mutation of *rrpA* resulted in reduced killing of *Galleria mellonella* larvae and enhanced biofilm formation independent of *rrpB* status. The oxidative and aerobic stress responses of *rrpB*$^-$ and *rrpB*$^+$ strains suggest adaptation of *C. jejuni* within different hosts and niches that can be linked to specific MLST clonal complexes.

Keywords: *Campylobacter jejuni*, oxidative stress response regulation, aerobic stress response regulation, transcription factors, restriction modification system

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INTRODUCTION

Campylobacter jejuni is the leading cause of bacterial foodborne diarrhoeal disease worldwide with an estimated 400 million human infections occurring each year (Ruiz-Palacios, 2007). The predominance of *C. jejuni* can be attributed to the ability to survive in the environment as well as within avian and mammalian hosts despite the microaerophilic nature of this bacterium

(Byrne et al., 2007). *C. jejuni* has evolved specific adaptation mechanisms to survive under atmospheric oxygen conditions (Kim et al., 2015). In addition to aerobic stress such as the exposure to increased levels of oxygen under atmospheric conditions, *C. jejuni* can also encounter stress conditions within the host, specifically oxidative stress in the form of reactive oxygen species (ROS) during *in vivo* survival (Fang, 2004; Palyada et al., 2009). ROS is a collective term that describes the chemical species generated upon incomplete reduction of oxygen (Imlay, 2003) with examples including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (D'Autreaux and Toledano, 2007). The accumulation of ROS in the bacterial cytoplasm and periplasm leads to damage of nucleic acids, proteins and membrane structures (Atack and Kelly, 2009).

Bacteria that can survive either within a host or in the environment like *C. jejuni* require variable responses to survive the stresses associated with exposure to different levels of ROS (Kim et al., 2015). Therefore it is not surprising that *C. jejuni* contains a number of regulatory proteins involved in the oxidative stress response such as PerR (Handley et al., 2015), Fur (van Vliet et al., 2000), and CosR (Hwang et al., 2011). The *C. jejuni* NCTC 11168 genome also contains two MarR-type transcriptional regulators that have previously been designated as RrpA and RrpB (Gundogdu et al., 2011, 2015). Using *C. jejuni* 11168H [a hypermotile derivative of the original sequenced strain NCTC 11168 that shows higher levels of cecal colonization in a chick colonization model (Karlyshev et al., 2002; Jones et al., 2004)] we have shown that both RrpA and RrpB play a role in oxidative and aerobic stress responses with auto-regulatory activity typical of MarR-type transcriptional regulators (Gundogdu et al., 2011, 2015). In addition, RrpA has also been shown to bind upstream of *katA* suggesting that RrpA directly influences the expression of catalase (KatA). Both 11168H *rrpA* and *rrpB* mutants exhibited reduced KatA activity. However, a 11168H *rrpAB* double mutant exhibited higher levels of resistance to hydrogen peroxide oxidative stress, but similar levels of KatA activity compared to the wild-type strain. Neither the 11168H *rrpA* mutant nor the 11168H *rrpB* mutant exhibited any significant difference in sensitivity to either cumene hydroperoxide or menadione oxidative stresses, but both mutants exhibited reduced cytotoxicity in the *Galleria mellonella* model of infection and enhanced biofilm formation. However, the 11168H *rrpAB* double mutant exhibited wild-type levels of both cytotoxicity in the *G. mellonella* model of infection and biofilm formation. Together these data indicate a role for both RrpA and RrpB in the *C. jejuni* oxidative and aerobic stress responses, enhancing bacterial survival both within a host and in the environment, but also prompted further investigations in order to understand the specific roles of RrpA and RrpB.

Traditional typing methods have failed to identify *C. jejuni* strains from different sources that cause disease in humans (Champion et al., 2005). Human infections are largely attributed to undercooking of poultry products or poor food hygiene practices involving handling of such produce (Sheppard et al., 2009). *C. jejuni* can survive in many different niches with the organism isolated from avian, animal, human, and environmental sources (Young et al., 2007). However, whole genome phylogenetic analysis of *C. jejuni* strains using microarrays has identified different clades and subclades linked to the source of the isolate (Champion et al., 2005; Stabler et al., 2013). Previously we identified differences in the distribution of *rrpA* and *rrpB* regulators amongst 111 *C. jejuni* strains with *rrpA* present in over 95% and *rrpB* in approximately only 50% of these strains (Gundogdu et al., 2011). A more recent analysis of 270 *C. jejuni* strains identified nine clusters (C1–C9) based on genotype and multilocus sequence typing (MLST) data and indicated that clusters C1–C6 were dominated by livestock-associated clonal complexes, whilst clusters C7–C9 contained the majority of the water and wildlife associated clonal complexes (Stabler et al., 2013). Using the original data from this study, *rrpA* was identified in 129/133 (96.99%) strains within the C1–C6 subclades and in 130/137 (94.89%) strains within the C7–C9 subclades. In contrast *rrpB* was identified in 102/133 (76.67%) strains within the C1–C6 subclades and only in 19/137 (13.87%) strains within the C7–C9 subclades. In total *rrpA* was identified in 259/270 (95.92%) strains whilst *rrpB* was identified in only in 121/270 (44.81%) strains (Supplementary Figure 1; Supplementary Table 1).

The discovery of the varied distribution for *rrpA* and *rrpB* has led us to further explore the potential reasons as to why certain strains have one or both regulators. To try and understand the reasons for this, we have investigated the presence or absence of *rrpA* and *rrpB* in 4,232 *C. jejuni* and *C. coli* genome sequences. Analysis of 4,232 *Campylobacter* genomes showed that whilst *rrpA* is present in over 99% of *C. jejuni* strains, the presence of *rrpB* is restricted and appears to correlate with livestock-associated MLST clonal complexes. Further analysis showed that the presence of *rrpB* is linked to a hypervariable region containing the IF subtype of the type I Restriction-Modification (*hsd*) system, whereas $rrpB^-$ strains contain the IAB subtype *hsd* system. Further investigation of the phenotypes of different *C. jejuni* $rrpB^-$ and $rrpB^+$ strains identified a link between the presence of the MarR-type transcriptional regulator RrpB with the ability of *C. jejuni* to adapt and survive in different environmental niches. The oxidative and aerobic stress response of $rrpB^-$ and *rrpB*+ strains suggests adaptation of *C. jejuni* within different hosts and niches that can be linked with specific MLST clonal complexes.

MATERIALS AND METHODS

Comparative Genomics of *rrpA* and *rrpB* within *Campylobacter* Genomes

A total of 4,232 complete and draft *Campylobacter* genome sequences (3,746 *C. jejuni* and 486 *C. coli*) were obtained from public collections (Jolley and Maiden, 2010; Cody et al., 2013) and are listed in Supplementary Table 2 with accession numbers/pubMLST IDs and assembly status. These genomes were previously used for identification of DNase-genes (Brown et al., 2015), CRISPR repeats and *cas* genes (Pearson et al., 2015) and the fucose utilization operon (Dwivedi et al., 2016). Genomes

included were between 1.5 and 2.0 Mbp and had at least 5 of the 7 MLST alleles identifiable using BLAST. The genomes were phylogenetically clustered using FFPry feature frequency profiling with a word length of 18 (Van Vliet and Kusters, 2015) and further analyzed by MLST (Pearson et al., 2015). Genomes were provisionally annotated using Prokka (Seemann, 2014) and searched for the presence of the predicted RrpA and RrpB proteins using BLASTP (Supplementary Table 2). Presence of the corresponding genes was also assessed at the DNA level using the MIST program (Kruczkiewicz et al., 2013) and the BLAST+ (v2.28) suite. Both DNA and amino acid comparisons were done using the *rrpA* genes/RrpA proteins from *C. jejuni* NCTC 11168, *C. jejuni* 81116, *C. jejuni* 414, and *C. coli* 76639, and the *rrpB* genes/RrpB proteins of *C. jejuni* NCTC 11168 and *C. coli* 2544. Conservation of flanking genes was assessed using the output from the comparative genomics software package Roary (Page et al., 2015) and the provisional Prokka annotation of the 4,232 *C. jejuni* and *C. coli* genome sequences (Supplementary Tables 2 and 3).

Bacterial Strains and Growth Conditions

Campylobacter jejuni strains (**Table 1**) were grown at 37°C in a microaerobic chamber (Don Whitley Scientific, United Kingdom) containing 85% N_2, 10% CO_2 and 5% O_2 either on blood agar (BA) plates containing Columbia agar base (Oxoid, United Kingdom) supplemented with 7% (v/v) horse blood (TCS Microbiology, United Kingdom) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella broth (Oxoid) with shaking at 75 rpm. *C. jejuni* strains were grown on BA plates for 24 h prior to use in all assays unless otherwise stated. *Escherichia coli* XL-2 Blue MRF' competent cells (Stratagene, United Kingdom) were used for cloning experiments and were grown at 37°C in aerobic conditions either on Luria-Bertani (LB) agar plates (Oxoid) or in LB broth (Oxoid) with shaking at 200 rpm. Antibiotics were added at the following concentrations; ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (50 μg/ml for *E. coli* studies or 10 μg/ml for *C. jejuni* studies). All reagents were obtained from Fisher Scientific (United Kingdom) unless otherwise stated.

Construction of *C. jejuni* Mutants

Campylobacter jejuni mutants were constructed as described previously (Gundogdu et al., 2011). Briefly, genes or gene fragments were amplified from *C. jejuni* genomic DNA using the appropriate gene specific primers (**Table 2**). PCR products were ligated with pGEM-T Easy vector (Promega, United Kingdom) and then transformed into XL-2 Blue MRF' cells. If required, inverse PCR mutagenesis (IPCRM) was performed to introduce a unique *Bgl*II site into the cloned gene. A kanamycin cassette (Kan^R) was then ligated into the unique *Bgl*II site within the cloned gene (Trieu-Cuot et al., 1985; van Vliet et al., 1998). These constructs were electroporated into competent *C. jejuni* cells and putative clones were confirmed by PCR and sequencing as described previously (Gundogdu et al., 2011).

Oxidative Stress and Aerobic Growth Assays

Oxidative stress and aerobic growth assays were performed as described previously (Gundogdu et al., 2011, 2015). Briefly, bacterial cells were harvested into 1 ml PBS and diluted to an OD_{600} of 1. For oxidative stress assays, bacterial cells were exposed to H_2O_2 at final concentrations of 25, 50, or 100 mM for 15 min, menadione at a final concentration of 100 mM for 60 min and cumene hydroperoxide at 0.05% (w/v) for 15 min, all at 37°C under microaerobic conditions. Serial dilutions were prepared and 10 μl of the 10^{-1}–10^{-6} dilutions spotted onto BA plates, incubated for 48 h and colonies counted. For growth curves, 10 ml Brucella broth was pre-incubated in a 30 ml flask at 37°C under microaerobic conditions for 24 h. Bacterial cells grown on BA plates for 24 h were used to inoculate pre-incubated Brucella broth at an OD_{600} of 0.1 and grown for up to 24 h at 37°C under microaerobic and aerobic conditions. OD_{600} readings were performed at selected time points. In addition bacterial colony forming units (CFUs) were assessed at time point 16 h under microaerobic and aerobic conditions.

Galleria mellonella Infection Model

Galleria mellonella infection assays were performed as described previously (Champion et al., 2010; Gundogdu et al., 2015). Briefly, *G. mellonella* larvae (LiveFoods Direct, United Kingdom) were stored at 16°C on wood chips. Ten larvae for each experiment were infected with a 10 μl inoculum of a 24 h *C. jejuni* culture diluted to OD_{600} 0.1 by micro-injection (Hamilton, Switzerland) in the right foremost leg, giving an infectious dose of approximately 10^6 CFU (Champion et al., 2010). Controls were injection with PBS and no injection. Larvae were incubated at 37°C with survival recorded at 24 h.

Biofilm Assays

Biofilm assays were performed as described previously (Gundogdu et al., 2015). Briefly, bacterial cells were harvested into Mueller Hinton broth, then inoculated to an OD_{600} of 0.1 into 10 ml Mueller Hinton broth pre-incubated in a 25 ml flask at 37°C under microaerobic conditions for 24 h prior to inoculation, then grown for 5 h at 37°C under microaerobic conditions with shaking at 75 rpm. The OD_{600} was readjusted to 0.1, then 1 ml of culture was added to a 24 well polystyrene plate (Corning, U.S.A) and incubated at 37°C under either aerobic or microaerobic conditions stationary for 72 h. The wells were washed twice with PBS, dried for 20 min at 37°C followed by addition of 1% (w/v) crystal violet (Sigma–Aldrich) for 15 min. The wells were washed three times with PBS, then destained with 10% (v/v) acetic acid / 30% (v/v) methanol. Absorbance (A_{595}) was measured using a SpectraMax M3 microplate reader (Molecular Devices, USA).

Statistical Analyses

The data is presented as mean ± SD. All experiments were performed with at least three biological replicates. Each biological replicate was performed in three technical replicates. Statistical analyses were performed using Prism software (GraphPad

TABLE 1 | *Campylobacter jejuni* strains used in this study.

Strain	Description	Reference
11168H	A hypermotile derivative of the original sequence strain NCTC 11168 that shows higher levels of caecal colonization in a chick colonization model. C3 clade. MLST clonal complex ST-21.	Karlyshev et al., 2002; Jones et al., 2004
81-176	Highly virulent and widely studied laboratory strain of *C. jejuni*. MLST clonal complex ST-42.	Korlath et al., 1985
81116	Genetically stable strain which remains infective in avian models. C9ii clade. MLST clonal complex ST-283.	Wassenaar et al., 1991
M1	M1 (laboratory designation 99/308) is a rarely documented case of direct transmission of *C. jejuni* from chicken to a person, resulting in enteritis. C9ii clade. MLST clonal complex ST-45.	Friis et al., 2010
11168H *rrpB* mutant	Isogenic 11168H *rrpB* mutant with insertion of a 1.4 kb Kan^R cassette	Gundogdu et al., 2011
11168H *rrpB* complement	*rrpB* complement constructed by the insertion of the *rrpB* CDS into the *Cj0233* pseudogene in the 11168H *rrpB* mutant (pDENNIS complementation vector used)	Gundogdu et al., 2011
81-176 *rrpB* mutant	Isogenic 81-176 *rrpB* mutant with insertion of a 1.4 kb Kan^R cassette	This study
81-176 *rrpB*complement	*rrpB* complement constructed by the insertion of the *rrpB* CDS into a rRNA gene in the 81-176 *rrpB* mutant (pRRC complementation vector used)	This study
11168H *rrpA* mutant	Isogenic 11168H *rrpA* mutant with insertion of a 1.4 kb Kan^R cassette.	Gundogdu et al., 2015
11168H *rrpA* complement	*rrpA* complement constructed by the insertion of the *rrpA* CDS into a rRNA gene in the 11168H *rrpA* mutant (pRRC complementation vector used).	Gundogdu et al., 2015
81-176 *rrpA* mutant	Isogenic 81-176 *Cj1546* mutant with insertion of a 1.4 kb Kan^R cassette	This study
81-176 *rrpA* complement	*rrpA* complement constructed by the insertion of the *rrpA* CDS into a rRNA gene in the 81-176 *rrpA* mutant (pRRC complementation vector used)	This study
81116 *rrpA* mutant	Isogenic 81116 *rrpA* mutant with insertion of a 1.4 kb Kan^R cassette	This study
81116 *rrpA* complement	*rrpA* complement constructed by the insertion of the *rrpA* CDS into a rRNA gene in the 81116 *rrpA* mutant (pRRC complementation vector used)	This study
M1 *rrpA* mutant	Isogenic M1 *rrpA* mutant with insertion of a 1.4 kb Kan^R cassette	This study
40917	Clinical bloody diarrhea isolate HS type 21 Water and wildlife associated strain. C9ii clade. MLST clonal complex ST-45.	*Campylobacter* Reference Lab, UK
12241	Ovine isolate HS type 50 Water and wildlife associated strain. C9ii clade. MLST clonal complex ST-206.	*Campylobacter* Reference Lab, UK
64555	Clinical bloody diarrhea isolate HS type 31 Water and wildlife associated strain. C8 clade.	*Campylobacter* Reference Lab, UK
47693	Isolated from chicken isolate HS type 27 Water and wildlife associated strain. C9ii clade. MLST clonal complex ST-45.	*Campylobacter* Reference Lab, UK
62914	Clinical vomiting isolate HS type – untypeable Water and wildlife associated strain. C7 clade.	*Campylobacter* Reference Lab, UK
44119	Clinical septicaemia isolate HS type – 18 Water and wildlife associated strain. C7 clade.	*Campylobacter* Reference Lab, UK
32787	Clinical asymptomatic isolate HS type – 18 Water and wildlife associated strain. C9i clade.	*Campylobacter* Reference Lab, UK
Hi80614	Human isolate Water and wildlife associated strain. C9i clade.	*Campylobacter* Reference Lab, UK
Hi41100305	Human isolate Water and wildlife associated strain. C9i clade.	*Campylobacter* Reference Lab, UK
31481	Clinical asymptomatic isolate HS type – 37 Water and wildlife associated strain. C8 clade.	*Campylobacter* Reference Lab, UK
47886	Clinical septicaemia isolate HS type – untypeable Livestock associated strain. C1 clade.	*Campylobacter* Reference Lab, UK

(Continued)

TABLE 1 | Continued

Strain	Description	Reference
30280	Clinical diarrhea isolate HS type – 16 Livestock associated strain. C2 clade.	*Campylobacter* Reference Lab, UK
13713	Ox liver portion isolate HS type – 2 Livestock associated strain. C1 clade.	*Campylobacter* Reference Lab, UK
11973	Chicken isolate HS type – 2 Livestock associated strain. C1 clade.	*Campylobacter* Reference Lab, UK
G1	Clinical GBS isolate HS type – 1 Livestock associated strain. C5 clade. MLST clonal complex ST-21.	Guy's Hospital, UK
Bovine27	Bovine isolate Livestock associated strain. C2 clade.	University of Bristol, UK
12912	Ox liver portion isolate HS type – 50 Livestock associated strain. C4 clade.	Campylobacter Reference Lab, UK
13040	Chicken isolate HS type – 50 Livestock associated strain. C6 clade.	Campylobacter Reference Lab, UK
40209	Chicken isolate HS type – 5 Livestock associated strain. C4 clade.	Campylobacter Reference Lab, UK
91B1	Chicken isolate Livestock associated strain. C5 clade.	Oxford University, UK
Hi41380304	Human isolate Livestock associated strain. C3 clade.	Campylobacter Reference Lab, UK
11168H *perR* mutant	Obtained from *Campylobacter* mutant bank http://crf.lshtm.ac.uk/wren_mutants.htm	LSHTM mutant bank
11168H *sodB* mutant	Obtained from *Campylobacter* mutant bank http://crf.lshtm.ac.uk/wren_mutants.htm	LSHTM mutant bank
11168H *ahpC* mutant	Obtained from *Campylobacter* mutant bank http://crf.lshtm.ac.uk/wren_mutants.htm	LSHTM mutant bank
11168H *katA* mutant	Obtained from *Campylobacter* mutant bank http://crf.lshtm.ac.uk/wren_mutants.htm	LSHTM mutant bank

Software). All statistical analyses were performed comparing two data sets directly assuming a normal distribution using a two-way student's *t*-test ($^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$).

RESULTS

Presence of *rrpA* and *rrpB* in a Collection of 4,232 *C. jejuni* and *C. coli*

To assess the distribution of *rrpA* and *rrpB* in *C. jejuni* and *C. coli* we utilized a collection of 4,232 *Campylobacter* genome sequences (3,746 *C. jejuni* and 486 *C. coli*) from public databases (Cody et al., 2013; Brown et al., 2015), which were phylogenetically clustered using FFPry feature frequency profiling (Van Vliet and Kusters, 2015; Dwivedi et al., 2016) and further analyzed for MLST sequence type and clonal complex (Pearson et al., 2015). The vast majority of *C. jejuni* strains contain *rrpA* whilst the presence of *rrpB* is more restricted (**Figure 1**; Supplementary Table 2 – Sheet 2). The presence or absence of *rrpB* appears to correlate with MLST clonal complex, especially ST-21 and ST-61. Most *C. coli* strains contain neither *rrpA* nor *rrpB*, with only 12 *C. coli* genomes encoding an RrpA ortholog and only a single *C. coli* genome encoding an RrpB ortholog (Supplementary Table 2). Variation in RrpA and RrpB was observed with isolates from *C. jejuni* MSLT clonal complex ST-607 having a shorter RrpA protein lacking the N-terminal 27 amino acids, while a proportion of *C. jejuni* ST-353 isolates have a RrpB protein either lacking the N-terminal 29 amino acids, the C-terminal 29 amino acids or a combination of 29 N-terminal amino acids and 9 C-terminal amino acids (Supplementary Figure 2). There was no apparent link between the truncated versions and source of the isolates (Supplementary Table 2), suggesting these are different forms of RrpA and RrpB circulating within clonal complexes.

Further analysis of the *C. jejuni* and *C. coli* genome sequences investigated the distribution of genes flanking *rrpA* and *rrpB*

TABLE 2 | Oligonucleotide primers used in this study.

Primer Name	Sequence
11168H and 81-176 *Cj1556*-F	ATCATTCTCTTTGTCCTAT
11168H and 81-176 *Cj1556*-R	TAAGATGGATTCTAAACTATTG
11168H Comp-*Cj1556*-F	CCCCCATGGATAAGGATTTATAATGAAAAAATATCATTCTCT
11168H Comp-*Cj1556*-R	CCCGCTAGCTTAAACGATATTTTTATAGCTAT
81-176 Comp-*Cj1556*-F	CCCTCTAGAATAAGGATTTATAATGAAAAAATATCATTCTCT
81-176 Comp-*Cj1556*-R	CCCTCTAGATTAAACGATATTTTTATAGCTAT
11168H and 81-176 *Cj1546*-F	TACTAGGATTTTCATGAG
11168H and 81-176 *Cj1546*-R	AGATGTTAAATCTCACTGCT
11168H and 81-176 *Cj1546* IPCR-F	GGGAGATCTCTCTTAAGGTATTGGTTA
11168H and 81-176 *Cj1546* IPCR-R	GGGAGATCTCGATGGTTTAATTATCAG
11168H and 81-176 Comp-*Cj1546*-F	CCCTCTAGACTAAAGGAATGTTAAATGACTAAAGAGAATTCTCCG
11168H and 81-176 Comp-*Cj1546*-R	CCCTCTAGATTAATTCAAGCATTTTTTCCC
11168H and 81-176 *Cj1546*-F checking primers	TTCTCCGTGCAATTTCG
11168H and 81-176 *Cj1546*-F checking primers	CCATTTGCTCATAGCTTGTAA
81116 *Cj1546*-F	ATGAGTTTGATCTACTTCG
81116 *Cj1546*-R	AGATATTAAATCTCACTGCT
81116 *Cj1546* IPCR-F	GGGAGATCTCTCTTAAGGTATTGGTTA
81116 *Cj1546* IPCR-R	GGGAGATCTTGATGGTTTAATTATCAG
81116 Comp-*Cj1546*-F	CCCTCTAGACTAAAGGAATGTTAAATGACTAAAGAGAATTCTCAG
81116 Comp-*Cj1546*-R	CCCTCTAGATTATCAAGCATTTTTTTCC
81116 *Cj1546*-F checking primers	ATGACTAAAGAGAATTCTC
81116 *Cj1546*-R checking primers	CCATTTGCACATAGCTTG
Kan^R forward-out	TGGGTTTCAAGCATTAGTCCATGCAAG
Kan^R reverse-out	GTGGTATGACATTGCCTTCTGCG
Cat^R forward-out	CGATTGATGATCGTTGTA
Cat^R reverse-out	TACAGCAGACTATACTG

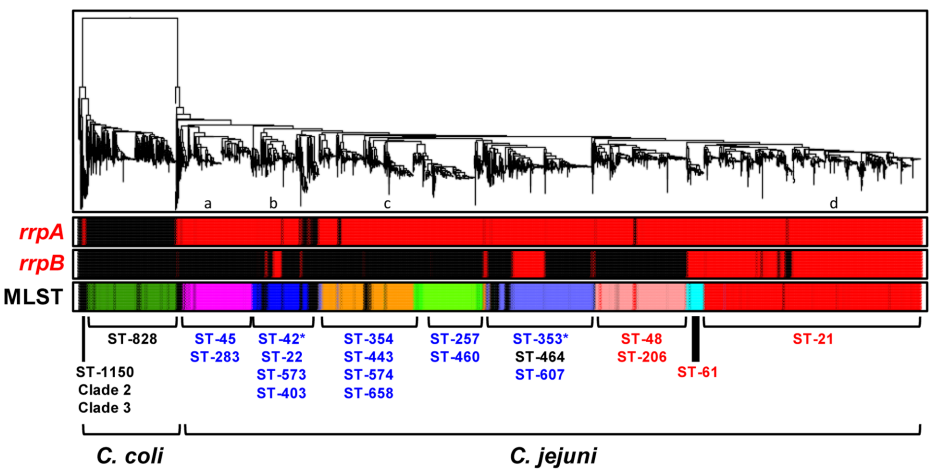

FIGURE 1 | Prevalence of the *rrpA* and *rrpB* genes among 3,746 *Campylobacter jejuni* and 486 *C. coli* genomes. Genomes were phylogenetically clustered using FFPry feature frequency profiling with $L = 18$ (Van Vliet and Kusters, 2015). The first two rows labeled '*rrpA*' and '*rrpB*' indicate genomes possessing the respective genes, which are shown in red, while those lacking the pathway are shown in black. The third bar shows the primary combinations of MLST clonal complexes for *C. jejuni* and *C. coli*, with red-labeled clonal complexes representing livestock-associated lineages, blue-labeled clonal complexes representing water and wildlife-associated lineages (Stabler et al., 2013), with the exception of the ST-61 clonal complex, which has been described as cattle (livestock)-associated (Kwan et al., 2008; Rotariu et al., 2009). The asterisks at ST-42 and ST-353 indicate that these clonal complexes may have a proportion of livestock-associated isolates. The association of some clonal complexes such as ST-464 was not reported previously and these are in black font. The lowercase letters indicate the approximate position of reference strains 81116 and M1 (a), 81-176 (b), RM1221 (c) and NCTC 11168 (d).

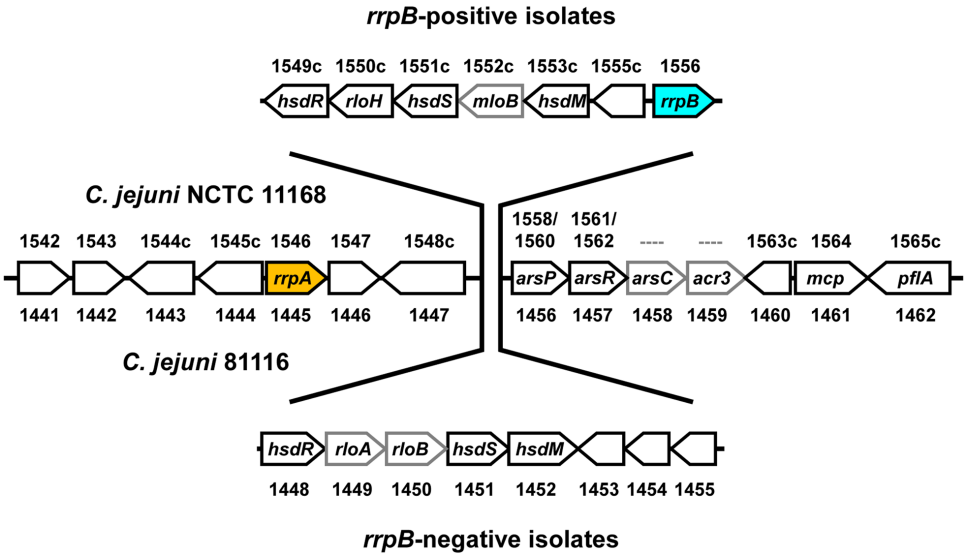

FIGURE 2 | NCTC 11168 type (*rrpB*$^+$ isolate) and 81116 type (*rrpB*$^-$ isolate) differ in a hypervariable region containing a type I Restriction-Modification (*hsd*) system. The genetic structure around *rrpA* is conserved in *C. jejuni* strains, however, *rrpB* appears to be part of a transferable hypervariable region linked with variation in the type I Restriction-Modification (*hsd*) system, suggesting a mechanism for the variable distribution of *rrpB*. The figure shows the *C. jejuni* NCTC 11168 (*rrpB*$^+$) and 81116 (*rrpB*$^-$) genes and gene numbers (Supplementary Table 3).

and identified two conserved flanking regions with a more variable central region (**Figure 2**). The upstream flanking region contains *rrpA* whilst the downstream flanking region contains an arsenic resistance operon, an MCP-gene and a paralyzed flagella gene *pflA*. The hypervariable central region contains a type I Restriction-Modification (*hsd*) system. The NCTC 11168 version of this hypervariable central region contains *rrpB* whilst the 81116 version does not (Supplementary Table 3). The 81116 version of this hypervariable central region is representative of all the other *C. jejuni* ST strains analyzed in this study. Analysis of the *C. coli* genome sequences (which have primarily a structure very similar to 81116) has versions mostly without *rrpA* and *rrpB*.

Campylobacter jejuni rrpB$^-$ Wild-Type Strains Exhibit Increased Resistance to Oxidative Stress Compared to *C. jejuni rrpB*$^+$ Wild-Type Strains

Characterization of the role of RrpA and RrpB in the *C. jejuni* 11168H wild-type strain has shown that both *rrpA* and *rrpB* mutants exhibit increased sensitivity to H_2O_2 when compared to the wild-type strain (Gundogdu et al., 2011, 2015). Based on the observed variation of *rrpA* and *rrpB* amongst *C. jejuni* isolates (Gundogdu et al., 2011), we utilized the Stabler et al. (2013) study to select 25 wild-type strains (**Table 1**) (13 *rrpB*$^+$ and 12 *rrpB*$^-$ strains) for testing sensitivity toward H_2O_2 (**Figure 3**). *rrpB*$^-$ strains (**Figure 3B**) displayed significantly greater resistance to H_2O_2 when compared to *rrpB*$^+$ strains (**Figure 3A**). The variation in the presence of *rrpA* and *rrpB* identified from the microarray data (Champion et al., 2005) for these 25 strains was also confirmed using PCR (data not shown).

Mutation of *rrpA* Results in Increased Sensitivity to Peroxide Stress in Strains 11168H and 81-176 (*rrpB*$^+$), But Not in Strains 81116 and M1 (*rrpB*$^-$)

To further investigate the link between oxidative stress resistance and the varied distribution of *rrpA* and *rrpB*, *rrpA* and *rrpB* single mutants were constructed in the 11168H and 81-176 wild-type strains (*rrpB*$^+$) and *rrpA* mutants were also constructed in the 81116 and M1 wild-type strains (*rrpB*$^-$). The mutants obtained did not have any observable deficiency in motility or growth rate under standard conditions (data not shown). Compared to the respective wild-type strains, both the 11168H and 81-176 *rrpA* mutants exhibited an increased sensitivity to 25 mM H_2O_2, however, the 81116 and M1 *rrpA* mutants exhibited wild-type levels of survival (**Figure 4**). Neither the 11168H and 81-176 wild-type strains nor the respective *rrpA* mutants survived exposure to higher concentrations of H_2O_2 (50 or 100 mM), however, the 81116 and M1 wild-type strains and the respective *rrpA* mutants all exhibited only slightly increased sensitivity to these higher concentrations of H_2O_2 (**Figure 4**). 11168H and 81-176 *rrpB* mutants exhibited the same increased sensitivity to 25 mM H_2O_2 as the *rrpA* mutants (**Figure 5**), but did not survive the higher concentrations of H_2O_2 (data not shown). Cumene hydroperoxide and menadione stress assays were also performed on the 11168H, 81-176, 81116, M1 wild-type strains and the respective *rrpA* and *rrpB* mutants (Supplementary Figure 3). There were no significant differences in sensitivity to either cumene hydroperoxide or menadione between any of the wild-type strains and respective mutants, with the exception that the 81116 *rrpA* mutant appeared to be more resistant to cumene hydroperoxide stress than the 81116 wild-type strain.

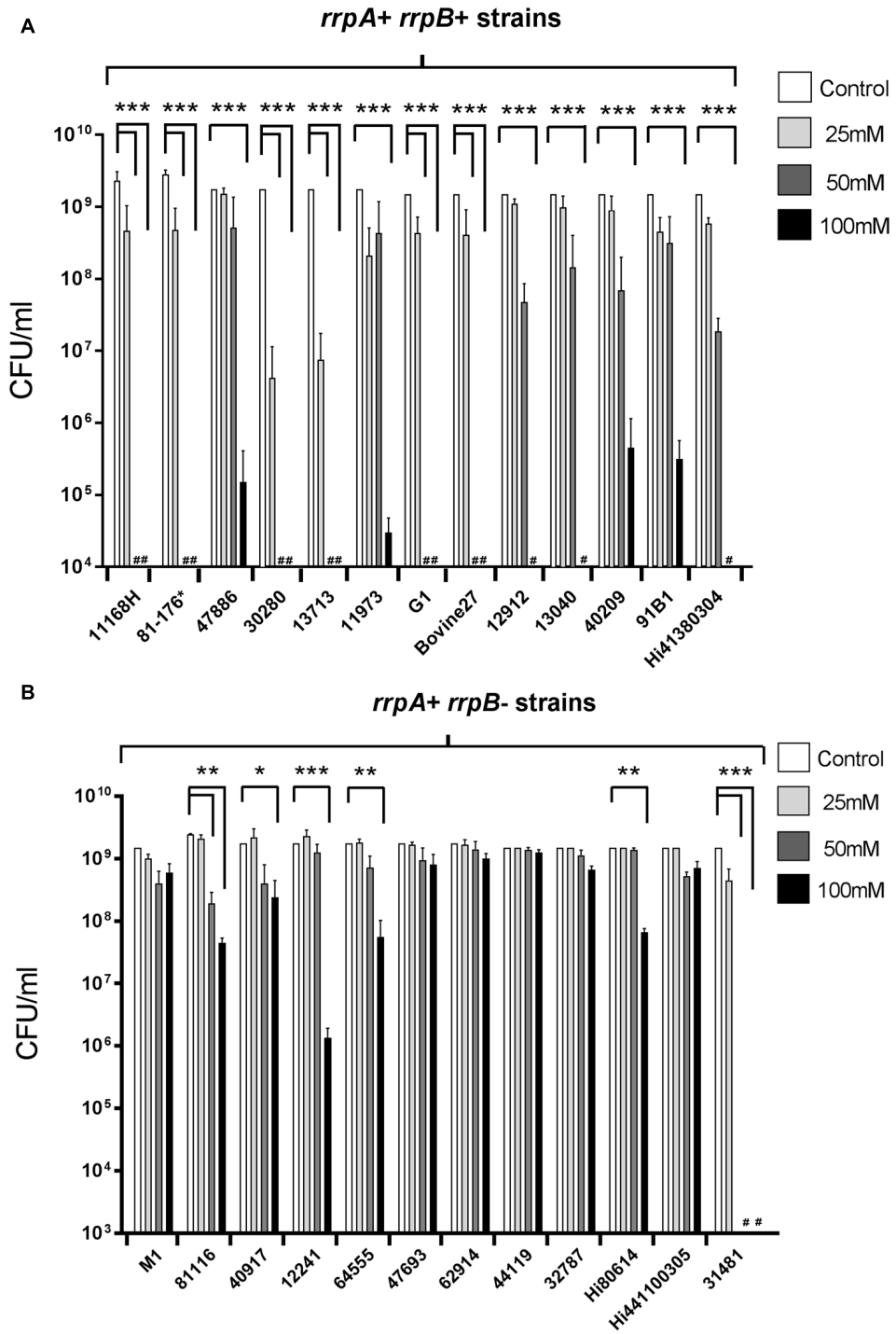

FIGURE 3 | Effect of oxidative stress on the survival of *C. jejuni rrpB*$^+$ (A) and *rrpB*$^-$ (B) wild-type strains. *rrpB*$^+$ strains are 11168H, 81-176, 47886, 30280, 13713, 11973, G1, Bovine27, 12912, 13040, 40209, 91B1, and Hi41380304. *rrpB*$^-$ strains are M1, 81116, 40917, 12241, 64555, 47693, 62914, 44119, 32787, Hi80614, Hi441100305, and 31481. These strains were selected from the Stabler et al. (2013) study (**Table 1**). *C. jejuni* strains were incubated with 25 mM, 50 or 100 mM H_2O_2 for 15 min at 37°C under microaerobic conditions. *For 81-176 denotes that this strain was not included in Stabler et al. (2013), but was selected here as a common laboratory strain. Bacterial survival was subsequently assessed. # Symbol indicates lack of growth. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) between strains.

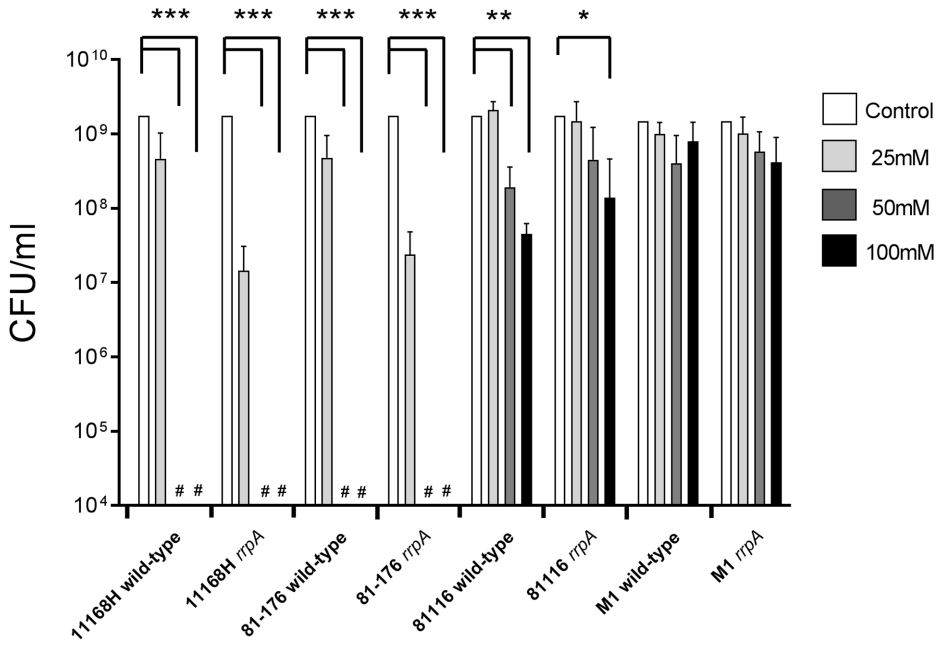

FIGURE 4 | Effect of oxidative stress on the survival of *C. jejuni* wild-type strains 11168H, 81-176, 81116, and M1 and respective *rrpA* mutants. *C. jejuni* strains were incubated with 25, 50, or 100 mM H_2O_2 for 15 min at 37°C under microaerobic conditions. Bacterial survival was subsequently assessed. # Symbol indicates lack of growth. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) between wild-type and mutant strains.

Mutation of *rrpA* Reduces Growth under Aerobic Stress Conditions in *rrpB*$^+$ Strains, But Not in *rrpB*$^-$ Strains

In *C. jejuni* 11168H, *rrpA* and *rrpB* mutants exhibit reduced growth under aerobic stress conditions (Gundogdu et al., 2011, 2015). The ability of *rrpA* and *rrpB* mutants to grow under aerobic stress conditions was compared to the respective four wild-type strains. No differences in bacterial growth kinetics (Supplementary Figure 4A) or in CFU counts after 16 h (**Figure 6A**) was observed under microaerobic conditions. However, differences were observed under aerobic conditions. All four *rrpA* mutants displayed significantly reduced growth kinetics during the logarithmic phase under aerobic conditions (Supplementary Figure 4B). However, compared to the respective wild-type strains, only the 11168H and 81-176 *rrpA* mutants exhibited a reduction in CFU counts after 16 h, whilst the 81116 and M1 *rrpA* mutants did not (**Figure 6B**).

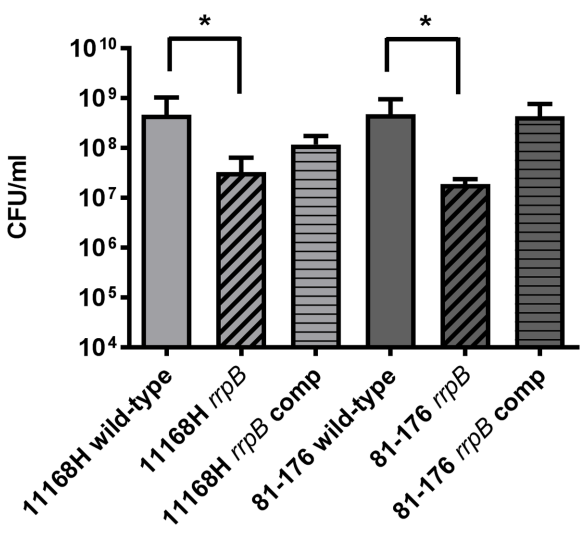

FIGURE 5 | Effect of oxidative stress on the survival of *C. jejuni* wild-type strains 11168H and 81-176, *rrpB* mutants and *rrpB* complements. *C. jejuni* strains were incubated with 25 mM H_2O_2 for 15 min at 37°C under microaerobic conditions. Bacterial survival was subsequently assessed. Asterisks denote a statistically significant difference (* = $p < 0.05$) between wild-type and mutant strains.

Mutation of *rrpA* Results in Decreased Cytotoxicity in the *Galleria mellonella* Larvae Model of Infection

Galleria mellonella larvae have been used as a model to study infection with many different enteric pathogens including *C. jejuni* (Champion et al., 2009). Insect larvae possess specialized phagocytic cells, termed haemocytes (Bergin et al., 2005; Mylonakis et al., 2007). Haemocytes mimic the functions of phagocytic cells in mammals and are able to degrade bacterial pathogens as well as generate bactericidal compounds such as superoxide via a respiratory burst (Lavine and Strand, 2002; Bergin et al., 2005). Both 11168H *rrpA* and *rrpB* mutants have been shown to exhibit reduced cytotoxicity in this model of infection compared to the wild-type strain (Gundogdu

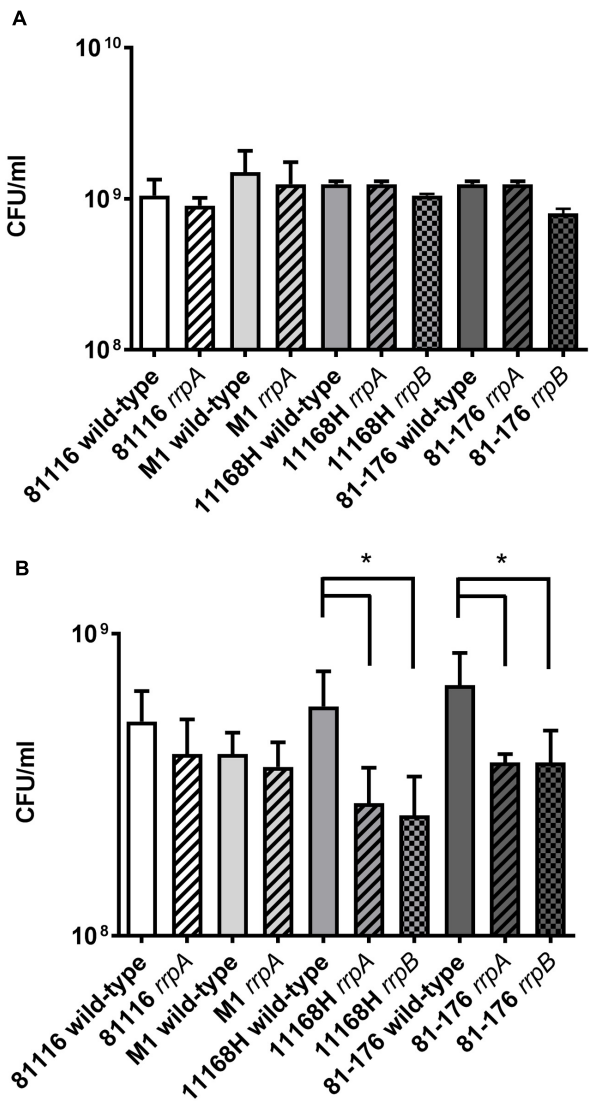

FIGURE 6 | Growth assays on *C. jejuni* 11168H, 81-176, 81116 and M1 wild-type strains and respective *rrpA and rrpB* mutants. Brucella broth was pre-incubated at 37°C under microaerobic conditions for 24 h, then inoculated with a bacterial suspension to a final OD_{600} of 0.1 and grown for 16 h with shaking at 75 rpm at 37°C under either microaerobic conditions **(A)** or aerobic conditions **(B)**. Bacterial CFU were also assessed at 16 h. Asterisks denote a statistically significant difference (* = $p < 0.05$) between wild-type and mutant strains.

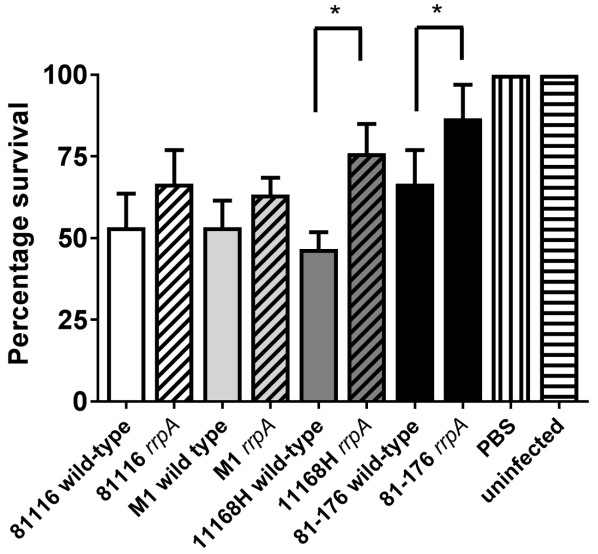

FIGURE 7 | *Galleria mellonella* infection model on *C. jejuni* 11168H, 81-176, 81116, and M1 wild-type strains and respective *rrpA and rrpB* mutants. *G. mellonella* larvae were injected with a 10 μl inoculum of a 24 h *C. jejuni* culture diluted to OD_{600} 0.1 by micro-injection in the right foremost leg, giving an infectious dose of approximately 10^6 CFU. Larvae were incubated at 37°C with survival and appearance recorded at 24 h intervals. PBS and no injection controls were used. For each experiment, 10 *G. mellonella* larvae were infected and experiments were repeated in triplicate. Asterisks denote a statistically significant difference (* = $p < 0.05$) between wild-type and mutant strains.

et al., 2011, 2015). The cytotoxicity of these mutants in *G. mellonella* larvae compared to the respective wild-type strains was investigated. Infection with either the 11168H or 81-176 *rrpA* mutants resulted in a statistically significant decrease in cytotoxicity to *G. mellonella* larvae compared to infection with the respective wild-type strains (**Figure 7**). Infection with the 81116 or M1 *rrpA* mutants also resulted in a decrease in cytotoxicity to *G. mellonella* larvae compared to infection with the respective wild-type strains, although this decrease was not statistically significant (**Figure 7**).

Mutation of *rrpA* Results in Increased Biofilm Formation

Several studies have demonstrated that *C. jejuni* exhibits increased biofilm formation under increased oxidative stress conditions (Fields and Thompson, 2008; Reuter et al., 2010). Previously *C. jejuni* 11168H *rrpA* and *rrpB* mutants exhibited an enhanced ability to form biofilms under both aerobic and microaerobic conditions (Gundogdu et al., 2015). All four *rrpA* mutants exhibited an increase in biofilm formation under both aerobic (**Figure 8A**) and microaerobic (**Figure 8B**) conditions after 72 h compared to the respective wild-type strains, which was statistically significant for the 11168H and 81116 *rrpA* mutants under aerobic conditions and for the 11168H, 81-176, and M1 *rrpA* mutants under microaerobic conditions. In addition both the 11168H *rrpB* mutant (Gundogdu et al., 2015) and 81-176 *rrpB* mutant (data not shown) exhibit increased biofilm formation under aerobic and microaerobic conditions when compared to the respective wild-type strains.

DISCUSSION

Campylobacter jejuni will be exposed to ROS both during colonization or infection of a host and in the environment as well as during the course of normal bacterial metabolism. It remains a conundrum as to how this microaerophilic pathogen is so widely dispersed, highly prevalent and able to survive

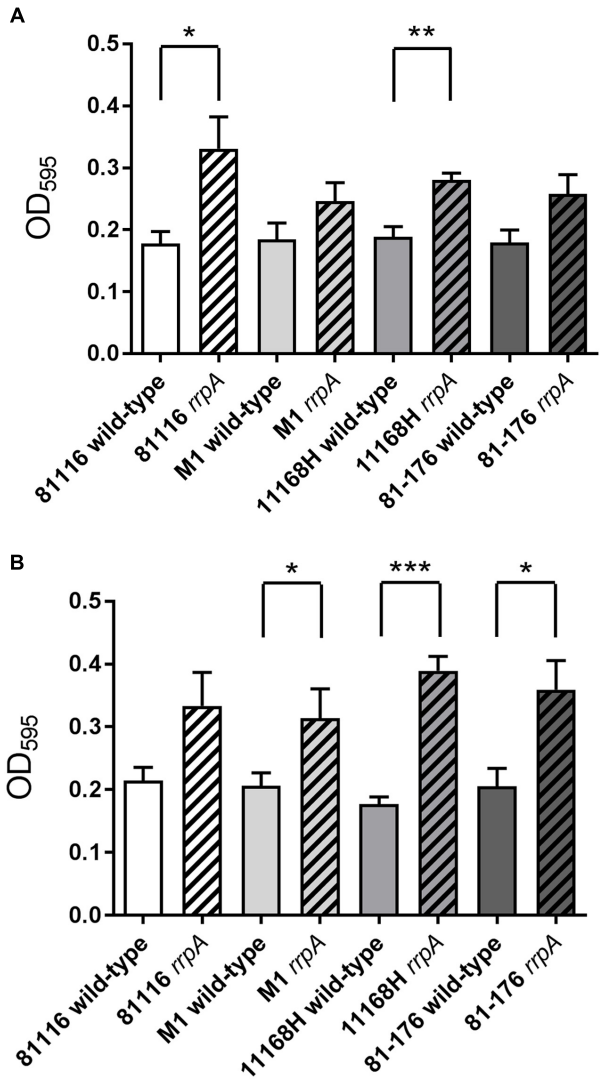

FIGURE 8 | Biofilm assay on *C. jejuni* 11168H, 81-176, 81116, and M1 wild-type strains and respective *rrpA and rrpB* mutants. *C. jejuni* 11168H wild-type strain and mutants were grown for 72 h under aerobic **(A)** and microaerobic **(B)** growth conditions at 37°C without shaking, rinsed three times with PBS, followed by crystal violet staining. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) between strains.

in the ambient environment. *C. jejuni* contains a number of different mechanisms for counteracting the effects of oxidative stress and the control of the *C. jejuni* oxidative stress response is complex involving multiple inter-linked levels of regulation (Atack and Kelly, 2009). The re-annotation of the *C. jejuni* NCTC 11168 genome sequence (Gundogdu et al., 2007) identified both RrpA and RrpB as putative MarR-type transcriptional regulators which were subsequently shown to be involved in the *C. jejuni* peroxide and aerobic stress response (Gundogdu et al., 2011, 2015). All the other regulators of the *C. jejuni* oxidative stress response, such as PerR, Fur, CosR, CsrA, CprRS, and RacRS, are all conserved (>99.8%) amongst all the *C. jejuni* and *C. coli* wild-type strains in this study (van Vliet et al., 1999; Atack and Kelly, 2009; Palyada et al., 2009; Hwang et al., 2012). Analysis of the distribution of *rrpA* and *rrpB* in 3,746 *C. jejuni* and 486 *C. coli* genomes from public databases confirmed that the vast majority of *C. jejuni* strains contain *rrpA*, whilst the presence of *rrpB* is more restricted, with the distribution linked to MLST clonal complex. The majority of *C. jejuni* strains that contain both *rrpA* and *rrpB* are from ST-21 and ST-61. ST-21 strains are often associated with human infections (Sheppard et al., 2009) and ST-61 strains with infections of livestock (Kwan et al., 2008; Rotariu et al., 2009).

Analysis of the distribution of *rrpA* and *rrpB* from the microarray data study of 270 *C. jejuni* strains (Stabler et al., 2013) identified water and wildlife strains predominantly as $rrpB^{-}$, whereas livestock-associated strains were predominantly $rrpB^{+}$ (Supplementary Figure 1; Supplementary Table 1). This distribution of *rrpA* and *rrpB* was also reflected in the analysis of the whole genome sequence data (**Figure 1**) where red-labeled clonal complexes represent livestock-associated lineages and blue-labeled clonal complexes represent water and wildlife-associated lineages. It is, however, not always clear whether each clonal complex is associated with a single host as clonal complexes are regularly isolated from multiple animal species and thus attribution to a single host reservoir is difficult using MLST data alone (Dearlove et al., 2016). One example is the ST-48 complex that has been isolated from humans, cattle and sand from beaches (Dingle et al., 2002). Another example is ST-257 and ST-61 that have been isolated from chicken and ruminants, yet ST-257 is designated as a water and wildlife-associated lineage, whereas ST-61 is denoted as a livestock-associated lineage (Sheppard et al., 2011). These livestock and water and wildlife descriptions may be somewhat generalized and investigating the properties of individual STs will be more important to understand the source. ST-21, ST-206, and ST-48 may form a "complex group" of related genotypes that are widely distributed, perhaps reflecting the "generalist" ability to colonize a wide range of hosts (Dingle et al., 2002; Sheppard et al., 2011).

The location of *rrpA* and *rrpB* close to a type I Restriction-Modification (*hsd*) system suggests an explanation as to why *rrpA* is present in almost all *C. jejuni* strains, whilst the presence of *rrpB* is restricted and appears to correlate with MLST clonal complexes. Restriction-modification (R-M) systems are ubiquitous in the bacterial world and provide a defense against foreign DNA and bacteriophages (Wilson and Murray, 1991). Foreign DNA is cleaved by endonucleases but host DNA avoids damage due to methylation. R-M systems have been classified into four distinct groups, type I, type II, type III, and type IV. A type I R-M locus was identified in the genome sequence of NCTC 11168 (*Cj1549–Cj1553*) (Parkhill et al., 2000). The type I enzyme is a bi-functional, multi-subunit complex consisting of HsdR, HsdM, and HsdS. Further analysis of the type I R-M system from 73 *C. jejuni* strains (including NCTC 11168, 81-176, and 81116) assigned some *hsd* systems to the classical type IC family but also identified two additional type I R-M families, termed type IAB and type IF (Miller et al., 2005). 81116 contains a type IAB *hsd* system, whilst both NCTC 11168 and 81-176 contain a type IF *hsd* system. This study also found evidence for

extensive rearrangements within the *C. jejuni hsd* loci of the same family but suggested that the low sequence similarity between the IC, IAB, and IF families would make inter-family recombination events unlikely. The NCTC 11168 version of the hypervariable central region identified in this study, which contains the type IF *hsd* system and *rrpB*, represents the majority of ST-21, ST-61, ST-42, and ST-353 strains. 81-176 also contains the type IF *hsd* system and *rrpB*. However, the 81116 version of this hypervariable central region, which represents the majority of all the other *C. jejuni* ST strains analyzed in this study, contains a type IAB *hsd* system and does not contain *rrpB*. The lack of recombination between *C. jejuni* strains containing the type IF *hsd* system and *rrpB* with other *C. jejuni* strains containing a different family of type I *hsd* system could be the explanation for the restricted distribution of *rrpB* amongst *C. jejuni* strains. This region of the *C. jejuni* genome has previously been identified as hypervariable region 14, one of 16 hypervariable regions (Taboada et al., 2004). The presence of intervening ORFs between *hsdR* and *hsdS* (referred to as *rlo* genes for R-linked ORF) and also between *hsdS* and *hsdM* (referred to as *mlo* genes for M-linked ORFs) are a distinct feature of the *C. jejuni hsd* loci (Miller et al., 2005). *H. pylori* ModH from the type III R-M system has been shown to have a regulatory role (Srikhanta et al., 2011) suggesting that R-M systems may also have a role in the regulation of virulence gene expression (Vasu and Nagaraja, 2013). Recently the same hypervariable region in *C. coli* has been shown to contain a novel streptomycin resistance gene (Olkkola et al., 2016). The genomics-based screening also showed that there are different alleles of RrpA and RrpB (Supplementary Figure 2) leading to a N-terminal truncation, a C-terminal truncation or both. The truncated version of RrpB was predominantly observed in isolates of the ST-353 clonal complex, while truncated versions of RrpA were rare in most clonal complexes, but dominant in ST-607 isolates (Supplementary Table 2). Future studies could utilize these truncated versions to test for the role of the N-terminal and C-terminal regions in functionality of RrpA and RrpB.

Campylobacter jejuni rrpB$^-$ strains exhibit a pattern of greater resistance to peroxide stress when compared to *rrpB*$^+$ strains. This pattern was the same when comparing the 11168H, 81-176, 81116, and M1 *rrpA* mutants against the respective wild-type strain where *rrpA* mutants in *rrpB*$^-$ strains were more resistant to peroxide stress compared to *rrpA* mutants in *rrpB*$^+$ strains. In addition, when comparing bacterial growth of 11168H, 81-176, 81116, and M1 *rrpA* mutants against the respective wild-type strain under aerobic conditions, all four *rrpA* mutants displayed a reduced growth rate during the logarithmic phase. Both the 11168H and 81-176 *rrpA* mutants exhibited lower CFUs at 16 h compared to the respective wild-type strains. Even though both 81116 and M1 *rrpA* mutants also displayed slower growth rates compared to the respective wild-type strains when comparing respective CFU counts at 16 h, these differences were not statistically significant. The general pattern that *rrpB*$^+$ strains exhibit increased sensitivity to peroxide and aerobic stresses is rather counterintuitive. Possibly having only a single Rrp regulator is more efficient in responding to such stresses. Certainly *rrpA* is the most conserved amongst *C. jejuni* strains. Even though the oxidative stress assays were performed on a relatively small number of selected strains, it is interesting to speculate why livestock-associated strains such as NCTC 11168 (ST-21) would be more sensitive to peroxide and aerobic stress, whilst water and wildlife-associated strains such as 81116 (ST-283) and M1 (ST-45) would be more resistance to such stresses is difficult to explain. One possible hypothesis is that the ability to survive aerobic stress is more important for the latter strains. The variation in the presence of *rrpA* and *rrpB* between different wild-type strains may play an important role in the ability of *C. jejuni* to adapt and survive in different environmental niches.

Infection with *rrpA* mutants from both *rrpB*$^+$ and *rrpB*$^-$ strains resulted in an increase in survival of *G. mellonella* larvae compared to infection with the respective wild-type strains. We have previously demonstrated that infection of *G. mellonella* with the 11168H *rrpA* mutant leads to increased survival of the *G. mellonella* larvae (Gundogdu et al., 2015). Here we demonstrate that the 81-176 *rrpA* mutant (*rrpB*$^+$) also exhibits a similar statistically significant reduction in larval cytotoxicity. Both 81116 and M1 *rrpA* mutants (*rrpB*$^-$) exhibit reduced larval cytotoxicity, but not to a significant degree. This suggests the *rrpA* mutants (like the *rrpB* mutants) are more susceptible to the host immune mechanisms resulting in reduced bacterial survival within *G. mellonella*. This also suggests that RrpA may in fact play a role in the oxidative stress response of *rrpB*$^-$ strains such as 81116 and M1, but at a different level compared to *rrpB*$^+$ strains, such as 11168H and 81-176. Certainly the enhanced resistance of the 81116 and M1 *rrpA* mutants to peroxide stress compared to the 11168H and 81-176 *rrpA* mutants is not reflected by increased virulence in the *G. mellonella* larvae infection model.

Campylobacter jejuni forms biofilms (Joshua et al., 2006; Gundogdu et al., 2011) and this may be an important factor in the survival of *C. jejuni* both within hosts and in the environment. Though our understanding of the specific mechanisms underlying biofilm formation in *C. jejuni* is still limited (Svensson et al., 2008), *C. jejuni* lacks the classical two component regulatory systems involved in biofilm formation that are present in other bacteria such as GacSA in *Pseudomonas aeruginosa* (Parkins et al., 2001). Biofilm formation has been linked to responses to oxidative and aerobic stress as *C. jejuni* biofilm formation is increased under aerobic conditions (Reuter et al., 2010) and mutation of genes encoding oxidative stress response proteins results in changes in biofilm formation (Oh and Jeon, 2014; Gundogdu et al., 2015). Analysis of biofilm formation demonstrated that *rrpA* mutants from both *rrpB*$^+$ and *rrpB*$^-$ strains exhibited an increased biofilm phenotype compared to the respective wild-type strain under both microaerobic and aerobic conditions. Again the enhanced resistance of the 81116 and M1 *rrpA* mutants to peroxide stress compared to the 11168H and 81-176 *rrpA* mutants is not reflected by a decrease in biofilm formation.

The basis of *C. jejuni* survival is dependent upon the ability to sense and respond to the different environments encountered within hosts and in the environment. In this study we identified bioinformatically that over 99% of *C. jejuni* strains contain *rrpA*, whilst *rrpB* is restricted and appears to correlate with livestock-associated MLST clonal complexes. There exists a conserved genetic structure for *rrpA*, whilst *rrpB* seems to be

part of a transferable hypervariable region linked with variation in the type I R-M (*hsd*) system, giving an explanation for the more restricted distribution of *rrpB* amongst *C. jejuni* strains. *rrpB*$^{-}$ strains possess an increased level of resistance to peroxide and aerobic stress compared to *rrpB*$^{+}$ strains. So whilst all the other oxidative stress response regulators such as PerR, Fur, CosR, CsrA, CprRS, RacRS, and RrpA appear to be conserved in *C. jejuni*, variation in the presence of RrpB between different wild-type strains may play an important role for altered oxidative stress responses through the concerted actions of these multiple regulators in this microaerophilic pathogen. This highlights the potential of genetic variation in the natural population in the adaptation to different environmental niches.

AUTHOR CONTRIBUTIONS

OG, DdS, BW, and ND designed this study. OG, DdS, BM, and AE performed all the lab-based experimental work. OG, DdS, and AvV performed all the bioinformatic analysis. OG, DdS, BW, AvV, and ND wrote the manuscript.

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frontiers in Microbiology

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Profiling of *Campylobacter jejuni* Proteome in Exponential and Stationary Phase of Growth

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Campylobacter jejuni has been reported as a major cause of bacterial food-borne enteritides in developed countries during the last decade. Despite its fastidious growth requirements, including low level of oxygen and high level of CO_2, this pathogen is able to persist in the environment without permanent loss of its viability and virulence. As *C. jejuni* is not able to multiply outside a host, the cells spend significant amount of time in stationary phase of growth. The entry into the stationary phase is often correlated to resistance to various stresses in bacteria. The switching between exponential and stationary phases is frequently mediated by the regulator sigma S (RpoS). However, this factor is absent in *C. jejuni* and molecular mechanisms responsible for transition of cells to the stationary phase remain elusive. In this work, proteomic profiles of cells from exponential and stationary phases were compared using 2-D electrophoresis (2DE) fingerprinting combined with mass spectrometry analysis and qRT-PCR. The identified proteins, whose expression differed between the two phases, are mostly involved in protein biosynthesis, carbon metabolism, stress response and motility. Altered expression was observed also in the pleiotropic regulator CosR that was over-expressed during stationary phase. A shift between transcript and protein level evolution of CosR throughout the growth of *C. jejuni* was observed using qRT-PCR and (2DE). From these data, we hypothesized that CosR could undergo a negative autoregulation in stationary phase. A consensus sequence resulting from promoter sequence alignment of genes potentially regulated by CosR, including its own upstream region, among *C. jejuni* strains is proposed. To verify experimentally the potential autoregulation of CosR at the DNA level, electrophoretic mobility shift assay was performed with DNA fragments of CosR promoter region and rCosR. Different migration pattern of the promoter fragments indicates the binding capacity of CosR, suggesting its auto-regulation potential.

Keywords: foodborne pathogen, *Campylobacter jejuni*, growth, exponential phase, stationary phase, CosR, regulation

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INTRODUCTION

Campylobacter jejuni is continuously reported as the main cause of bacterial food-borne infections in developed countries (EFSA and ECDC, 2015). The disease caused by this pathogen, namely campylobacteriosis, is triggered mainly by consumption of contaminated food or water, although a direct transmission from infected animals to human hosts can occasionally occur (Bronowski et al., 2014). It manifests as an acute inflammatory diarrhea with symptoms common to other bacterial enteritides—abdominal pain, fever and watery diarrhea often accompanied with the presence of blood and leukocytes in stool (Blaser and Engberg, 2008). In most cases, campylobacteriosis is self-limiting and does not require specific therapy, however severe autoimmune disorders, such as Guillain-Barré and Miller-Fisher syndromes (Salloway et al., 1996; Nachamkin, 2002), reactive arthritis (Pope et al., 2007), and inflammatory bowel disease (Rodriguez et al., 2006) may appear. These late-onset complications, together with long convalescence time and high occurrence of campylobacteriosis, are the reasons why the disease is ranked as an infection with one of the highest annual burden (Batz et al., 2012; Gibney et al., 2014; Mangen et al., 2015). Although various strategies have been adopted by member states of EU in order to decrease numbers of campylobacteriosis (Lin, 2009; Saxena et al., 2013), the prevalence of this disease remains very high. It is therefore necessary to identify genetic and environmental factors affecting the persistence of *C. jejuni* in the environment, in order to develop new methods mitigating the campylobacteriosis cases.

As a pathogen with fastidious growth requirements, *C. jejuni* puzzles scientists with its ability to withstand broad range of stresses encountered during its lifecycle. In other pathogens represented by *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp., general stress response is regulated by sigma factor RpoS that is also responsible for switching the growth to stationary phase (Duval et al., 2015). The entry to the stationary phase requires cooperation of the sigma factor and many other regulators (Llorens et al., 2010). When switching to the stationary phase, growth rate of cells dramatically decreases as a result of reduced protein synthesis. The overall role of RpoS is to ensure adaptation and resistance of the cells to challenging environments. It directly regulates 10% of *E. coli* genes (Weber et al., 2005) that play role in morphological changes of the cells, resistance to broad range of stresses (oxidative and osmotic stress, heat shock, pH changes, etc.), virulence, metabolic processes, and the GASP (growth advantage in stationary phase) phenotype (Martinez-Garcia et al., 2001; Raiger-Iustman and Ruiz, 2008).

Unlike other Gram-negative bacteria, *C. jejuni* lacks several stress response genes, including the sigma factor RpoS (Parkhill et al., 2000; Garenaux et al., 2008). Despite its small genome, this strictly microaerobic pathogen had to develop other mechanisms allowing its survival in stressful conditions, such as lack of nutrients in aquatic environments, or high concentration of oxygen when exposed to air or to an oxidative attack of macrophages. The molecular mechanisms responsible for its survival in food, persistence in the environment and virulence have not yet been fully understood. Similarly, no information concerning the transition of *C. jejuni* cells from exponential to stationary phase are available nowadays. Functional replacement of the sigma factor RpoS has not yet been described and the molecular mechanisms facilitating a cellular switch from exponential to stationary phase remain unknown. Therefore, in this work, proteomic profiles of the cells from exponential and early stationary phase of growth were compared using a 2-D electrophoresis (2DE) and quantitative real-time PCR (qRT-PCR), in order to contribute to better understanding of the molecular changes occurring during cellular transition from exponential to stationary phase.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

In this work, *C. jejuni* strain 81-176 isolated from a raw milk outbreak (Korlath et al., 1985) was used. Cells were resuscitated from a stock on Karmali agar plates (Oxoid) at 42°C for 48 hours (h) in stainless steel jars filled with gas mixture containing 5% O_2, 10% CO_2, and 85% N_2 (microaerobic atmosphere). Grown cells were subcultured microaerobically in BHI (Merck) for 48 h at 42°C and 110 rpm, and then used for preparation of final suspension. The final suspension was cultivated at 42°C, with shaking at 110 rpm in microaerobic atmosphere for 7 h to harvest proteins in exponential phase and for 18 h for stationary phase. All experiments were performed in 3 biological replicates.

Protein Isolation Purification

Proteins were extracted and purified as described previously (Bieche et al., 2012; Haddad et al., 2012). Briefly, cells were harvested by centrifugation at 7000 g, 4°C for 20 min and washed consecutively with 200 mM glycine (Sigma-Aldrich) and 100 mM Tris-HCl pH 7.0 (Sigma-Aldrich). Pellets were resuspended in 10 ml of a 10 mM Tris-HCl pH 7.0 and the cells were disrupted by series of 6 × 30 s sonication at 20 kHz with 6 min intervals on ice (Vibracell 72434, Bioblock Scientific). Cell debris was removed by two consecutive centrifugations at 10 000 g, 4°C for 20 min. Then, cytoplasmic proteins were separated from membrane fractions by ultracentrifugation at 188 000 g for 1 h at 4°C. The cytoplasmic protein fraction in the supernatant was treated with protease inhibitor cocktail tablets COMPLETE (Roche Diagnostics) and nuclease solution with final concentrations of 18 mg/ml RNAse and 9 mg/ml DNAse (Sigma-Aldrich). Protein samples were dialyzed using cellulose membrane tubing with a cut-off at 12 kDa (Sigma-Aldrich) against MilliQ water at 4°C with shaking for 3 days by refreshing the dialysis bath each day. Total protein concentration was determined using the Micro BCA™ Protein Assay Kit (Perbio-Science).

Two-Dimensional Gel Electrophoresis

A quantity of 100 μg of proteins isolated after 7 and 18 h of cultivation was concentrated using Concentrator 5301 (Eppendorf, France) at room temperature. Samples were mixed with rehydration buffer containing 6 M urea, 2 M thiourea, 4% CHAPS, 0,4% dithiothreitol (DTT), and 2% Bio-Lyse 3/10 Ampholyte (Bio-Rad) and few grains of bromophenol blue (BB).

Proteins were then loaded into 17 cm IPG strips (pH 4-7, Bio-Rad) by active rehydration at 50 V for 12 h. After that, the isoelectric focusing (IEF) was performed using the Bio-Rad IEF program as follows: from 50 to 250 V for 3 h, from 250 to 6,000 V for 3 h and at 6,000 V until reaching 54,000 Vh. Strips were then equilibrated in migration buffer containing 6 M urea, 50 mM Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, 2% DTT, 4% iodoacetamide, and few grains of BB. The second dimension was performed using SDS-PAGE with 12% acrylamide gels. The IPG strips were immobilized to the gel using 1% low-melting point agarose (Bio-Rad). The migration ran at 40 mA/gel using Protean II xi cell (Bio-Rad) at 14°C, until the bromophenol blue reached the base of the gels. Proteins in gels were finally silver stained and scanned with a GS-800 densitometer operated with the QuantityOne® software (Bio-Rad). Three independent experiments were performed, with two technical replicates for each of them.

Statistical Analysis and Protein Identification

The image analysis was performed using the Progenesis Samespots® software (NonLinear Dynamics). For the statistical analysis of the results, three independent experiments were performed, each with two technical replicates. Differences between the two conditions from the independent experiments and replicates were validated by Principal Component Analysis (PCA) and differences among matched spot intensities were statistically validated by ANOVA test at a 5% significance level. Lower and higher abundant proteins were taken into account only if the mean difference in spot intensities passed the threshold of 1.5. Predominant proteins whose amount differed significantly between the phases (q- and p-value $\leq$ 0.05, power $\geq$ 0.8, fold difference of spot intensities $\geq$ 1.5) were excised from BioSafe colloidal Coomassie blue (Bio-Rad) stained gels containing in total 700 µg of proteins. The excised proteins were analyzed after trypsin digestion with a mass spectrometer MALDI-TOF Voyager DE super STR (Applied Biosystems) at the PAPPSO platform of the INRA Center in Jouy-en-Josas (France). Gel plugs were first washed twice with 50% (v/v) acetonitrile, 25 mM ammonium carbonate in water, and then dried in a vacuum speed concentrator. Aliquot (10 µl) of a trypsin solution (Promega, 12.5 ng/µl in 50 mM NH_4HCO_3) was added to each sample and digestion was performed for 6 h at 37°C. A 1 µl aliquot of each supernatant was spotted directly onto the MALDI plate then dried at room temperature before adding 1 µl of the matrix solution (α-cyano-4-hydroxycinnamic acid, 3 mg/ml in 50% (v/v) acetonitrile 0.1% (v/v) trifluororacetic acid). Mass spectra were acquired on a Voyager-DE-STR (Applied Biosystems, Framingham, MA), equipped with a nitrogen laser (Laser Science, Franklin, MA). Tryptic autodigestion ion peaks, $(M + H)^+ = 2211.104$ and 842.509 Da, were used for internal calibration of spectra. The peptide mass lists were used for protein identification in a *C. jejuni*, strain 81-176, amino acid sequence database (UniprotKB, 09.09.2010), using the Protein Prospector v 3.2.1 software set with the following parameters: mass tolerance < 20 ppm, four peptides required to match, one missed cleavage, oxidized methionine and carbamidomethylated cysteine, respectively as possible and fixed modifications. Identification was evaluated with regard to sequence coverage, Mowse score, and compatibility between theoretical and experimental molecular weight and isoelectric point. Genes of proteins significantly over-expressed in one of the growth phases were then chosen for qRT-PCR experiments to verify their expression on translational level.

RNA Isolation and Reverse Transcription

Cells were harvested from cultures cultivated for 4, 7, 12, 16, 18, and 24 h by centrifugation at 3,300 g at 4°C for 6 min, pellets were homogenized with 1 ml of lysis solution Extract-All (Eurobio) according to manufacturer's guidelines. After that, 200 µl of chloroform were added to the suspension allowing separation of cell components. Water phase containing RNA was removed, precipitated with 500 µl of isopropanol and dissolved again in 50 µl of RNase-free water after washing in 75% cool ethanol. Samples were then treated with DNase I (Sigma-Aldrich) and purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Possible DNA contamination was detected by PCR detecting house-keeping gene *flaA* (Nachamkin et al., 1993). The integrity of RNA was verified using the Experion System (Bio-Rad). Its concentration and purity was measured using NanoDrop 2,000 (Thermo Scientific).

For reverse transcription 100 ng of RNA were mixed with 0.5 µl of 1 µM Random Hexamer Primer (Promega) and 4.5 µl of RNase-free water. This mixture was incubated 5 min at 70°C followed by 5 min on ice. After that 15 µl of Master Mix were added to mixture (5 µl of 5 × RT buffer, 1 µl 25 mM dNTP and 1 µl of M-MLV H^- reverse transcriptase; Promega) and it was incubated 10 min at room temperature, 50 min at 48°C and finally 15 min at 70°C to stop the reaction.

Quantitative PCR (q-PCR)

Quantitative real-time PCR assay was performed using the AB7300 Realtime PCR system with SYBR Green-I Master Mix (Applied Biosystems). Primers (Eurobio) were designed with the on-line Primer3 software (www.simgene.com; **Table 1**) and their quality was checked both virtually, using FastPCR software, and by PCR. The efficiency of the primer pairs was calculated with the formula $E = [10^{(1/-s)}] \times 100$, where "s" is the slope of the standard curve. The efficiency of designed primers ranged from 101.27 to 105.38%.

The composition of q-PCR mix and amplification program were the same as described by Bieche et al. (2012) Amplification efficiency was determined from standard curve of serially diluted cDNA. The expression level of examined genes was calculated with AB 7,300 software against endogenous control gene *rrs* to obtain relative quantity.

Purification of Recombinant CosR

In order to produce sufficient amount of His_6-tagged recombinant CosR (rCosR), restriction enzymes sites NdeI and BamHI were added to *cosR* sequence during its amplification using specific primers NtagCosR-NdeI (5'-GCACACATA TGAGAATTTTAGTTATAGAAG-3′) and NtagCosR-BamHI

TABLE 1 | Primers used in the experiments.

Experiment	Gene	Primer	5′ to 3′	Product size (bp)	T_a (°C)
qRT-PCR	*acnB*	AcnB Fw	AGCGGACTTGTAGCTTTTGC	98	60
		AcnB Rev	ACTCCAGCTTGCAATTCTCC		
	betA	BetA Fw	CACTGGGATGCGGATAATCT	105	59
		BetA Rev	AGCACAGCGATAAGCCAAAG		
	cheW	CheW Fw	GGTGAGACAAATGGAACTGGA	109	59
		CheW Rev	AAGTTTCAGGTGGTGGATCG		
	cosR	CosR Fw	TTTGAAAGCTGGAGCTGATG	100	59
		CosR Rev	GGTTCCGCCAAGTCTTAGTC		
	dnaK	DnaK Fw	CTTTCTTGGGCGAGAGTGTT	101	60
		DnaK Rev	TCCTGCTATCGTTCCTGCTT		
	flaA	FlaA Fw	AAAGCAGCAGAATCGCAAAT	110	58
		FlaA Rev	TTTGCTTGAGCCATTGCATA		
	fumC	FumC Fw	TGCGGTTGAGCAAGTAGAGA	118	59
		FumC Rev	TTGTAAATGCGTGCGTCCTA		
	oorA	OorA Fw	AGCGGTCCAGGAATTTCTTT	109	60
		OorA Rev	GAAGACCTGTCGAAGGACCA		
	rbr	Rbr Fw	GGCGAATCTATGGCAAGAAA	106	58
		Rbr Rev	TTTCATTTTCAGCCGCTTCT		
	trxB	TrxB Fw	CAGGGATAGGCTGTGCAGTT	146	60
		TrxB Rev	GGTTCCTTCGCATCCCTTAT		
EMSA		CosRpromF	TGGTATATTAGATTTCGAAAGAAG	374	ND
		CosRpromR	CTTCTATAACTAAAATTCTCAT		
		CosRintF	TTTTGATATTCTACTCGCAAGA	484	ND
		CosRintR	AAAAGAGACATCATAACCTTTCA		
		KatF2	CGTGCATCCCAGTGTTCTAT	337	ND
		KatR2	TTTTGCGCCTGCGCTTAATG		

(5′-TACAGGATCCAAGGTGCAAAATTGTTA-3′), to obtain NdeI-CosR-BamHI amplicons. These amplicons were inserted using NdeI and BamHI enzymes and subcloned into plasmid pET-15b (Novagen, France) to obtain a *N*-terminal His_6 tagged protein under the control of T7 promoter. The resulting plasmid was transformed to competent *E. coli* expression strain BL21(DE3) (Novagen, France). Overexpression of rCosR was obtained from an overnight culture at 28°C in LB supplemented with 50 μg/ml of ampicillin, and 0.125 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cells were then harvested by centrifugation at 5,000 *g* for 10 min, washed with TE buffer (Sigma-Aldrich, France) and resuspended for 30 min in a lysis buffer containing 50 mM TrisHCl, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol and 1 mg/ml of lysozyme. The lysate was centrifuged and rCosR was purified using Ni-NTA agarose columns (Qiagen, France) according to the manufacturer's instructions. Columns were equilibrated using lysis buffer before protein binding and then washed sequentially with buffer W1 (lysis solution with 1% glycerol, 0.5% triton and 20 mM imidazole) and buffer W2 (lysis buffer with 20 mM imidazole). The recombinant protein was eluted using 400 mM imidazole and 6 M urea and purified by dialysis according to the manufacturer's instructions. Aliquots of rCosR were stored at −80°C.

Bioinformatic Analysis of the CosR DNA-Binding Box

A bioinformatic analysis was performed to specify the consensus sequence of the CosR DNA-binding box proposed by Hwang et al. (2011, 2012). For this purpose, sequences of 5′ 400-nt upstream regions of 7 genes previously reported as binding CosR were retrieved from the platform MicroScope (Microbial Genome Annotation & Analysis Platform; http://www.genoscope.cns.fr/agc/microscope/home/). To inspect the conservation of the CosR binding box, sequences of nine different fully sequenced *C. jejuni* strains were used in the analysis (**Supplementary Table 1**). Resulting consensus sequence logos were created using the WebLogo platform (http://weblogo.threeplusone.com; Crooks et al., 2004).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out to identify putative CosR binding sites. DNA fragments covering promoter regulatory regions or gene internal regions of *cosR* and *katA* were PCR amplified using designed primers (**Table 1**). To demonstrate the DNA-binding properties of CosR, concentration range of purified recombinant protein CosR-His_6 (1–10 μmol/l) were mixed with 2 μmol of

purified PCR fragments in a total volume of 20 µl containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 10 mM KCl, 10 µg/ml sheared salmon sperm DNA, and 10% glycerol. Sonicated salmon sperm DNA was applied as a nonspecific competitor. All samples were incubated at room temperature for 30 min following by the separation of the complexes using electrophoresis on a native 6% polyacrylamide gel in 0.5 × TBE.

RESULTS

Considering the lack of information concerning the transition of *C. jejuni* from exponential to stationary phase, we compared the proteomic profiles of cells harvested after 7 and 18 h of microaerobic cultivation at 42°C.

The comparison of the 2-DE proteomic profiles revealed numerous differences between the two phases (**Figure 1**). In total, 42 spots were identified as 24 different proteins with 11 proteins more abundant in the exponential phase (17 spots), 10 proteins more abundant in the stationary phase (12 spots) and 3 proteins whose isoforms were overexpressed in both exponential and stationary phase (13 spots; **Table 2**). The identified proteins are involved mostly in metabolism (AcnB, BioB, FumC, HisD, MetK, and OorA), general and oxidative stress response (BetA, ClpB, CosR, DnaK, GroEL, Rbr, and TrxB), translation (FusA, RplL, Tig, Tsf, Tuf, and TypA), and motility (CheW and FlaA).

Subsequently, 10 differently expressed proteins were selected for qRT-PCR analysis according to their potential to play role in the phase switch. The selected proteins participate in pathways important for the survival of *C. jejuni*, such as carbon metabolism (AcnB, FumC, and OorA), general and oxidative stress response (BetA, DnaK, Rbr, and TrxB), motility (FlaA, CheW) and gene expression regulation represented by the essential two-component regulator CosR. When comparing the level of the transcripts, 6 genes were more transcribed in exponential phase (*betA*, *cheW*, *cosR*, *fumC*, *oorA*, and *trxB*), while 3 genes were more transcribed in stationary phase (*acnB*, *flaA*, and *rbr*). There was no difference in the expression of *dnaK* (**Figure 2**). In most of the cases, the level of transcription corresponded with the level of protein expression. However, in three cases opposite trends were found between the 2-DE and qRT-PCR data, where the proteins were found to be overexpressed in the stationary phase, while the amount of mRNA was higher in the exponential phase. Those include OorA subunit of enzyme involved in carbon metabolism, CheW protein regulating chemotaxis, and regulator CosR.

As the pleiotropic regulator CosR is essential for *C. jejuni* growth and regulates several pathways, CosR was selected for further analysis. To evaluate the changes of *cosR* transcript level throughout the growth of *C. jejuni*, total mRNA was extracted from the cells at different time-points of their cultivation and *cosR* specific transcript level was analyzed using qRT-PCR. The amount of *cosR* transcripts evolved during the growth of the strain 81-176 with an overall fold difference of 5.3. The highest level detected in cells was reached at 7 h of cultivation during the exponential phase and declined constantly afterwards in the stationary phase (**Figure 3**).

The comparison of the data obtained by qRT-PCR with the 4.0-fold protein increase in the early stationary phase led us to a hypothesis that CosR could undergo a negative autoregulation

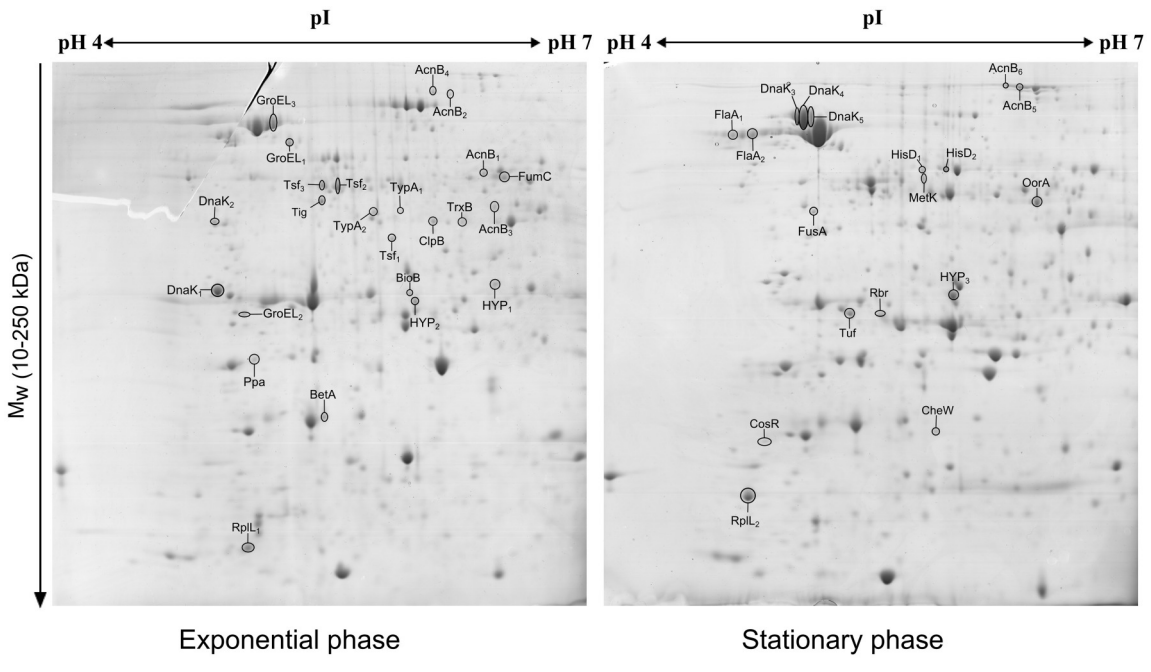

FIGURE 1 | 2D electrophoretic profiles of *C. jejuni* proteins isolated after 7 h (exponential phase) and 16 h (early stationary phase) with highlighted overexpressed proteins. A total of 700 µg of cytosolic proteins were separated on IPG strip (pH 4-7) followed by second separation by SDS-PAGE in 12% acrylamide gel stained by Coomassie blue.

TABLE 2 | Identification of spots differently expressed in exponential phase (7 h) and early stationary phase (16 h) of *C. jejuni* growth.

Access No.	Protein	Respective gene	Theoretical Mw (kDa)/pI	Fold[a]	*P*-value	MOWSE Score[b]	MP/PC[c]
METABOLISM							
gi|121612675	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	*acnB*	92.69/5.89	−3.3	5.96E−04	3.78E + 04	12/20%
				−2.6	1.31E−03	4.37E + 04	11/16%
				−2.0	6.59E−05	2.04E + 02	5/8%
				−2.6	6.85E−05	8.95E + 05	13/18%
				+3.4	1.19E−05	1.09E + 06	15/22%
				+3.1	6.90E−04	8.25E + 07	18/26%
gi|121613366	Histidinol dehydrogenase	*hisD*	46.40/5.52	+4.2	4.03E−08	2.86E + 04	11/26%
				+2.8	1.18E−04	7.94E + 02	8/20%
gi|121612907	Biotin synthase	*bioB*	30.98/5.58	−3.3	4.85E−04	1.42E + 04	8/28%
gi|121613034	Fumarate hydratase	*fumC*	50.65/6.12	−2.2	4.16E−05	7.73E + 03	9/19%
gi|121612575	S-adenosylmethionine synthetase	*metK*	44.23/5.45	+3.4	3.50E−05	3.22E + 02	6/16%
gi|121613267	2-oxoglutarate-acceptor oxidoreductase subunit OorA	*oorA*	40.93/5.85	+3.0	3.22E−07	1.03E + 05	12/39%
STRESS RESPONSE							
gi|121612439	DNA-binding response regulator	*cosR*	25.54/5.27	+4.0	1.27E−05	1.47E + 03	8/37%
gi|121612363	Putative oxidoreductase	*betA*	63.66/8.75	−2.7	5.90E−06	1.35E + 02	8/12%
gi|121612692	Rubrerythrin	*rbr*	20.97/5.56	+5.3	1.51E−06	8.61E + 01	7/29%
gi|121612116	Pyridine nucleotide-disulphide oxidoreductase	*trxB*	33.78/5.93	−1.8	3.33E−06	1.63E + 05	12/43%
gi|121613623	ATP-dependent chaperone ClpB	*clpB*	95.55/5.47	−3.2	1.93E−04	2.60E + 04	15/13%
gi|121613084	Chaperone DnaK	*dnaK*	67.44/4.98	−4.3	6.65E−05	2.69E + 02	4/8%
				−2.5	3.17E−04	1.48E + 02	6/13%
				+6.8	3.20E−06	2.82E + 02	7/19%
				+3.5	4.84E−04	1.12E + 03	7/20%
				+3.4	9.69E−04	6.01E + 02	7/11%
gi|121612249	Chaperonin GroEL	*groEL*	57.97/5.02	−6.3	2.35E−06	1.61E + 04	14/32%
				−3.8	4.24E−05	3.52E + 01	6/7%
				−2.4	2.23E−03	1.70E + 04	13/34%
MOTILITY							
gi|121612156	Purine-binding chemotaxis protein	*cheW*	19.51/5.48	+3.9	1.18E−05	1.26E + 02	6/38%
gi|121612545	Flageline	*flaA*	59.54/5.61	+5.0	1.77E−04	8.27E + 04	9/19%
				+3.1	2.98E−03	4.21E + 02	7/15%
TRANSLATION							
gi|121612720	Elongation factor G	*fusA*	76.75/5.07	+4.3	3.05E−06	1.75E + 03	10/15%
gi|121613176	50S ribosomal protein	*rplL*	13.02/4.70	−3.8	1.01E−03	4.67E + 01	5/37%
				+2.8	4.53E−04	1.34E + 03	4/40%
gi|121612935	Trigger factor	*tig*	51.02/5.60	−2.5	1.06E−04	2.93E + 04	18/31%
gi|121612430	Elongation factor Tu	*tuf*	43.59/5.11	+3.1	4.40E−05	6.30E + 05	8/19%
gi|121612368	Elongation factor Ts	*tsf*	39.54/5.24	−4.6	7.18E−06	4.73E + 03	9/29%
				−3.2	2.51E−04	6.74E + 02	10/27%
				−2.1	5.03E−03	7.23E + 03	7/23%
gi|121612682	GTP-binding protein	*typA*	66.48/5.23	−2.5	4.81E−05	6.22E + 00	5/6%
				−1.9	7.77E−04	8.55E + 00	8/12%
VARIOUS							
gi|121613702	Inorganic pyrophosphatase	*ppa*	19.33/4.79	−1.8	6.24E−04	6.64E + 01	5/31%
gi|121613455	Putative zinc ribbon domain protein	CJJ81176_0729	27.74/5.60	−2.0	5.25E−03	2.96E + 03	8/27%
				−1.9	1.13E−02	1.19E + 04	14/50%
gi|121612896	Putative glutathione synthetase ATP-binding domain-like	CJJ81176_0107	38.79/5.23	+4.4	1.25E−07	1.72E + 02	8/15%

The fold changes are normalized on the level of protein abundance detected in exponential phase.

[a] *Positive values indicate a fold change for higher protein abundance in stationary phase. Negative values indicate a fold change for lower protein abundance in stationary phase.*

[b] *Each protein was identified with a mass tolerance < 20 ppm using at least four peptides*

[c] *MP/PC: Number of matched proteins/protein coverage*

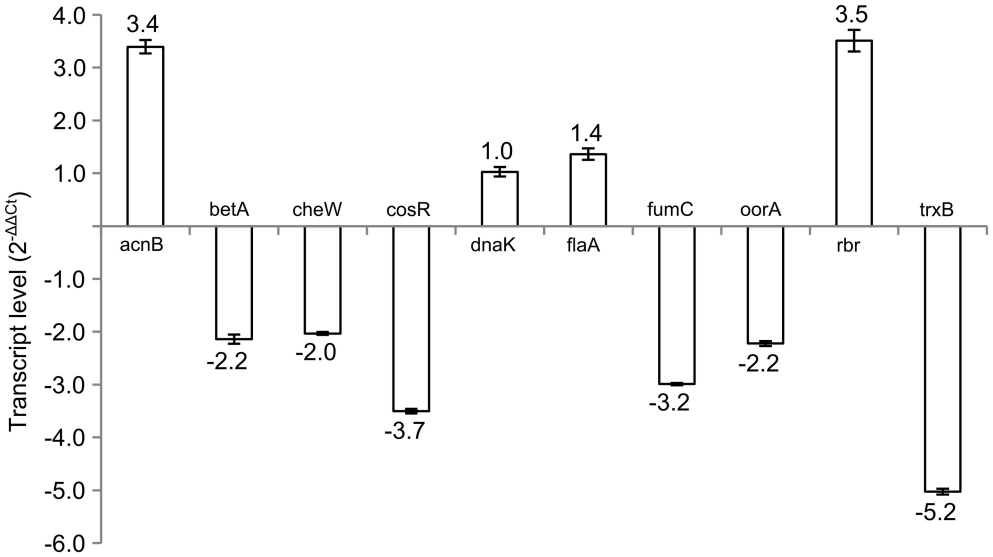

FIGURE 2 | Transcript levels of selected genes in early stationary phase (16 h) of *C. jejuni* growth normalized to the level of transcript from exponential phase (7 h).

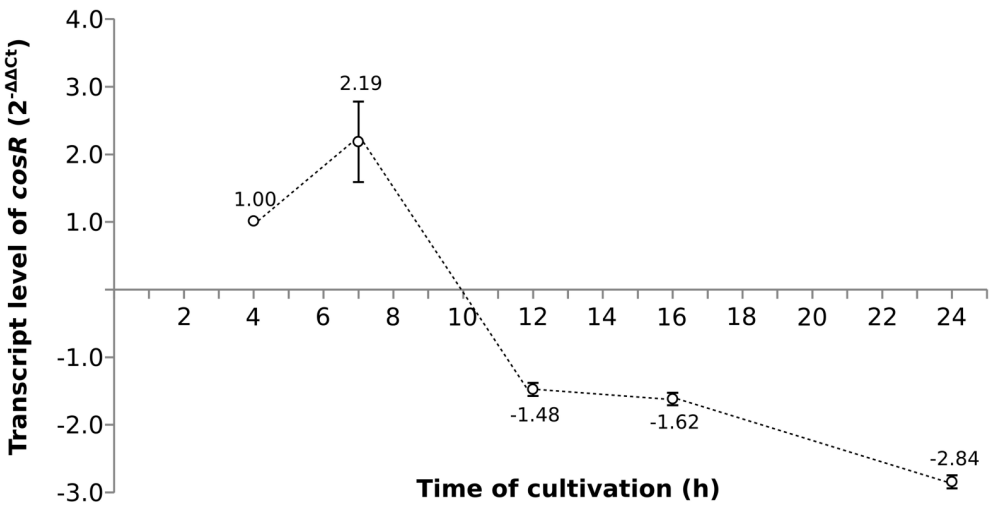

FIGURE 3 | The dynamics of *cosR* transcript level throughout the growth of *C. jejuni* normalized to the transcript level at 4 h.

by binding to its own promoter region. The hypothesis was strengthened by identification of the CosR box described by Hwang et al. (2011, 2012) in *C. jejuni* 81-176 genome upstream from the *cosR* gene. Interestingly, the sequences of the CosR-binding boxes of 4 genes responsible for oxidative stress response are conserved among the analysed strains (**Figure 4A**) with the consensus sequence 5′-wwhhwwwwAAAwTTAdwwTTT-3′ which is similar to the one described by Hwang et al. (2012). To further refine the consensus sequence of the CosR box, 400-nt upstream sequences of the 6 genes previously described as binding CosR (*ahpC*, *cmeA dps*, *katA*, *luxS*, and *sodB*) by Hwang et al. (2011, 2012) and the one of CosR itself were compared among 9 fully sequenced *C. jejuni* strains (**Supplementary Table 1**). The refine CosR box resulted in the consensus sequence 5′-wdnnhdwnwhwwTTwnhhTTd-3′ (**Figure 4B**). In order to verify whether CosR can bind to its own promoter region, EMSA was performed (**Figure 5**). Regions containing the promoter region of *cosR* and an internal part of *cosR* gene were amplified and subjected to a concentration range of purified recombinant rCosR. Promoter region of *katA* from NCTC 11168 was used as a positive control validating the EMSA assays, as rCosR was previously shown to be able to bind to this DNA promoter region (Hwang et al., 2011). A mobility shift was observed when the DNA fragments of *cosR* and *katA* promoters

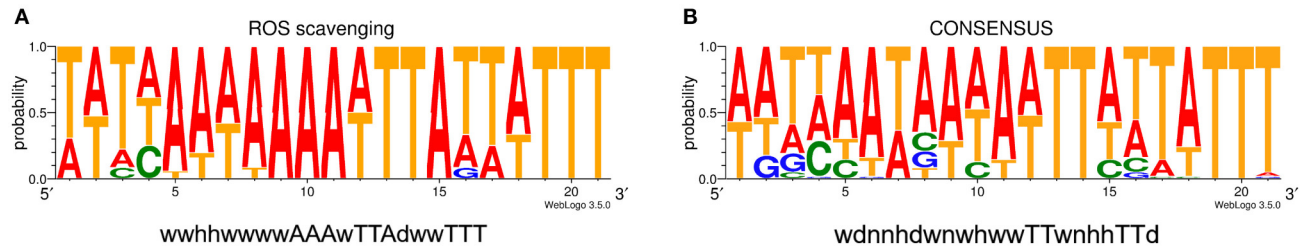

FIGURE 4 | Promoter sequence alignment. (A) Consensus sequence logo from upstream sequence of the promoter region of genes responsible for ROS scavenging (*ahpC*, *cosR*, *katA* and *sodB*). **(B)** Consensus sequence logo of CosR-binding box of the upstream sequences of genes with CosR-binding capacity (*ahpC*, *cmeA*, *cosR*, *dps*, *katA*, *luxS*, and *sodB*). Both logos are based on sequences from 9 strains listed in **Supplementary Table 1**. D–A, T or G; H–A, T or C; N–A, T, C or G; W–A or T.

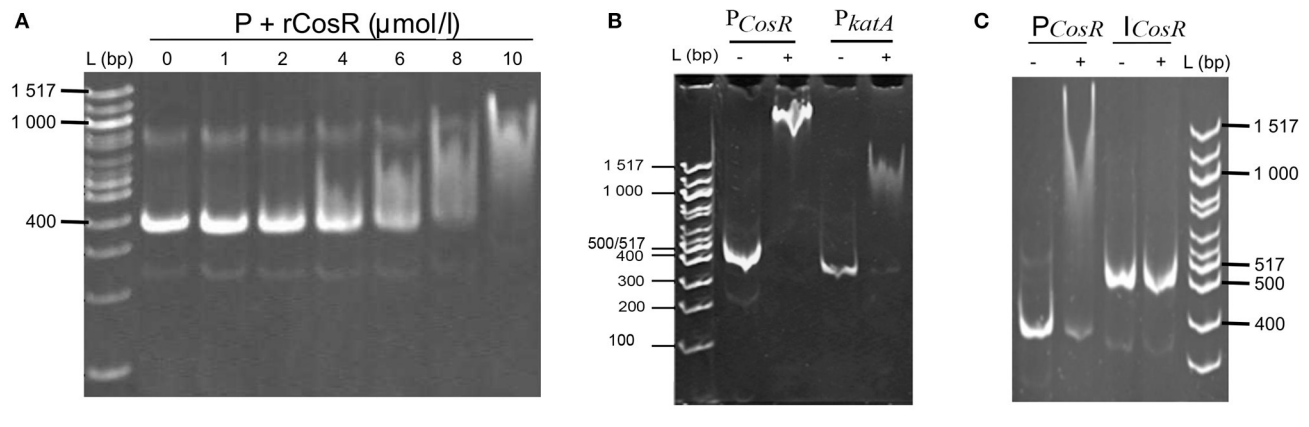

FIGURE 5 | *In vitro* interactions of rCosR with promoter regions. (A) Titration of a 374-bp *cosR* promoter DNA with rCosR. **(B)** Shift mobility of 374-bp *cosR* promoter DNA of *C. jejuni* 81–176 and the positive control using a 337-bp *katA* promoter DNA of *C. jejuni* NTCTC 11168 performed with 10 µmol/l rCosR. **(C)** Mobility of the promoter and internal DNA fragments of *cosR* with (+) or without (–) the addition of 10 µmol/l of the purified *rCosR* protein. L corresponds to 100 bp molecular weight ladder (NE Biolabs).

were used, while no motility shift was observed for the internal DNA region of *cosR*, indicating the potential capacity of CosR to bind to its own promoter.

DISCUSSION

Campylobacter jejuni is repeatedly reported as the major cause of food-borne bacterial enteritides worldwide, causing severe economic and sociological burden. This generally stress-sensitive bacterium, that supposedly cannot multiply outside a host, is able to survive harsh conditions encountered in the environment (Whiley et al., 2013). Despite the concern of this pathogen, relatively little is known about the molecular mechanisms responsible for its ability to persist in meat processing facilities, on carcasses and in the food environment. Enhanced bacterial resistance to various stresses is often connected to the transition of the cells into different phases of growth. For example in the case of *E. coli*, cells in stationary phase were found to be more resistant to high hydrostatic pressure, mild heat, oxidative and osmotic stress, and chlorine disinfectants (Kaur et al., 1998; Benito et al., 1999; Cherchi and Gu, 2011). However, *Campylobacter* cells were reported as generally more resistant in exponential phase of growth, with dynamic emergence of resistant subpopulations in the stationary phase (Kelly et al., 2001, 2003). Molecular mechanisms responsible for the transition between the growth phases have not yet been fully described. Unlike other bacteria, *C. jejuni* lacks sigma factor RpoS regulating the switch from one phase to another (Parkhill et al., 2000). According to several authors, post-transcriptional regulator CsrA could contribute to modulation of gene expression in *C. jejuni* in response to growth phase. Indeed, in *E. coli* and *S. enterica* Thyphimurium, CsrA participates in the regulation of genes inducible in stationary phase (Romeo, 1998; Lawhon et al., 2003). Similar data were recently reported also for *C. jejuni* 81-176 where CsrA strongly influences expression of proteins in stationary phase and might therefore compensate the lack of RpoS (Fields et al., 2016). Another potential regulator that might participate in the transition could be the stringent response regulator *spoT*, as it was previously reported as important for survival of cells in stationary phase (Gaynor et al., 2005). As regulations during phase transition in *C. jejuni* remain unexplained at the molecular level, proteomic fingerprinting of cells isolated from exponential and stationary phase were

compared in this work, to identify the main protein changes occurring between the two main growth phases of *C. jejuni* 81-176.

The first group of identified proteins is involved in cellular stress response. This group contains mostly proteins participating in oxidative stress and protein folding. Interestingly, there is no clear pattern of phase-depending overexpression of these proteins. However, two out of three chaperons were overexpressed in exponential phase, suggesting higher abundance of misfolded and aggregated proteins in the exponential phase, probably caused by the request of functional *de novo* proteins for rapid biosynthesis of cellular material during cell multiplication. The overexpression of stress-related proteins is not surprising, as toxic metabolites accumulate during growth in batch cultivation. The presence of toxic metabolites together with starvation occurring in the stationary phase could be the reasons why the cells overexpressed proteins involved in the stress response. The overexpression of stress response proteins, reported previously by Wright et al. (2009), could speed up the adaptation process of *C. jejuni* when changing the environment. Such adaptation would also require adjustment of metabolism reflecting the changes in the availability of nutrients during the cultivation of the cells. Indeed, in this work, we also observed altered expression of several proteins playing role in *C. jejuni* metabolism, mostly of those involved in the citric acid cycle (TCA). Asaccharolytic bacterium *C. jejuni* uses TCA as the main carbon source through gluconeogenesis (Velayudhan and Kelly, 2002). Unlike other bacteria, the TCA cycle of *C. jejuni* involves enzymes containing oxygen-sensitive Fe-S clusters, such as Oor enzymes (Atack and Kelly, 2009) that are rapidly inactivated after exposure to ambient air (Kendall et al., 2014). Higher abundance of OorA observed in the stationary phase could be explained by successful detoxification of ROS with the help of TrxB and BetA which are among the overexpressed protein in the exponential phase.

Another group of proteins with altered expression between the two growth phases is involved in protein biosynthesis. The proteins overexpressed in the exponential phase were mostly elongation factors responsible for correct synthesis of new peptides. The proteins whose abundance is higher in the stationary phase play role in binding of tRNA to ribosomes and their translocation from A to P site. These results suggest high importance of protein biosynthesis in *C. jejuni* regardless of the physiological state of the cells, although they are in contrast with the decline of ribosomal proteins in the stationary phase reported previously (Wright et al., 2009).

Interestingly, the cells in stationary phase overexpressed proteins involved in motility (flagellar protein FlaA) and chemotaxis (regulator CheW). Similar overexpression was already reported both for cells in stationary phase (Wosten et al., 2004; Wright et al., 2009; Fields et al., 2016) and in biofilms (Kalmokoff et al., 2006). The overexpression of FlaA in stationary phase is mediated by CsrA (Fields et al., 2016) and could be explained by multifunctionality of *C. jejuni* flagella. Besides their role in motility, flagellum apparatus works as a type III secretion system (Konkel et al., 2004) and it was found to be crucial for *C. jejuni* adhesion and biofilm formation (Svensson et al., 2014). In our case the protein regulating chemotaxis was also overexpressed, suggesting that the chemotaxis-driven motility is enhanced in stationary phase, facilitating the migration of cells toward nutrient-rich environment.

The transcript analysis revealed that for most of the tested genes, the level of the transcription was correlated to the variation in protein abundance, indicating a regulation of the corresponding proteins at the transcriptional level. However, in three cases (CosR, CheW, and OorA) the level of transcripts was higher in the exponential phase while the protein abundance was significantly lower as compared to the amount in the stationary phase. Such difference could be explained by post-trancriptional or post-translation modifications (Maier et al., 2009). The translation can be delayed when Shine-Dalgarno sequence of mRNA sequence is not completely complementary to the rRNA sequence (Maier et al., 2009) or when the mRNA undergoes conformational change, e.g., by binding of small metabolites (Nahvi et al., 2004). Another reason can be the involvement of short regulatory sRNA that is influencing stability of mRNA and translation process by targeting or blocking mRNA binding to ribosomes (Maier et al., 2009). In addition, overall number of available ribosomes and half-life of translated proteins also play role in final mRNA-protein ratio. Among the proteins presenting an opposite trend between transcript level and protein amount, only CosR is identified as a DNA regulator. This recently discovered orphan two component system (TCS), whose sequence is conserved among *Campylobacter* and *Helicobacter* species, affects transcription of 93 different genes, including 17 genes essential for *C. jejuni* viability (Hwang et al., 2012). It is involved in the regulation pathway of ROS detoxification (Hwang et al., 2011), efflux pump expression (Hwang et al., 2012), and also influences biofilm formation and maturation of *C. jejuni* (Oh and Jeon, 2014; Turonova et al., 2015). The *cosR* gene is present in all *C. jejuni* strains (Gundogdu et al., 2016). It is an essential gene for *C. jejuni* with the absence of a consensus catalytic aspartic acid replaced by an asparagine. It shares 60% amino acid identity with Hp1043, a response-regulator element of TCS from *Helicobacter pylori* (Beier and Frank, 2000; Schar et al., 2005; Stahl and Stintzi, 2011). The interchange of *C. jejuni cosR* with homologous gene from *H. pylori* does not impair its functionality, indicating a probable conserved function of the gene throughout strains and closely related bacterial genera (Muller et al., 2007). As proteomics analyses using time-point profiling are restricted, dynamics of transcript levels and protein abundance of CosR were explored.

The results indicated a maximum transcribed amount of *cosR* after 7 h of cultivation in exponential phase followed by a decline up to 24 h of cultivation, while the abundance of the protein was 4-times higher at the early stationary phase. The transient upregulation of the transcript level and the protein amount during the growth led us to a hypothesis that CosR could potentially undergo negative autoregulation. Negative autoregulation, or negative autogenous regulation, is one of the three common motifs used for bacterial regulation of transcription (Shen-Orr et al., 2002), where transcription factor negatively regulates its own transcription (Savageau, 1974). This type of autoregulation has been previously detected in

E. coli, where it concerns over 40% of all transcription factors, including global regulators Crp, Hns, Ihf, and Lrp (Shen-Orr et al., 2002). The kinetics of transcription is generally slow, faster kinetics can be achieved by shortening the lifetime of a product by degradation, which is inconvenient due to metabolic cost (Rosenfeld et al., 2002). As it was proven both mathematically and experimentally, negative autoregulation speeds up the transcription response (Rosenfeld et al., 2002; Shen-Orr et al., 2002). However, autoregulation requires active binding of the transcription factor to the promoter region of the operon. Previous studies of binding of CosR–DNA sequences in upstream regions of some genes revealed the presence of a specific CosR-binding box (Hwang et al., 2011, 2012). The resulting consensus sequence of the box was estimated from sequences of 5 genes in *C. jejuni* NCTC 11168. The authors considered some of the nucleotides to be identical although they were not present in all cases (4/5 for positions 3, 9, 11, 14, 20, and 21). The only identical nucleotides present in all 5 examined regions were on positions 5, 10, 15, and 19. Our analysis show lower level of similarity of the CosR boxes. When we compare the sequences of genes responsible for ROS scavenging (*ahpC*, *cosR*, *katA*, and *sodB*), the CosR-binding box is conserved and similar to the one reported by Hwang et al. (2012). This consensus sequence was refined using 63 sequences from upstream promoter regions of all genes previously described to bind CosR and the own promoter region of CosR. The resulting consensus sequence indicated a well conserved motif at the end of the consensus sequence which might be valuable for further analyses of the CosR regulon. To detect whether CosR could potentially bind to its own promoter, DNA shift mobility analysis was used. The data indicated a shift mobility of CosR in presence of its own promoter region sequence, which indicates that CosR is also able to bind its own promoter region. Therefore, it might have a potential to undergo a negative autoregulation. The autoregulation of CosR could be also influenced by the post-transcriptional regulator CsrA, which was recently reported to affect CosR expression (Fields et al., 2016). However, due to broad spectrum of genes under CrsA regulation it is not clear whether the observed difference in CosR expression is caused by direct or indirect regulation. Further analyses are therefore required to decipher a potential role of CsrA in cosR expression. Taken together, these data suggest a potential binding of CosR to its own promoter, although additional analyses are required to confirm the *in vivo* protein-DNA complexation. Future analyses should focus on this potential complexation of CosR with its own promoter and its impact on gene expression.

In summary, our proteomic data show that the transition from exponential to stationary phase is accompanied with numerous changes in the gene expression. The main functional groups of differently expressed proteins involve those playing role in metabolism, stress response, translation, and motility, suggesting active adaptation of the cells to the environment. Our transcriptomic and proteomic analyses also pinpointed the dynamics of the CosR expression, leading to the hypothesis of negative autoregulation of this pleiotropic regulator. Altogether, we outlined mechanisms that *C. jejuni* could use during the growth.

AUTHOR CONTRIBUTIONS

OT conceived the study. OT, NH, MH, and HT designed the experiments. HT and MH performed the experiments, DC performed the protein identification. HT and MH analyzed the results and HT prepared the draft manuscript. NH, JP, and OT contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00913/full#supplementary-material

Supplementary Table 1 | CosR-binding box sequences of genes used in the bioinformatic analysis.

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Cj0440c Affects Flagella Formation and *In Vivo* Colonization of Erythromycin-Susceptible and -Resistant *Campylobacter jejuni*

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Campylobacter jejuni is one of the most common foodborne pathogen worldwide. A putative transcriptional regulator, *Cj0440c*, was up-regulated in the erythromycin-resistant *C. jejuni*, however, the precise role of *Cj0440c* is yet to be determined. The aim of this study was to determine the biological functions of *Cj0440c*. The *Cj0440c* isogenic mutants were constructed from erythromycin-susceptible *C. jejuni* NCTC 11168 (S) and -resistant *C. jejuni* 68-ER (R), designating as SM and RM, respectively. The isogenic Cj0440c mutants (SM and RM) and parental strains (S and R) were subjected to microarray and qRT-PCR analysis to examine the transcriptional profile changes contributed by *Cj0440c*. The antimicrobial susceptibility, flagellar morphology, *in vitro* growth and *in vivo* colonization in chickens were carried out to analyze the biological function of *Cj0440c*. The results showed that 17 genes were down-regulated in SM compared to S, while 9 genes were down-regulated in RM compared to R. The genes with transcriptional change were mainly involved in flagella biosynthesis and assembly. Using transmission electron microscopy, we found that the filaments were impaired in SM and lost in RM. The chicken colonization experiments showed that *Cj0440c* mutants (SM and RM) had reduced colonization ability in chickens when compared with corresponding parental strains (S and R). In conclusion, *Cj0440c* regulates flagella biosynthesis and assembly, and consequently affect the *in vivo* colonization of erythromycin-susceptible and -resistant *C. jejuni*.

Keywords: ***Campylobacter jejuni, Cj0440c*, flagella, colonization, erythromycin resistance**

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INTRODUCTION

Campylobacter jejuni has been recognized as one of the most important pathogens, which can cause infectious diarrhea and severe forms of disease such as Guillain-Barre Syndrome or Miller Fischer Syndrome (Samuel et al., 2004; Hughes and Cornblath, 2005; Riddle et al., 2006). The CDC estimated that in 2009 the number of *Campylobacter* infection was 13.02 per 100,000 people (Silva et al., 2011). The cost of human Campylobacteriosis in the United States is estimated at $1.3 to 6.8 billion dollars annually (Scharff, 2012; Epps et al., 2013). Macrolides (e.g., erythromycin) are

the most important drugs of choice for clinical treatment of *Campylobacter* infections (Gibreel et al., 2005). Unfortunately, macrolides-resistant *Campylobacter* have emerged and impose a global public health concerns (Gibreel and Taylor, 2006; ECDC et al., 2009). In earlier study we demonstrated that the transcription level of *Cj0440c* was increased in high-level erythromycin-resistant *C. jejuni* (Hao et al., 2013).

Bioinformatic analyses suggested that *Cj0440c* is a putative transcriptional regulator and encodes a TENA/THI-4 family protein, however, the molecular function of this family is yet to be determined. The gene *Cj0440c* is located downstream of the *Cj0437–Cj0439* operon *(mfr,* methylmenaquinol:fumarate reductase) which plays an important role in the susceptibility to hydrogen peroxide (H_2O_2) (Parkhill et al., 2000; Weingarten et al., 2009; Kassem et al., 2012) and upstream of *Cj0441* (*acpP*, acyl carrier protein) which is a universal and highly conserved acyl donor for synthesis of fatty acid, endotoxin and acylated homoserine lactones for the quorum sensing in *C. jejuni* (Byers and Gong, 2007). Both the downstream and upstream genes of *Cj0440c* were essential for the growth, survival, colonization and pathogenesis in *Campylobacter*. Although *Cj0440c* is located on the opposite DNA coding strand, it may divergently transcribed with its up-and-downstream genes, and likely to act as a transcriptional regulator and play an important role in gene regulation and the biological function in *C. jejuni*. The biological functions of *Cj0440c* in *C. jejuni* are largely unknown.

In the present study, *Cj0440c*-inactivated mutation was constructed in both erythromycin-susceptible (S) and -resistant *C. jejuni* (R), the transcriptional profile and relative *in vitro* and *in vivo* phenotype determination were implemented to decipher the function and regulation mechanism of *Cj0440c*.

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Growth Conditions

The *C. jejuni* NCTC11168 (designated as **S**) was kindly provided by Chinese Center for Disease Control and Prevention. *C. jejuni* strains were routinely cultured in Mueller-Hinton (MH) medium at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) in the anaerobic incubator (YQX-II, Shanghai, China) (Mace et al., 2015). The *Escherichia coli* DH5α was grown aerobically in Luria-Bertani medium at 37°C. The erythromycin-resistant *C. jejuni* strain 68-ER (designated as **R**) was descendant of *C. jejuni* NCTC11168 resulting from *in vitro* step-wise selection by erythromycin. Plasmids pGEM-T (Promega, Madison, WI, USA) and pMW10 was kindly provided by China Agricultural University and used for mutant vector construction.

Construction of Isogenic Δ*Cj0440c* Mutants

The DNA fragment containing *Cj0440c* gene and its flanking regions was amplified from *C. jejuni* NCTC 11168 genome using *Pfu* polymerase (Promega) with primers of Cj0440cF_2 and Cj0440cR_2 (**Table 1**) and was cloned into pGEM-T easy vector (Promega,) to generate plasmid pCJ0440c. Primers pCj0440cU and pCj0440cL (**Table 1**) carrying endonuclease restriction sites of KpnI and XbaI were used to inversely amplify DNA fragment from the vector of pCJ0440c using Taq and Pfu polymerase (8:1). A kanamycin resistance cassette (*kan*) was amplified from pMW10 plasmid with Pfu polymerase (Promega) using primers KanF and KanR (**Table 1**) which have the same restriction sites of KpnI and XbaI. The amplified DNA fragments of inverse PCR and *kan* were digested with KpnI and XbaI and purified with a PCR clean-up kit (Generay, Shanghai, China). The digested inverse PCR product was ligated to the *kan* cassette using T4 DNA ligase (Takara, Dalian, China) to obtain the construct plasmid pCJ0440c-Kan, which was then transformed into *E. coli* DH5α. The purified plasmid of pCJ0440c-Kan was introduced into S and R via electroporation according to the method described previously (Jeon et al., 2011). Insertional mutants, named SM and RM, respectively, were selected on MH agar plates with 25 μg/ml kanamycin and 50 μg/ml ampicillin. Both PCR and sequencing analysis of the *Cj0440c* mutants (SM and RM) confirmed that the mutation resulted in deletion of 200 bp of coding sequence in *Cj0440c* and simultaneous insertion of the *kan* gene into the same location.

TABLE 1 | Primers used for construction of *Cj0440c* mutant and for real-time qPCR.

Primer name	Primer Sequence (from 5′ to 3′)	Product size (bp)
Primers used for construction and confirmation of *Cj0440c* mutant		
Cj0440c-F2	AATACCAGAAGCCGAAAC	2315
Cj0440c-R2	GAGGGTGAAATAGAAGGG	
pCj0440c-U	GGGGTACCAGATCATCCTTACAAGGAAT *Kpn*I site	5100
pCj0440c-L	GCTCTAGATTCATAGCAAAACGAAGT *Xba*I site	
Kan-F	GCTCTAGAAATGGGCAAAGCAT *Xba*I site	1203
Kan-R	GGGGTACCATAATGCTAAGACAAT *Kpn*I site	
Primers used for real-time qPCR		
Cj1339cF	TCCATTAAACGGTTGATATCTGCTT	125
Cj1339cR	AAGGCTATGGATGAGCAACTTAAAAT	
Cj1328F	CTTTTAGCGATGCTTTTGAAGACTTA	126
Cj1328R	CGCCACATAAATGCACTAAAGG	
Cj1294F	GGCGTAAAACACGCTTGTGTATT	79
Cj1294R	TTTCTTGGACACCTAGTGCTGTGTA	
Cj1338cF	TTACCATTGTTGATAGCTTGACCTAAA	75
Cj1338cR	TGCTTCAGGGATGGCGATA	
Cj0043F	GGGTTCTCCTGTTGCAAGTGA	75
Cj0043R	GCCCCTAAAACCCCAAAAAAT	
Cj0697F	TGGTTCAGACCAAAGATGGA	138
Cj0697R	TGCCAGCATTCTGAGGATTA	
Cj1242F	AAGACATTGATCTTGGTGCTG	143
Cj1242R	ATTGTTTGTGGCATTTCCTG	
Cj1385F	GGAAACTGGGACTTGGTAGGAA	83
Cj1385R	TGAGTATGGATGAAATCAGGGAATT	
Cj1464F	CGAGTAAAATCGCAGAGCAG	69
Cj1464R	TCGCAGCAGCTGTAGCTTT	
q16SF	GCTCGTGTCGTGAGATGTTG	199
q16SR	GCGGTATTGCGTCTCATTGTAT	

TABLE 2 | Transcriptional difference in the mutant SM comparing to its parental strain S determined by microarray.

Function class	Gene name	Gene function	Fold change
Target gene	*Cj0440c*	Putative transcriptional regulator	−24.3
Cell motility/signal transduction	*Cj1339c/flaA*	Flagellin	2.1
	Cj1338c/flaB	Flagellin	−3.5
	Cj1729c/flgE2	Flagellar hook protein FlgE	−2.2
	Cj0887c/flgL	Flagellar hook-associated protein FlgL	−2.4
	Cj1466/flgK	Flagellar hook-associated protein FlgK	−3.3
	Cj0043/flgE	Flagellar hook protein	−3.5
	Cj1462/flgI	Flagellar basal body P-ring protein	−3.0
	Cj0698/flgG	Flagellar basal body rod protein FlgG	−3.2
	Cj0687c/flgH	Flagellar basal body L-ring protein	−3.2
	Cj0697/flgG2	Flagellar basal-body rod protein	−3.6
	Cj0041/fliK	Putative flagellar hook-length control protein	−4.5
Carbohydrate metabolism	*Cj1327/neuB2*	*N*-acetylneuraminic acid synthetase	−3.1
	Cj1328/neuC2	UDP-*N*-acetylglucosamine 2-epimerase	−2.4
Amino acid/energy metabolism	*Cj1293/pseB*	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	−2.2
	Cj1294/pseC	C4 aminotransferase specific for PseB product	−2.5
Hypothetical proteins	*Cj1026c*	Putative lipoprotein	−2.1
	Cj1242	Hypothetical protein	−2.3
	Cj1632c	Putative periplasmic protein	5.7

Only flaA and Cj1632c were up-regulated.

TABLE 3 | Transcriptional difference in the mutant RM comparing to its parental strain R determined by microarray.

Function class	Gene name	Gene function	Foldchange
Target gene	*Cj0440c*	Putative transcriptional regulator	−35.2
Cell motility/signal transduction	*Cj1338c/flaB*	Flagellin	−2.3
	Cj0547/flaG	Flagellar protein FlaG	−2.3
	Cj0548/fliD	Flagellar capping protein	−2.1
	Cj0042/flgD	Flagellar basal body rod modification protein	−2.7
Energy metabolism	*Cj1385/katA*	Catalase	−2.5
Hypothetical proteins	*Cj1464/flgM*	Hypothetical protein	−4.4
	Cj1465	Hypothetical protein	−3.8
	Cj1242	Hypothetical protein	−2.0
	Cj0391c	Hypothetical protein	−2.1

RNA Microarray and Data Analysis

The transcriptional difference between *Cj0440c* mutants and their parental strains (SM&S and RM&R) was examined by microarray (CapitalBio Corporation, Shanghai, China). Briefly, the strains were separately grown in MH broth for 24 h at 42°C under microaerophilic conditions with shaking. Immediately after the incubation, twice volume of RNA protective reagent (Qiagen, Germantown, MD, USA) was added to the culture (with same OD_{600} of 0.3) to stabilize mRNA. The total RNA from each sample was extracted using RNeasy Protect Mini Kit (Qiagen) and purified using Nucleo®Spin RNA clean-up kit (Macherey-nagel, Germany). The RNA quality and quantity was determined by formaldehyde denatured gel electrophoresis and Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized from the extracted RNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was labeled by Cy5 or Cy3 dye and co-hybridized onto one microarray slide (NimbleGen 4 K × 72K), scanned by Axon Instruments Gene Pix 4000B (Union City, CA, USA) and read by Gene Pix Pro 6.0 (Axon Instruments). Microarray data were analyzed using Array Star software. The genes with False Discovery Rate (FDR)-corrected q-values < 0.01 and fold change >2 were selected as differentially expressed genes and subjected to NCBI gene annotation, KEGG pathway analysis and STRING 9.05 protein network analysis.

Microarray Data Accession Number

The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database[1] and assigned accession number GSE49255 and GSE49256.

[1]http://www.ncbi.nlm.nih.gov/geo/database

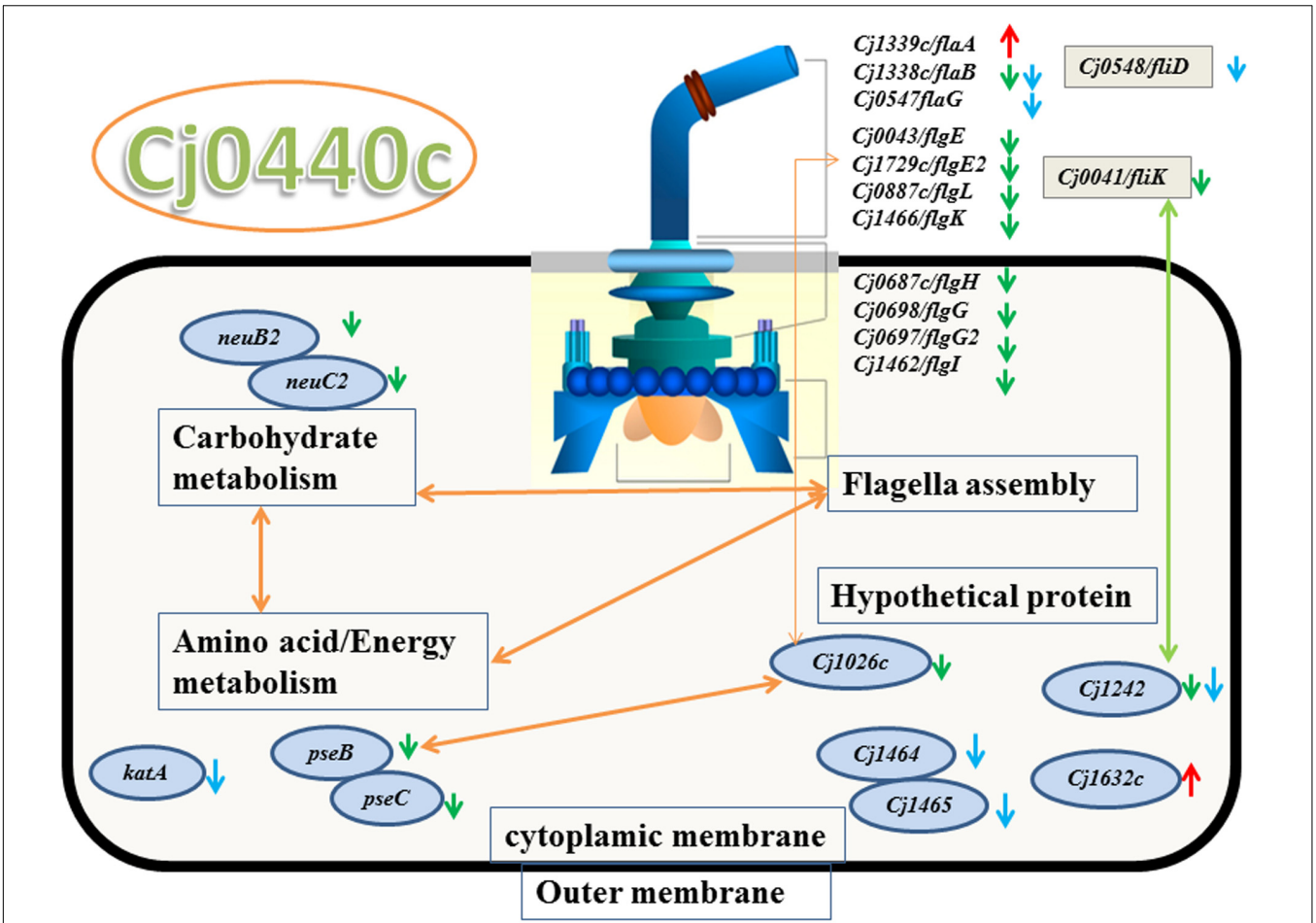

FIGURE 1 | Significant genes in *Cj0440c*-inactivated mutants and their relationship based on KEGG and STRING protein network analysis. Green arrow and red arrow were genes down-regulated and up-regulated in SM, respectively. Genes with blue arrow were down-regulated in RM. The yellow double sided arrow means positive relationship between these genes and green double sided arrow means negative relationship between these genes.

qRT-PCR

The same batches cDNA of *Cj0440c* mutants (SM and RM) and their parental strains (S and R) used in microarray were subjected to qRT-PCR analysis to confirm the transcriptional difference of some respective genes identified by microarray following method described in previous study (Hao et al., 2010). Briefly, the PCR amplification was performed in IQ5 Multicolor Real-time PCR Detection System (Bio-Rad). The cycling conditions were as follows: 3 min of pre-incubation at 95°C, followed by 30 cycles of 10 s at 95°C and 40 s at 60°C. The primer sets used for specific genes are listed in **Table 1**. 16S rDNA was used as an internal control for normalization. The experiment was done in triplicate to obtain the average value of fold change. The student's *t*-test was performed to analyze the significant difference between mutants and their parental stains.

Antimicrobial Susceptibility Test

Minimum inhibitory concentrations (MICs) of nine antimicrobial agents (azithromycin, erythromycin, tylosin, ciprofloxacin, olaquindox, chloromycetin, tetracycline, gentamicin, and ceftriaxone) were determined using agar dilution method recommended by Clinical and Laboratory Standards Institute (CLSI). *C. jejuni* ATCC 33560 was used as a quality control strain.

Transmission Electron Microscopy

The presence and length of flagella on the four *C. jejuni* strains (S, SM, R and RM) were examined using transmission electron microscopy according to a previously described method (Barrero-Tobon and Hendrixson, 2014; Matsunami et al., 2016). Briefly, bacterial suspensions were obtained after washing plate with 2 ml sterile phosphate-buffered saline and spotted on carbon-coated copper grids. The cells were stained with 2% phosphotungstic acid (pH 6.7) for 1 min. Samples were observed employing a HITACHI H-7650 transmission electron microscope (Hitachi, Japan).

In Vitro Growth Determination

To compare the growth kinetics of the mutants with that of the parental strains, a fresh culture (100 μL) of each *C. jejuni* strain

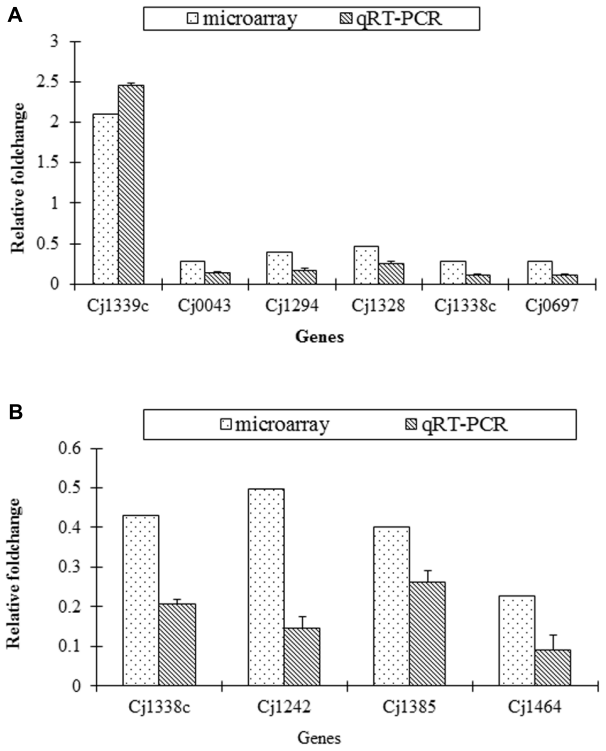

FIGURE 2 | Confirmation of transcriptional change of target genes in the mutants and the parental strains by qRT-PCR. (A) Fold change of six genes in SM versus S; **(B)** Fold change of four genes in RM versus R.

(0.5 McFarland) was inoculated into 100 mL MH broth and the cultures were incubated at 42°C under microaerobic conditions for 36 h with shaking. The growth kinetics was determined by measuring the absorbance in 600 nm (OD_{600}) at 0, 4, 8, 12, 20, 22, 24, 27, 31, 33, and 36 h post-inoculation.

Single and Competitive Colonization in Chicken

Newly hatched broiler chickens (White Leghorns) were purchased from Zhengda Limited Company (Wuhan, China). All the broiler chickens were examined for *C. jejuni* to ascertain that birds are free of *C. jejuni* prior to infection all the chickens (Hao et al., 2015).

These chickens were randomly assigned to seven groups with 6 to 10 chickens per group. One group was used as a control. Four groups were used for single colonization test in which 10^9 CFU *C. jejuni* strains (S, SM, R and RM) were individually inoculated via oral gavage into each group. Another two groups were used for pairwise competition test in which 10^9 CFU *C. jejuni* pairwise mixtures (S&SM or R&RM) were inoculated via oral gavage to each group. Fecal samples were collected from each bird at 3, 6, 9, and 12 days' post-infection. The CFU of S, SM, R and RM were determined using *Campylobacter* selective CCDA agar (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) with or without 25 μg/ml Kanamycin or 50 μg/ml erythromycin. Each sample was spread onto three respective selective plates to obtain the average CFU.

The significance of differences between mutant and parental strain in colonization at each sampling time point was determined by using Student's *t*-test, Welch's *t*-test to allow for non-constant variation across treatment groups, and the Wilcoxon rank-sum test to allow for non-normality (Guo et al., 2008; Luangtongkum et al., 2012; Xia et al., 2013). Differences were considered significant at a *P*-value of <0.01.

TABLE 4 | Minimum inhibitory concentration (MIC) of *Cj0440c* mutant strains and parental strains to different drugs.

Strains	MIC to different drugs (μg/mL)								
	ERY	TYL	AZI	TET	CIP	CHL	GEN	CRO	OLA
S	1	4	0.0625	0.5	0.125	2	0.5	16	2
SM	1	4	0.0625	0.5	0.125	2	0.5	16	1
R	256	256	32	0.5	0.125	2	0.5	16	2
RM	256	256	32	0.5	0.125	2	0.5	16	1

S was C. jejuni NCTC 11168; SM was Cj0440c deletion mutant of S; R was Erythromycin resistant strain selected from C. jejuni NCTC 11168; RM was Cj0440c deletion mutant of R. The drugs included erythromycin (ERY), tylosin (TYL), azithromycin (AZM), tetracydine (TET), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), ceftriaxone (CRO) and olaquindox (OLA).

Ethics Statement

The animal study was approved by Animal Ethics Committee of Huazhong Agricultural University (HZAUCH 2013-002) and the Animal Care Center, Hubei Science and Technology Agency in China (SYXK 2013-0044). All experimental procedures in this study were performed according to the guidelines of the committee on the use and care of the laboratory animals in Hubei Province, China. All the animals were monitored throughout the study for any signs of adverse effects.

RESULTS

Differentially Expressed Genes in SM and RM

The target gene *Cj0440c* was down-regulated in the *Cj0440c*-inactivated mutants (SM and RM). The other differentially expressed genes in *Cj0440c* mutants (SM and RM) compared to their parental strains (S and R) were shown in **Tables 2**, **3**. The relationship of these different genes was summarized in **Figure 1**.

A flagellin gene (*flaA*) and a gene (*Cj1632c*) encoding a putative periplasmic protein were up-regulated in SM as compared to S (indicated by red arrows in **Figure 1**). Among the down-regulated genes in SM (indicated by green arrows in **Figure 1**), 10 genes (*flaB*, *flgE*, *flgE2*, *flgG*, *flgG2*, *flgH*, *flgI*, *flgK*, *flgL*, and *fliK*) are possible involve in flagellar assembly; 2 genes (*pseB* and *pseC*) in carbohydrate metabolism; 2 genes ($neuB_2$ and *neuC*) in surface glycoprotein metabolism.

None of the genes were up-regulated genes were found in RM when compared the expression of different genes with R. Eleven down regulated genes in RM (indicated by blue arrows in **Figure 1**) are flagellar associated genes (*flgD*, *fliD*, *flaG* and *flaB*),

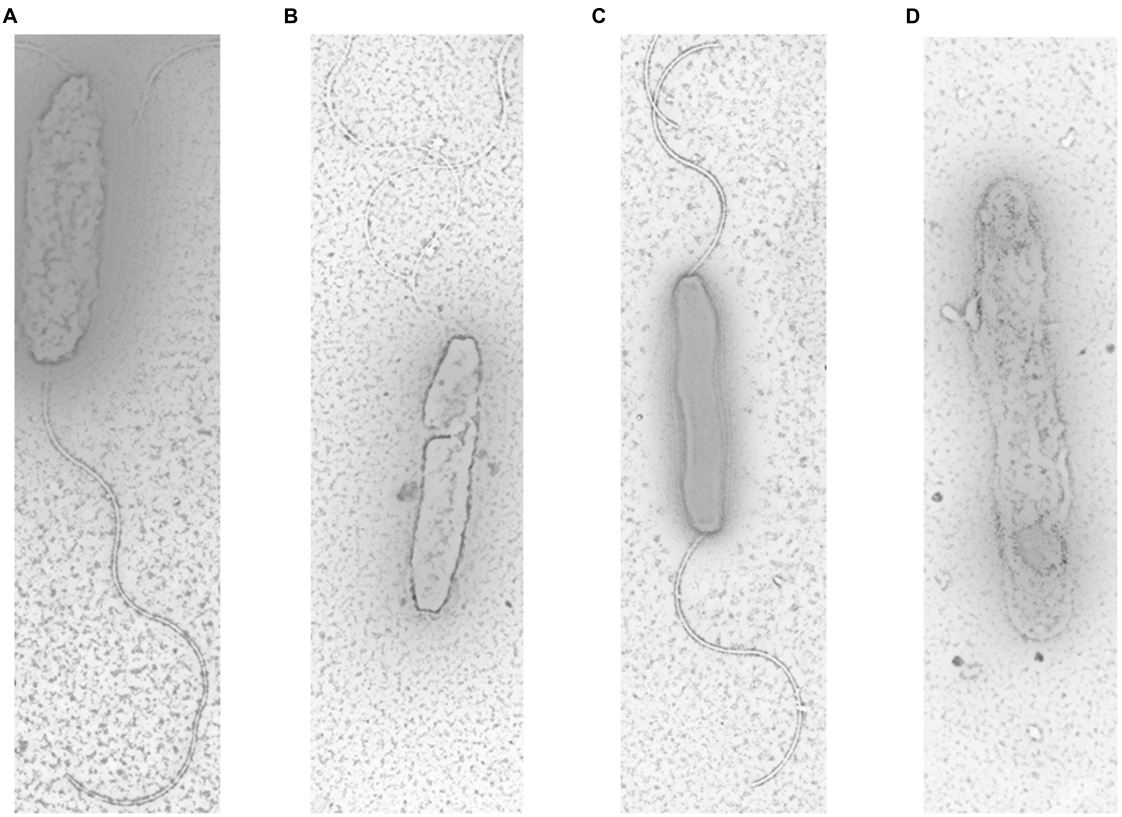

FIGURE 3 | Flagella morphology of S (A), SM **(B)**, R **(C)**, and RM **(D)** under transmission electron microscope. The magnification used for TEM images in the captionis are 1 μm.

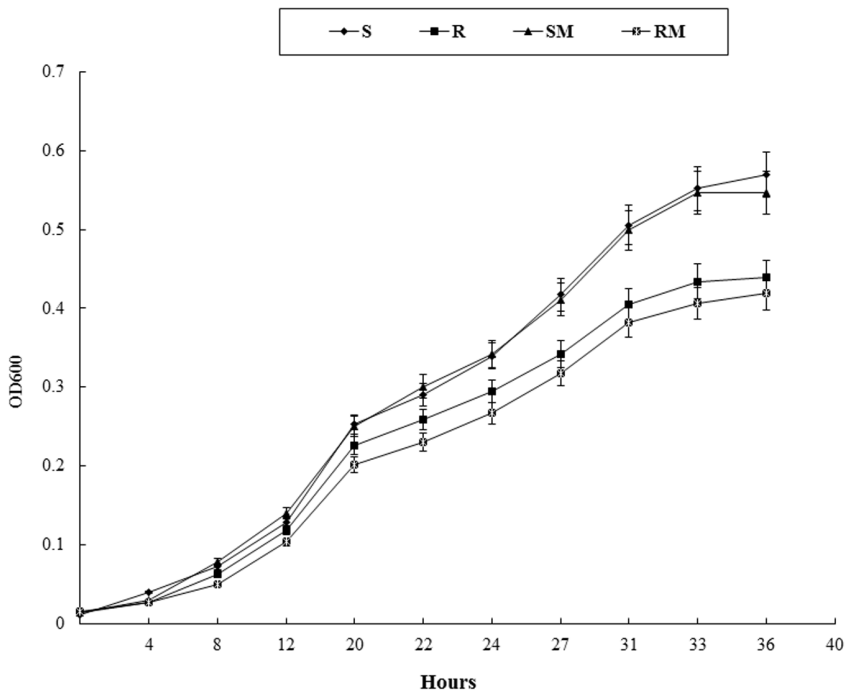

FIGURE 4 | The growth curve of *Cj0440c* mutants (SM and RM) and their parental strains (S and R).

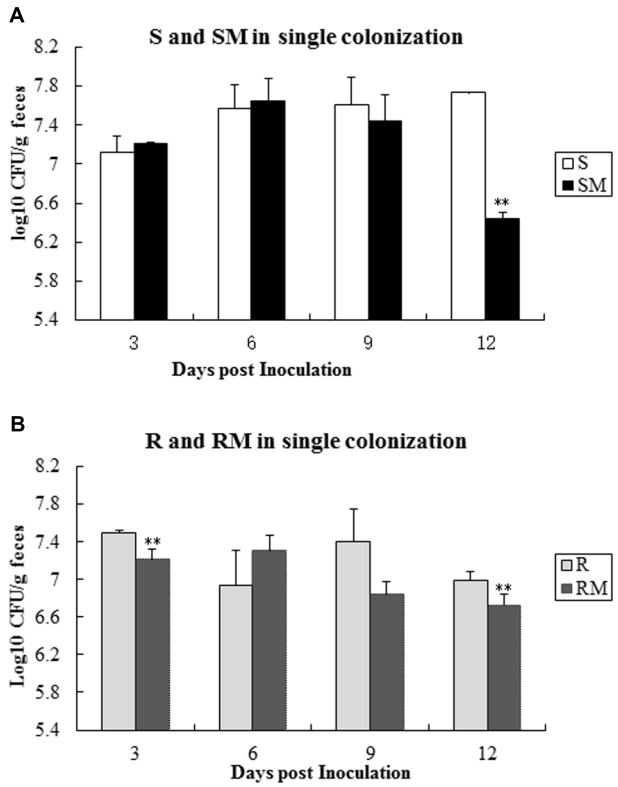

FIGURE 5 | Single colonization of four *C. jejuni* strains in chickens after oral inoculation. (A) Colonization of S and SM; **(B)** Colonization of R and RM. The asterisk (**) represent statistically significant difference with $P \leq 0.01$ comparing with parental strains, respectively.

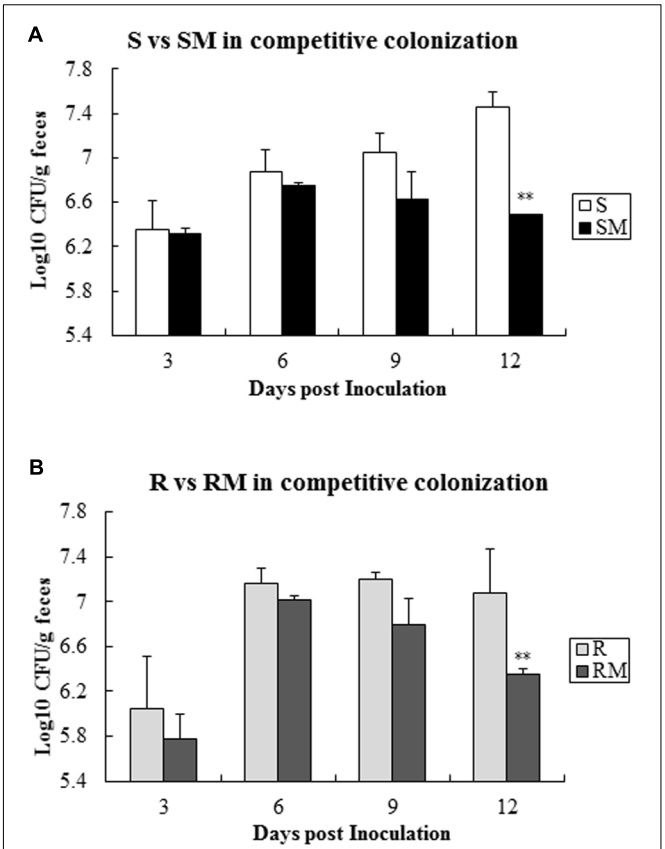

FIGURE 6 | Competitive colonization of *Cj0440c* mutants with their patent strains (SM&S and RM&R). (A) Competitive colonization of the pair of SM&S; **(B)** Competitive colonization of the pair of RM&R. The asterisk (**) represent statistically significant difference with $P \leq 0.01$ comparing with parental strains, respectively.

a catalase encoding gene (*katA*) and four genes with unknown function (*flgM*, *Cj1465*, *Cj0391*, and *Cj1242*).

When submitted to STRING 9.05 and KEGG pathway analysis, the result showed that 10 flagellar genes were interacted with other down-regulated genes (*pseB*/*C*, *neuB*$_2$/*C2* and *Cj1026c*) (**Figure 1**).

The transcriptional change of several representative genes detected in microarray was validated by qRT-PCR. The similar change of the selected genes was found both in microarray and qRT-PCR (**Figure 2**).

The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database[2] and assigned accession numbers GSE49255 (RM&R) and GSE49255(SM&S).

Antimicrobial Susceptibility of *Cj0440c* Mutants

As shown in **Table 4**, there was no significant difference between MIC of nine antimicrobial agents in *Cj0440c* mutants (SM and RM) comparing to their parental strains (S and R). Inactivation of *Cj0440c* did not affect antimicrobial susceptibility of *C. jejuni*.

[2]https://www.ncbi.nlm.nih.gov/geo/

Flagella Presence and Length

The electron micrographs of all tested strains were shown in **Figure 3**. The results showed that parental strains (S and R) had long, spiral and complete flagella filaments in two sides (**Figures 3A,C**). However, SM had shorter filaments in only one side (**Figure 3B**). No filaments of RM were detected in RM (**Figure 3D**). These findings indicated that *Cj0440c* may affect the formation of flagella in *C. jejuni*.

In Vitro Growth of *Cj0440c* Mutants

Growth kinetics of *Cj0440c* mutants (SM and RM) and their parental strains (S and R) were determined in MH broth. No significant difference in growth rate was observed between SM and S. The RM exhibited slower growth rate compared to its parental R, however, the difference was not significant (**Figure 4**).

In Vivo Colonization of *Cj0440c* Mutants

To determine the colonization capacity, broiler chickens were infected individually with four *C. jejuni* strains (S, R, SM and RM). All the strains were able to colonize in chicken intestinal tract, albeit at different rate. Comparing with the parental strains

(S or R), the *Cj0440c* mutants (SM and RM) showed a significant reduction in colonization on 12 days' post-inoculation (**Figure 5**).

When the two pairs of *C. jejuni* strains (SM&S and RM&R) were infected chickens with one pair at a time, *Cj0440c* mutants (SM and RM) exhibited lower colonization level compared to their parental strains (S and R) after 9 days' post-inoculation (**Figure 6**).

DISCUSSION

Campylobacter jejuni is a very common foodborne pathogen in the developed world. Its biology and pathogenicity is largely unknown (Young et al., 2007). *Cj0440c* is a putative transcriptional regulator and an increased transcriptional level expression was detected in the erythromycin-resistant *C. jejuni* (Hao et al., 2013). The gene may encode a TENA/THI-4/PQQC family protein. TENA is one of a number of proteins that enhance the expression of extracellular enzymes (e.g., alkaline protease, neutral protease and levansucrase) (Pang et al., 1991). The extracellular enzymes may be regulated by the master regulator of flagellar genes (e.g., *flhDC*) (Cui et al., 2008). THI-4 protein is involved in thiamine biosynthesis (Akiyama and Nakashima, 1996). This family also includes bacterial coenzyme pyrroloquinoline quinone (PQQ) synthesis protein C (PQQC) proteins. PQQ is the prosthetic group of several bacterial enzymes, including methanol dehydrogenase of methylotrophs and the glucose dehydrogenase (Toyama et al., 2002, 2007). In *E. coli*, PQQ biosynthesis may be affected by *tldD* gene which encodes a peptidase involved in processing of small peptides (Holscher and Gorisch, 2006). The *tldD* may lead to chromosomal DNA relaxation and subsequent derepression of *cdtB* and *lgeR* which may regulate the expression of some flagellar genes (Haghjoo and Galan, 2007). Therefore, the TENA/THI-4/PQQC family may have some indirect relationship with flagellar genes.

The flagella formation plays an important role in the pathogenesis of *Campylobacter* including motility, microcolony formation, biofilm formation, autoagglutination, protein secretion, adherence to host cell, and host invasion (Guerry et al., 2006; Guerry, 2007). The major groups of down-regulated genes in *Cj0440c* mutants (SM and RM) were involved in flagellar assembly, including 11 genes (*flaB*, *flgE*/*E2*/L/K/H/*G*/*G2*/*I*, *flgK*, *flgL*, *fliK*) in SM and 4 genes (*flaB*/*G*, *flgD*, *fliD*) in RM (**Figure 1**). The down-regulation of those flagella-associated genes in *Cj0440c* mutants can reasonably explain why SM and RM lose one or two sides of filament. The reduced colonization of *Cj0440c* mutants may result from the down-regulation of flagella genes.

A second group of genes (*pseB, pseC, neuB2* and *neuC2*) down-regulated in SM were involved in *O*-linked glycosylation which was also essential for flagellin assembly. The *pseB*/*C* in *C. jejuni* contribute in glycosylation modifications of flagellin, often by non-specifically modifying the surface-exposed Thr, Ser, and Tyr residues of filament proteins FlaA and FlaB (Ewing et al., 2009). While *neuB2*/*C2* requires in *O*-linked glycosylation which may contribute to flagella antigen diversity of *Campylobacter* (Linton et al., 2000; Sundaram et al., 2004; Tabei et al., 2009). The down-regulation of these glycosylation-associated genes in SM suggested that *Cj0440c* may play an important role in flagella assembly.

Several hypothetical genes (*Cj1026c, Cj1242, Cj1464, Cj1465* and *Cj0391c*) were down-regulated in SM or RM. The Cj1026c (FlgP) was essential for motility of *C. jejuni* and possible encode the promoter of *flaG* (Sommerlad and Hendrixson, 2007). The Cj1464 (FlgM) may regulate temperature-dependent FlgM/FliA complex formation and flagella length of *C. jejuni* (Wösten et al., 2010). The *Cj0391c* generally co-expressed with flagella-associated genes and involved in biofilm formation of *Campylobacter* (Kalmokoff et al., 2006). The down-regulation of these genes suggested that *Cj0440c* may be closely associated with flagella biosynthesis and assembly.

All our data showed that *Cj0440c* may have close relationship with flagella biosynthesis and assembly, however, the precise role of *Cj0440c* in flagella formation pathway is yet to be determined. Flagellar biogenesis in *C. jejuni* requires three distinct sigma factors, including σ^{80}, σ^{54} (or RpoN) and σ^{28} (or FliA) (McCarter, 2006; Anderson et al., 2010). The FlgS/FlgR two-component system is required for transcription of the RpoN regulon (Joslin and Hendrixson, 2009). The FliK likely part of a negative feedback loop that turns off expression of σ^{54}-dependent genes (Ryan et al., 2005; Kamal et al., 2007). The FlgM (anti σ^{28}) may negatively regulate the class III motility genes (Wang et al., 2005). The present study showed that the transcription of *fliK* was down-regulated in SM and the transcription of *flgM* (*Cj1464*) was down-regulated in RM. The down-regulation of *fliK* and *flgM* can influence the down-regulation of class II and class III motility. The roles of *Cj0440c* on flagellar genes are complex and further investigations are required.

The transcriptional change of majority parts of the genes was similar in both SM and RM except for few differences. The *flaA* and *Cj1632c* were up-regulated and *O*-linked glycosylation was down-regulated only in SM, while *katA*, encoding a sole catalase, was down-regulated in RM but not in SM. The flagellar filaments of *Campylobacter* spp. were composed primarily by FlaA and FlaB flagellin (Guerry et al., 1991). The *flaA* was merely up-regulated in SM but *flaB* was down-regulated in both SM and RM. Findings of our study suggested that that the role *Cj0440c* on transcription of FlaA and FlaB flagellin are different in Ery^s and in Ery^r *C. jejuni*. The *katA* involves in oxidative stress and ROS defense which was essential for intra-macrophage persistence and environmental stress survival of *Campylobacter* (Farr and Kogoma, 1991; Day et al., 2000; Vliet et al., 2002; Flint et al., 2012). The down-regulation of *katA* in RM suggested that *Cj0440c* may interact with *katA* to improve their survival capacity in environmental stress.

The macrolide-resistance in *C. jejuni* generally suffered a fitness cost, however, several other factors may compensate the adaptation weakness (Björkman and Andersson, 2000; Kugelberg et al., 2006; Nilsson et al., 2006; Hao et al., 2010, 2013; Luangtongkum et al., 2012). Our previous study showed that *Cj0440c* was over-expressed in the Ery^r *C. jejuni* (Hao et al., 2013). The result from the present study suggests that *Cj0440c* plays a role in compensating the fitness cost of erythromycin resistance

through the positive relationship with flagellar and other related genes.

CONCLUSION

Cj0440c regulates expression of genes involved in flagella biosynthesis and assembly which consequently affects the growth or colonization of *C. jejuni in vitro* and *in vivo* environment.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XF, HH, YW, XW, and ZY. Performed the experiments: XF and HH. Analyzed the data: XF, HH, JH, SF, GC, LH, and ZY. Contributed reagents/materials/analysis tools: ZY, ZL, MD, and HH. Wrote the paper: HH, XF, JH, SF, and ZY.

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Cj1199 Affect the Development of Erythromycin Resistance in *Campylobacter jejuni* through Regulation of Leucine Biosynthesis

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The aim of this study was to reveal the biological function of *Cj1199* which was overexpressed in the laboratory induced erythromycin resistant strains. The *Cj1199* deletion mutant (ΦCj1199) was constructed via insertional inactivation from its parent strain *Campylobacter jejuni* NCTC11168. The ΦCj1199 and NCTC11168 were then subjected to microarray and real-time PCR to find gene pathway of *Cj1199*. The antimicrobial susceptibility, antimicrobial resistance development, growth characteristics and leucine metabolism were examined to confirm the biological function of *Cj1199*. Our result showed that a total of 20 genes were down-regulated in ΦCj1199. These genes were mainly involved in leucine biosynthesis, amino acid transport and periplasmic/membrane structure. Compared to NCTC11168, ΦCj1199 was difficult to acquire higher-level erythromycin resistance during the *in vitro* step-wise selection. The competition growth and leucine-dependent growth assays demonstrated that ΦCj1199 imposed a growth disadvantage under pressure of erythromycin and in the leucine-free medium. In conclusion, *Cj1199* gene may directly regulate the leucine biosynthesis and transport and indirectly affect the development of erythromycin resistance in *C. jejuni*.

Keywords: *Cj1199*, *C. jejuni*, biological function, leucine biosynthesis, erythromycin resistance

INTRODUCTION

Campylobacter jejuni (*C. jejuni*), is an important foodborne pathogen whose infection often results in acute, self-limiting enteritis, but can lead to more serious complications such as Guillain-Barre Syndrome (Butzler, 2004; Hughes and Cornblath, 2005). Macrolides (mainly erythromycin), which can bind to the 50S subunit of bacterial ribosome and interfere with protein synthesis by inhibiting the elongation of peptide chains (Weisblum, 1995), are the valuable antimicrobials of choice to treat severe *Campylobacter* infections (Gibreel et al., 2005; EFSA, 2009). Point mutations in domain V of 23S rRNA (Taylor et al., 1997), in the ribosome protein L4/L22 and overexpression of efflux pumps are known as the major causes of resistance in *Campylobacter* (Lin et al., 2002; Gibreel et al., 2005). Despite the improved understanding of its general physiology and biochemistry, the molecular mechanisms involved in *Campylobacter* fitness and resistance development are largely unknown.

New approaches, like microarray and RNA-seq, have been used to determine the transcriptome changes associated with some gene mutations and the adaptive mechanisms of *C. jejuni* to erythromycin treatment (Chaudhuri et al., 2011; Xia et al., 2013). Our previous study, using a microarray analysis to find the transcriptional change involved in the development of macrolide resistance in *Campylobacter*, showed that a large number of genes were responsible to the development of macrolide resistance in *Campylobacter*, ranging from genes involved in energy metabolism to macromolecular metabolism to cell processes (Hao et al., 2013). In these differentially expressed genes, a gene of unknown function, *Cj1199,* was overexpressed in the erythromycin resistant *C. jejuni* mutants selected from their parent strains NCTC11168 and 81176. The expression level of *Cj1199* was dose dependent to the level of resistance in the series erythromycin resistant *C. jejuni* mutants selected from NCTC11168. Bioinformatic analyses suggested that *Cj1199* may encode putative iron/ascorbate-dependent oxidoreductase which may be involved in leucine biosynthesis and consequently affect the growth of the strains. Many previous studies revealed that leucine is a key amino acid of erythromycin resistant short peptides (E-Peptide) in *E. coli* (Dam et al., 1996; Tenson et al., 1997; Tripathi et al., 1998; Tenson and Mankin, 2001; Vimberg et al., 2004). Therefore, *Cj1199* may also be associated with E-Peptide mediated erythromycin resistance. The function of *Cj1199* on the development of macrolide resistance and fitness has yet to be determined. The objectives of this study were to decipher functionality and active network of this gene in *C. jejuni*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *C. jejuni* NCTC11168 was kindly provided by Chinese Center for Disease Control. *C. jejuni* strains were routinely cultured on Mueller-Hinton (MH) agar (Oxoid, Basingstoke, UK) or blood agar plates at 42°C under microaerophilic conditions. *E. coli* DH5α was grown aerobically in Luria-Bertani medium at 37°C. The Ham's F-12 nutrient powder mixture (SH30010, Thermo Fisher, Waltham, MA, USA) and Ham's F-12m nutrient powder mixture (RR13033.01, Thermo Fisher, Waltham, MA, USA) lacking leucine were used for leucine biosynthesis test.

Construction of *Cj1199* Deletion Mutant

A 2.5-Kb fragment containing *Cj1199* and its flanking region was amplified with primers of cj1199F and cj1199R (**Table 1**) by PCR and cloned into pGEM-T easy vector (Promega, Madison, WI, USA) to yield pL1199. A chloramphenicol resistance cassette (Cm^r marker) was amplified from pUOA18 using primers of ecatF and ecatR. Primers rF and rR were used to inversely amplify pL1199. The PCR product of the inverse amplification of pL1199 was digested with *Sma*I and *BamH*I and ligated with the Cm^r marker to yield plasmid pL1199T. The plasmid pL1199T was transformed into *E. coli* DH5α and then introduced to *C. jejuni* NCTC11168 by natural transformation (Jeon et al., 2010). The *Cj1199* deletion mutant (ΦCj1199) was selected on MH agar containing chloromycetin (20 μg/ml) and confirmed by PCR and DNA sequencing.

RNA Extraction

The cells of ΦCj1199 or *C. jejuni* NCTC11168 were grown in MH agar plate for 24 h at 42°C under microaerophilic conditions. For RNA extraction, RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA) was added to the cultures immediately after the incubation to stabilize mRNA. The total RNA from each sample was extracted using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA) following the manufacture's protocol. RNA samples were extracted from four independent experiments.

DNA Microarray and Data Analysis

The differential gene expression between ΦCj1199 and *C. jejuni* NCTC11168 was identified using DNA microarray which was supplied by CapitalBio Corporation (Santa Clara, CA, USA). Briefly, iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) was used for synthesis of cDNA from an RNA template via reverse transcription. The cDNA was then labeled with Cy5 or Cy3 dye. The labeled cDNA probes were co-hybridized onto one microarray slide (Roche NimbleGen 4 × 72K, Indianapolis, IN, USA). Hybridized slides were scanned using NimbleGen MS200, and the fluorescence intensities were collected with NimbleGen Scan Software. The linear normalization method was used for data analysis based on expression of the housekeeping genes. Normalized data was log transformed and loaded into MANOVA under R environment. Microarray spots with false discovery rate (FDR)-corrected q-values < 0.01 and fold change ≥ 2 in the T-test were regarded as differentially expressed genes. The differentially expressed genes were classified based on the genomic annotation in NCBI and then subjected to KEGG database for pathway analysis.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

The surplus cDNA of ΦCj1199 and *C. jejuni* NCTC11168 was subjected to real-time quantitative reverse transcriptase PCR (qRT-PCR) analysis to confirm the expression of some respective genes identified by DNA microarray. The primers (**Table 1**) of respective genes were designed using Primer 5 software. The qRT-PCR was performed using SYBR Green Ex Taq^{TM} kit (Takara, Madison, WI, USA) in IQ5 Multicolor Real-time PCR Detection System (Bio-Rad) following method described in previous study (Hao et al., 2010). The qRT-PCR was initiated with a 30 s denaturation at 95°C, followed by 38 cycles of amplification with 5 s of denaturation at 95°C, 30 s of annealing according to the melting temperatures of amplifications. The melting curve was performed from 65 to 95°C (1 s hold per 0.2°C increase) to check the specificity of the amplified product.

Determination of Antimicrobial Resistance (AMR)

Minimum inhibitory concentrations (MICs) of azithromycin (AZM), erythromycin (ERY), tylosin (TYL), ciprofloxacin (CIP), nalidixic acid (NAL), olaquindox (OLA), florfenicol (FFC),

TABLE 1 | Primers used for construction of Cj1199 mutant and for qRT-PCR

Primer name	Primer sequence	Product size (bp)
Primers used for construction and confirmation of Cj1199 mutant		
Cj1199F	TAGCGTATTTGATTTGCGTTTT	2536
Cj1199R	TATTTGCACCTATTCTTGGGTAA	
rF	ACCCCGGGAAATGGCTTAGTATACCCCCACT SmaI site	5140
rR	ACGGATCCGCTAGTATAACCTCTAAATTGAGG BamHI site	
ecatF	ACGGATCCAAAGAGTGACCGCCGAGA BamHI	810
ecatR	ACCCCGGGCAGTGCGACAAACTGGGA SmaI site	
16SF	CTGGCTCAGAGTGAACGC	533
16SR	CCCTTTACGCCCAGTGATT	
hipOF	AGAAGCCATCATCGCACCT	148
hipOR	TGCTGAAGAGGGTTTGGGT	
catF	AAAGAGTGACCGCCGAGATA	0/798[a]
catR	CAGTGCGACAAACTGGGATT	
Cj1199F	TGAACTTACCTATACTTGATT	954/1353[b]
Cj1199R	AAATACTTGCCACATCAG	
Primers used for real-time qRT-PCR		
cj0560F	GCATTTAGGTTTAGGCAAGAAG	159
cj0560R	GTCATCGTTTTAATCTCATCGCT	
cj0920F	CTATTGGTGTATTGGGTGTTGG	184
cj0920R	GCTTCAAACTGACCTCTTGGC	
cj1241F	AGTTACTGGAGTGCCGCTTG	147
cj1241R	AGATTGGTTGGGCTAGGATGT	
cj1388F	CAACTTCCTATCAACCCTGCTTC	221
cj1388R	GCACTTCTTGCTGGATAAGGAG	
cj1646F	CAGGCTGCTTACCAACTCG	275
cj1646R	GCATCGCTAATGGCAACG	
cj1717cF	GCACAGCAAAAGGCACAGG	232
cj1717cR	TCATCACTTCTTAAACTCTTCCAAT	
q16SF	GCTCGTGTCGTGAGATGTTG	199
q16SR	GCGGTATTGCGTCTCATTGTAT	

[a]*There should be no amplification product for NCTC11168, while 798 bp product for ΦCj1199;* [b]*Product length of NCTC11168 should be 954 bp, product length of ΦCj1199 should be 1353 bp.*

doxycycline (DOX), gentamicin (GEN), and levofloxacin (LEV) against ΦCj1199 and *C. jejuni* NCTC11168 were determined with broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). The concentrations of the antimicrobial agents tested ranged from 0.01 to 16 μg/mL. The microdilution plates (96 well plates) were incubated at 42°C for 24 h and the MIC was determined as the lowest concentration where visible bacterial growth was inhibited at the end of the incubation period. *C. jejuni* ATCC 33560 was used as a quality control strain.

For the minimal bactericidal concentration (MBC) of drugs against *C. jejuni* strains, 100 μL from each well was successively diluted 1:10 in MH broth. Then 10 μL was spread on MH agar plates and incubated at 42°C for 24 h for colony forming unit (CFU) counting. The MBC was defined as the lowest drug concentration that resulted in a 99.9% reduction in the bacterial density. The final result was expressed as mean of five independent experiments.

The mutant prevention concentration (MPC) of drugs was determined by agar method (Blondeau et al., 2001). The inoculum of *C. jejuni* was concentrated to 10^{10} CFU/mL. Bacterial suspensions were inoculated on the agar plates containing serial dilutions of drugs and cultured for 96 h. The MPC was the lowest drug concentration on agar plates without bacterial growth under micro-aerobic conditions.

Erythromycin Tolerance Tested by Flow Cytometry (FCM)

The concentration of bacteria was adjusted to a 0.5 McFarland density. Serial twofold dilutions of erythromycin were prepared with MH broth. The final concentrations of erythromycin ranged from 0.0625 to 8 μg/mL. A 100 μL aliquot of bacteria was added to 900 μL MH broth containing different concentrations of erythromycin and incubated at 42°C in a micro-aerobic condition for 3 h. Each dilution was then centrifuged and the supernatant was discarded. The pellet was washed twice with sterile PBS and propidium iodide (PI), a membrane-impermeable DNA-intercalating dye, was used to stain the erythromycin-treated bacterial cells. One milliliter of buffer and 5 μL of PI were added to each sample, and then incubated for 30 min at 4°C. FCM analysis was conducted on CyAn ADP^{TM} FC500 flow

cytometer with Summit^{TM} software (Beckman Coulter, Miami, FL, USA). The intensity of fluorescence of 10,000 cells labeled with PI was analyzed to obtain the mean channel fluorescence (MCF).

In vitro Selection of Erythromycin Resistance

ΦCj1199 and NCTC11168 were subjected to step-wise selection in MH agar plate containing erythromycin. In the first step of selection, cells were plated on MH agar containing 0.25 μg/mL erythromycin. After 3–5 days of incubation under microaerophilic condition at 42°C, colonies were selected and repeatedly incubated with same concentration of erythromycin up to five times. The mutants obtained from first-step selection were then subjected to next step selection by exposing the cells to a twofold increased concentration of erythromycin. The procedure of selection was repeated up to 10 times to obtain highly erythromycin-resistant mutants. All *in vitro*-selected mutants were subjected to MIC test using the broth microdilution method as described above.

Single Growth Curve and Pair-Wise Competition Experiments

Growth curves of ΦCj1199 and *C. jejuni* NCTC11168 were measured individually and repeated three times. Briefly, a fresh *C. jejuni* culture was diluted 100-fold into 10 mL MH broth and incubated for up to 36 h. Viable *C. jejuni* counts were determined at 4, 12, 24, and 36 h.

Pair-wise competition experiments were used to estimate the growth rate of ΦCj1199 compared with *C. jejuni* NCTC11168 in the erythromycin containing (1/2 MIC) or drug-free MH broth. Briefly, the same densities of the parent and mutant cultures were prepared before experiments. Equal volumes (0.1 mL) of adjusted cultures were mixed into 10 mL of fresh medium with or without erythromycin. After 24 h incubation, 0.1 mL bacterial culture was then transferred to another 10 mL fresh medium. The passage was repeated up to 10 times and the initial and final concentrations of parent and mutant were determined by standard plate counting on MH plate with (for the mutant population) or without 10 μg/mL chloromycetin (for the total population), respectively. The assays were repeated three times with three technical replicates.

Leucine-Dependent Growth Test

Cultures of ΦCj1199 and *C. jejuni* NCTC11168 were adjusted to the same densities at the beginning of the experiments. Equal volumes (0.1 ml) of adjusted cultures were added into 10 mL Ham's F-12 and F-12m individually. After 48 h incubation under microaerophilic conditions at 42°C, 0.1 mL of bacterial culture was then transferred to another 10 mL of fresh medium. The passage was repeated up to five times. Viable *C. jejuni* counts were determined by dilution and spatula method on MH plates (ICC, 1992). The experiment was performed in triplicate.

RESULTS

Differentially Expressed Genes in ΦCj1199

The 20 genes down-regulated in ΦCj1199 are shown in **Table 2**. Five genes (*leuA*, *leuB*, *leuC*, *leuD,* and *trpE*) were involved in amino acid biosynthesis. The *leuABCD* genes participated in leucine biosynthesis pathway. Five genes (*Cj0919c, Cj0920c, peb1A*, *pebC*, and *ceuC*) encoded transporters or binding proteins. The permease protein (Cj0919c-Cj0920c), substrate-binding protein (Peb1A), and ATP-binding protein (PebC) worked cooperatively in an ABC-type amino acid transport system. The *ceuC* encoded enterochelin uptake permease which was also transporters or binding protein. The Cj0246c was involved in signal transduction. Six genes (*Cj0560, Cj1356c, Cj0423, Cj0424, Cj0425,* and *Cj0200c*) encoded putative integral membrane or periplasmic proteins. Another three (*Cj1025c, Cj1722c,* and *Cj1388*) were hypothetical genes.

Only *Cj1241* gene encoding a putative major facilitator superfamily (MFS) transport protein was up-regulated in ΦCj1199. Using qRT-PCR to verify the microarray results, *Cj1241* gene was confirmed to be up-regulated, while other four respective genes (*Cj0920c, Cj1388, Cj0560,* and *Cj1717c*) were confirmed to be down-regulated (**Figure 1**).

Antimicrobial Susceptibility of ΦCj1199

The antimicrobial susceptibility of the parent and mutant strains are presented in **Table 3**. There was no significant difference between the MIC of 11 antimicrobials in the ΦCj1199 and its parent strain NCTC11168. Both of the strains exhibited very low MIC (MIC $\leq$ 0.5 μg/mL) to the 14, 15, and 16-membered ring macrolides (erythromycin, azithromycin, and tylosin). The MBC and MPC of five antimicrobials in ΦCj1199 were similar (erythromycin, azithromycin, tylosin, olaquindox, and cydox) with that in NCTC11168 (**Table 3**).

Erythromycin Tolerance of ΦCj1199

The erythromycin tolerance of ΦCj1199 to increased concentration of erythromycin was detected by flow cytometer analysis. As shown in **Figure 2**, the mean value of the fluorescence intensity was increased following the increase of erythromycin concentration. However, when the concentration of erythromycin reached to their MIC (0.125 μg/mL), the mean PI intensity of *C. jejuni* NCTC11168 was significantly decreased, while the mean PI intensity of ΦCj1199 kept at its highest level. Under the treatment of different concentrations of erythromycin, the mean PI intensity of ΦCj1199 was higher than that of parent strain, which indicated that ΦCj1199 had higher mortality and lower tolerance to erythromycin.

Development of Erythromycin Resistance *In vitro*

There was no significant difference between ΦCj1199 and NCTC11168 in the first and second steps of selections. When transferred to third step selection by 1 μg/mL erythromycin, ΦCj1199 descendants exhibited less growth

TABLE 2 | Differentially expressed genes in the mutant of ΦCj1199 comparing to its parent strain NCTC 11168.

Function class	Gene name	Gene function	Fold change
Signal tranduction	*Cj0246c*	putative MCP-domain signal transduction protein	0.3624
Transporters/ binding proteins	*Cj0919c*	putative ABC-type amino-acid transporter permease	0.4997
	Cj0922c/pebC	amino-acid ABC transporter ATP-binding protein	0.461
	Cj0921c/peb1A	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein	0.4597
	Cj0920c	putative ABC-type amino-acid transporter permease	0.3920
	Cj1353/ceuC	enterochelin uptake permease	0.4557
	Cj1241	putative MFS transport protein/Arabinose efflux permease	2.3237
Amino acid biosynthesis	*Cj1716c/leuD*	isopropylmalate isomerase small subunin	0.3996
	Cj1717c/leuC	isopropylmalate isomerase large subunin	0.378
	Cj1718c/leuB	3-isopropylmalate dehydrogenase	0.348
	Cj1719c/leuA	2-isopropylmalate synthase	0.3167
	Cj0345/trpE	putative anthranilate synthase componentI	0.4076
Cell envelop	*Cj0560*	putative MATE family transport protein	0.3564
	Cj1356c	putative integral membrane protein	0.4983
	Cj0423	putative integral membrane protein	0.4534
	Cj0425	putative periplasmic protein	0.4918
	Cj0424	putative acidic periplasmic protein	0.4483
	Cj0200c	putative periplasmic protein	0.4689
Hypothetical proteins and unknown function proteins	*Cj1025c*	hypothetical protein	0.4974
	Cj1722c	hypothetical protein	0.4727
	Cj1388	putative endoribonuclease L-PSP	0.3849

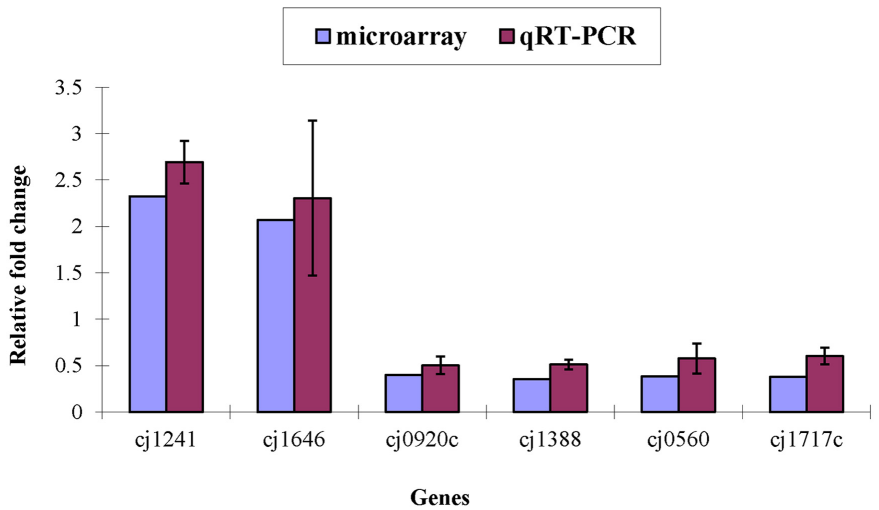

FIGURE 1 | Differential expression of target genes in ΦCj1199 and NCTC11168 determined by real-time qRT-PCR and microarray. The genes were randomly selected. RNA used for real-time qRT-PCR and microarray were prepared in the same time.

than NCTC11168 descendants. When transferred to fourth step of selection, ΦCj1199 descendants could not grow on MH agar containing 2 μg/mL erythromycin, while *C. jejuni* NCTC11168 descendants grew well and successfully promoted to fifth step of selection by 4 μg/mL erythromycin. The erythromycin MIC of mutants selected from NCTC11168 was equal to or higher than 128 μg/mL, which was significantly higher than erythromycin MIC (4 μg/mL) of mutants selected from ΦCj1199.

Growth Disadvantage of ΦCj1199

The ΦCj1199 and NCTC11168 were separately cultured in macrolide-free MH broth. No significant difference in growth rates was observed between the two strains in single cultures (**Figure 3A**).

When ΦCj1199 and NCTC11168 were mixed in pairs into drug-free MH broth, population of ΦCj1199 was decreased after the fifth passage and reduced by twofolds of magnitude after the 10th passage (**Figure 3B**).

TABLE 3 | Minimum inhibitory concentration (MIC)/minimal bactericidal concentration (MBC)/mutant prevention concentration (MPC) of wild-type and mutant strains to different types of drugs.

Drug	Strains					
	Mutant			NCTC 11168		
	MIC (μg/mL)	MBC (μg/mL)	MPC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MPC (μg/mL)
ERY	0.125	0.25	0.25	0.125	0.125	0.25
TYL	0.5	1	1	0.5	1	1
AZI	0.015	/	0.031	0.015	/	0.031
OLA	0.25	0.5	0.5	0.25	0.5	0.5
CYA	0.25	0.5	0.5	0.25	0.5	0.5
FFC	0.5	/	/	0.5	/	/
GEN	0.125	/	/	0.125	/	/
NAL	8	/	/	8	/	/
CIP	0.062	/	/	0.062	/	/
LEV	0.062	/	/	0.062	/	/
DOX	0.125	/	/	0.125	/	/

ERY, erythromycin; TYL, tylosin; AZM, azithromycin; FFC, florfenicol; GEN, gentamicin; NAL, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; DOX, doxycycline; OLA, olaquindox; CYA, cydox. The "/" means not detected.

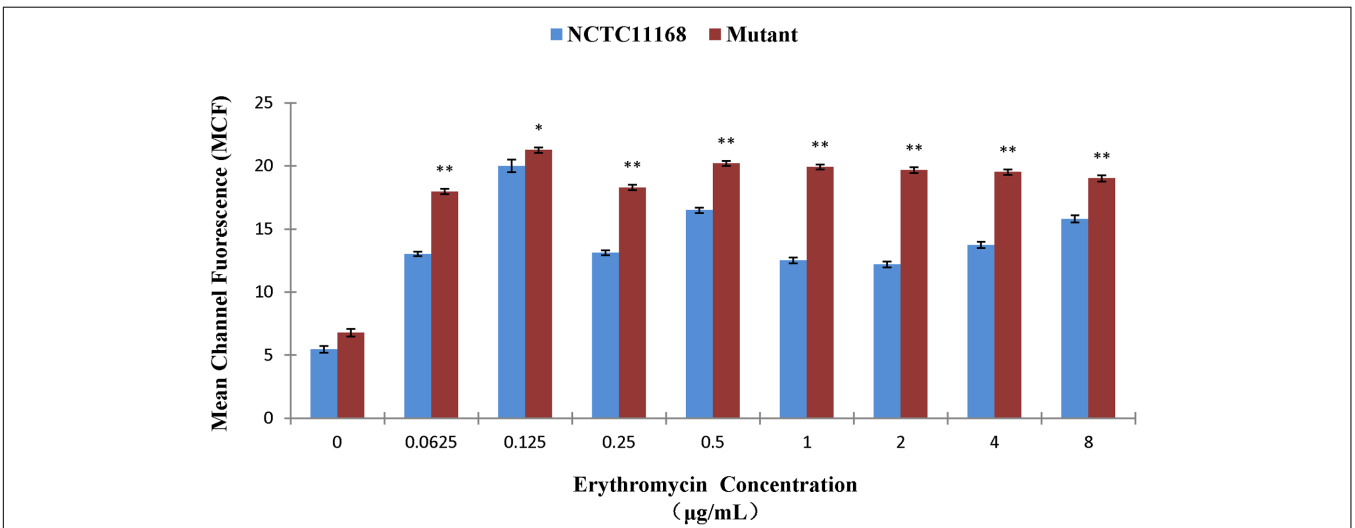

FIGURE 2 | Mean values of PI stain of ΦCj1199 and NCTC11168 cultured with different concentrations of erythromycin. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* NCTC11168, respectively.

In the mixture culture containing 1/2 MIC erythromycin, population of ΦCj1199 was reduced during passages. The ΦCj1199 was outcompeted by NCTC11168 in the drug-containing culture after the fourth passage (**Figure 3C**).

Leucine-Dependent Growth

There is no significant difference of the growth between ΦCj1199 and NCTC11168 in the medium F-12. Both of them grew well after five passages in the medium F-12 (**Figure 4**). However, the number of ΦCj1199 was remarkably decreased during the five times of passage in the medium F-12m. After the fifth passages in the medium F-12m, the number of ΦCj1199 mutant had been reduced by two orders of magnitude comparing with its parent strain NCTC11168 which kept at the level of 10^5 CFU/mL. Conclusively, in absence of leucine, the growth of ΦCj1199 was much slower than NCTC11168.

DISCUSSION

To our knowledge, this is the first study of the function of *Cj1199* on the development of macrolide resistance and biological characteristics of *C. jejuni*. Previously, *Cj1199*, a gene of unknown function, was up-regulated in the macrolide resistant *C. jejuni* mutants selected by step-wise increased erythromycin *in vitro* from their parent strains NCTC11168 and 81176 (Hao et al., 2013). Bioinformatic analyses suggested that *Cj1199* may encode putative iron/ascorbate-dependent oxidoreductase. This gene

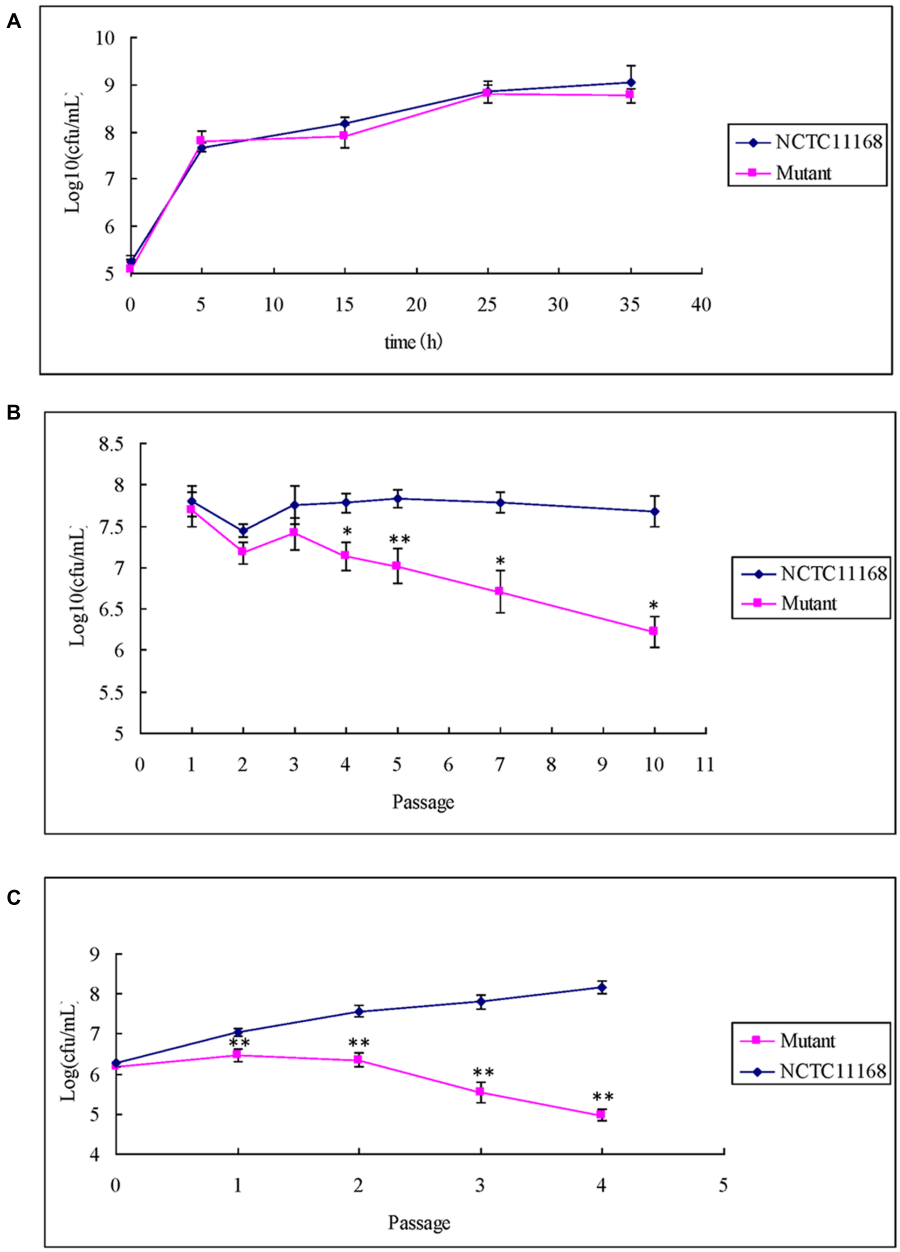

FIGURE 3 | Single growth curve and competitive growth of ΦCj1199 and NCTC11168 in the culture with or without erythromycin. (A) Bacterial culture grown in MH for 36 h. Viable colonies were determined at 5, 15, 25, and 35 h. **(B,C)** Pair-wise competition of C. *jejuni* NCTC11168 and mutant was determined during 5–10 passages in the mixed culture. The Initial and final concentrations were determined by standard plate count on MH or MH with chloromycetin. The asterisk (*) and (**) represent statistical significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* NCTC11168, respectively.

was located in the downstream of *luxS* and over-expressed in the *luxS* null mutation (He et al., 2008). Therefore, the function of *Cj1199* attracted our attention. The gene knockout method, microarray assay, antimicrobial susceptibility test, FCM, and growth determination were used to find out the function and molecular mechanism of *Cj1199*.

From the microarray data, four genes (*Cj0921c-Cj0919c-Cj0920c-Cj0922c*) involved in amino acid transporters were obviously down-regulated in ΦCj1199. The *Cj0921c* gene was known to encode a bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein (Peb1A; Pei and Blaser, 1993; Leon-Kempis Mdel et al., 2006). The Peb1A had higher affinity to both aspartate and glutamate, thus it could bind aspartate and glutamate as specific substrates to initiate the nutrient transport systems in gram-negative species (Pei and Blaser, 1993; Leon-Kempis Mdel et al., 2006). The glutamine could modulate bacterial metabolism of the arginine family as well as serine and aspartate families of amino acid and

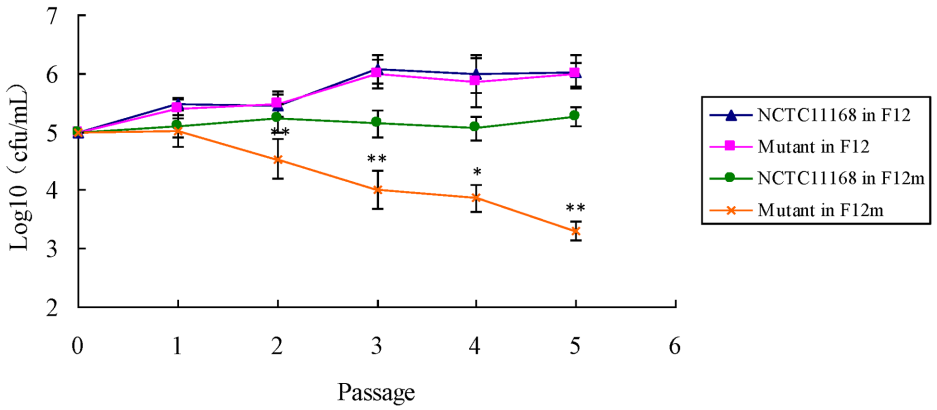

FIGURE 4 | The leucine-dependent growth test. The growth curve of NCTC11168 and ΦCj1199 in Ham's F12 and Ham's F12m were determined. The asterisk (*) and (**) represent statistically significant difference with $P \leq 0.05$ and $P \leq 0.01$ comparing with *C. jejuni* NCTC11168, respectively.

could reduce catabolism of most nutritionally essential amino acids including lysine, leucine, valine and serine, thus it may initiate signaling pathways related to amino acid metabolism in the intestinal bacteria (Dai et al., 2012, 2013). As shown in previous studies, *peb1A* was essential for the growth, invasion and intestinal colonization of *Campylobacter* species (Kervella et al., 1993; Pei et al., 1998; Leon-Kempis Mdel et al., 2006; Muller et al., 2007). Under a microaerobic environment, *peb1A* deficient mutants cannot grow on dicarboxylic amino acids which were the major nitrogen source (Leon-Kempis Mdel et al., 2006). In *C. jejuni*, the mutation in *peb1A* locus could reduce the interactions with epithelial cells and intestinal colonization of mice (Pei et al., 1998). There was no report about the characteristic of the other three genes (*Cj0919c-Cj0920c-Cj0922c*). The string 9.0 and KEGG analysis revealed that these four genes have close interaction with each other and they may work synergistically in the amino acid transport system. The gene cluster (*Cj0921c-Cj0919c-Cj0920c-Cj0922c*) may be essential for the uptake of nitrogen source which was indispensable for the growth and fitness of *Campylobacter* species. However, it is still unknown that how *Cj1199* effects the expression of *Cj0921c-Cj0919c-Cj0920c-Cj0922c* gene cluster.

The *leuABCD* gene cluster was involved in leucine biosynthesis (Labigne et al., 1992). The *leuA* encoded alpha-isopropylmalate synthase (alpha-IPMS) which was key enzyme to catalyze the first committed step in the leucine biosynthetic pathway (Yindeeyoungyeon et al., 2009). The *leuB* gene encoded 3-isopropylmalate dehydrogenase, while *leuC* and *leuD* genes encoded the large and small subunits of isopropylmalate isomerase, respectively (Tamakoshi et al., 1998). The *leuB* gene was reported to be essential for the survival in starvation, colony formation and growth in the Enterobacteriaceae (Matsumoto et al., 2011). In the sequences of *leuC* and *leuD*, cysteine residues for iron-sulfur binding and other amino acid residues involved in isomerase activity were highly conserved (Tamakoshi et al., 1998). The transcriptional derepresssion of a *leuC*-defective allele leaded to accumulation of Leu (+) mutations (Martin et al., 2011). Recent studies revealed that the *leuABCD* gene cluster also participated in the formation of norvaline and norleucine from 2-ketobutyrate and potentially contributed to non-polymeric carbon-chain elongation, which was essential in the synthesis of non-native metabolites in microorganisms (Sycheva et al., 2007; Shen and Liao, 2011). The coordinated expression of the functionally related *leuABCD* gene clusters may be mediated by LeuO and governed by transcription-driven DNA supercoiling (Chen et al., 2003; Wu and Fang, 2003). Our study indicated that the inactivation of *Cj1199* impacted the coordinated expression of *leuABCD* genes. However, the molecular mechanism involved in the regulation is still unknown. The leucine biosynthesis was versatile for many biogenic processes in prokaryotic and eukaryotic organisms. In the yeast and human cells, leucine biosynthesis regulated cytoplasmic iron-sulfur enzyme biogenesis in an atm1p (ABC transporter)-independent manner (Bedekovics et al., 2011). In many enteric bacteria and fungi, leucine, or a precursor of leucine can be used to regulate peripheral nitrogen metabolism with other cellular processes and can be selected directly or indirectly as a signal molecule in a wider metabolic context by very different mechanisms (Kohlhaw, 2003). Thus, leucine biosynthesis acts as a back door for effecting metabolism in cells (Kohlhaw, 2003).

Little is known about the gene cluster encoding putative membrane proteins (Cj0423-Cj0424-Cj0425). However, the *Cj0425* gene was considered to be an important contributor to the oxygen requirement and tolerance of *C. jejuni* (Kaakoush et al., 2007). The function of the other three genes involved in cell envelop (*Cj0560, Cj1356c,* and *Cj0200c*) is still unknown, but they may affect the fitness of ΦCj1199.

The down-regulation of the genes involved in amino acid transport and biosynthesis, and the genes encoding cell envelop may result in the disadvantage of nutrient utilization and growth in ΦCj1199. This hypothesis was confirmed by a series of competitive growth tests in the present study. Although there was no significant change in the short-term and single growth tests, the remarkable disadvantage of ΦCj1199 was observed in the long-term and competitive growth tests. Therefore, the *Cj1199* gene was essential for the fitness (growth) of *C. jejuni* by

controlling the expression of the several genes involved in amino acid transport and leucine biosynthesis pathway.

According to the previous literature, a mechanism of macrolide resistance based on the expression of a specific short peptide in the cell was discovered several years ago (Tenson and Mankin, 1995; Tenson et al., 1996). The Shine-Delgarno region of the mini-gene encoding the short peptide was located in the 23S rRNA secondary structure (Tenson and Mankin, 2001). The peptide rendering *C. jejuni* resistance to erythromycin was one of the C-peptides. It bound erythromycin and removed the drug from its binding site of the ribosome and then sequestered it in an inactive complex (Tripathi et al., 1998). C-peptides showed a very strong bias in amino acid composition with a prevalence of the hydrophobic amino acid residues Leu, Ile, Val, Ala, Phe, and Trp. The most conserved position was the third, which was most commonly occupied by leucine. Therefore, it was presumable that the obstruction of leucine biosynthesis may lead to the failure of short peptide synthesis in the environment containing erythromycin. However, the role of *Cj1199* on the formation of C-peptides was not verified. No difference of the MIC between ΦCj1199 and NCTC11168 was observed in our study. Considering the limited sensitivity of MIC determination by broth microdilution method, the erythromycin susceptibility was detected by FCM, the result indicated that the tolerance of ΦCj1199 to erythromycin was decreased under the treatment by a series concentration of erythromycin. Additionally, ΦCj1199 showed remarkable disadvantage on the development of erythromycin resistance *in vitro* step-wise selection. Therefore, Cj1199 may not directly impact on the erythromycin resistance, but it can contribute to the initial survival rate of the bacteria during erythromycin treatment and may increase the chances of acquiring resistance.

CONCLUSION

ΦCj1199 exhibited a weak competition of growth in a variety of competitive environments, especially in the medium with erythromycin. ΦCj1199 possessed lower risk for development of erythromycin resistance. Based on functions of differentially expressed genes, *Cj1199* has an impact on the biosynthesis of leucine and utilization of amino acid in gut, and thus may play an important role in the synthesis of short peptide and in the provision of energy and carbon. The genotype and phenotype change of ΦCj1199 indicates that *Cj1199* may contribute to the initial survival and colonization of the *C. jejuni* under the antibiotic treatment. *Cj1199* may impact the resistance acquirement or resistance development in a long term but not in a short term of growth. The role of *Cj1199* on the development of erythromycin resistance is indirectly dependent on the regulation of the expression of genes involved in amino acid biosynthesis and utilization. This study widened our understanding on the molecular mechanism of resistance and provides scientific reference for drug research and application.

ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FL, HH, MD, XW, and ZY. Performed the experiments: FL, HH, YW, and YS. Analyzed the data: FL, HH, MD, JH, SF, LH, and ZY. Contributed reagents/materials/analysis tools: ZY, MD, HH, and ZL. Wrote the paper: HH, FL, JH, SF, and ZY.

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The Periplasmic Chaperone Network of *Campylobacter jejuni*: Evidence that SalC (Cj1289) and PpiD (Cj0694) Are Involved in Maintaining Outer Membrane Integrity

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The outer membrane (OM) of Gram-negative pathogenic bacteria is a key structure in host–pathogen interactions that contains a plethora of proteins, performing a range of functions including adhesion, nutrient uptake, export of effectors and interaction with innate and adaptive components of the immune system. In addition, the OM can exclude drugs and thus contribute to antimicrobial resistance. The OM of the food-borne pathogen *Campylobacter jejuni* contains porins, adhesins and other virulence factors that must be specifically localized to this membrane, but the protein sorting mechanisms involved are only partially understood. In particular, chaperones are required to ferry OM proteins across the periplasm after they emerge from the Sec translocation system. The SurA-related chaperone PEB4 (Cj0596) is the only protein with a proven role in OM biogenesis and integrity in *C. jejuni*. In this work, we have constructed a set of isogenic deletion mutants in genes encoding both known and predicted chaperones (*cj0596*, *cj0694*, *cj1069*, *cj1228c,* and *cj1289*) using NCTC 11168H as the parental strain. These mutants were characterized using a range of assays to determine effects on growth, agglutination, biofilm formation, membrane permeability and hydrophobicity. We focused on Cj1289 and Cj0694, which our previous work suggested possessed both chaperone and peptidyl-proyl *cis/trans* isomerase (PPIase) domains. Mutants in either *cj1289* or *cj0694* showed growth defects, increased motility, agglutination and biofilm formation and severe OM permeability defects as measured by a lysozyme accessibility assay, that were comparable to those exhibited by the isogenic *peb4* mutant. 2D-gel comparisons showed a general decrease in OM proteins in these mutants. We heterologously overproduced and purified Cj0694 and obtained evidence that this protein was an active PPIase, as judged by its acceleration of the refolding rate of reduced and alkylated ribonuclease T_1 and that it also possessed holdase-type chaperone activity. Cj0694 is most similar to the PpiD class of chaperones but is unusual in possessing PPIase activity. Taken together, our data show that in addition to PEB4, Cj1289 (SalC; SurA-like chaperone) and Cj0694 (PpiD) are also key proteins involved in OM biogenesis and integrity in *C. jejuni*.

Keywords: *Campylobacter*, outer membrane, periplasmic chaperone, PpiD, SurA, PEB4, VirK, HtrA

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INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are leading causes of human bacterial enteric disease worldwide and these bacteria present a serious ongoing public health and economic problem (O'Brien, 2017). Campylobacters are part of the gut microbiota of many bird and animal species. For *C. jejuni*, undercooked chicken is the main source of human campylobacteriosis and is estimated to be the cause of up to 70% of infections in the UK alone (Sheppard et al., 2009). As a strategically important food-borne pathogen, novel interventions are required to reduce the numbers of campylobacters in the human food-chain. As well as increased bio-security measures, targeted interventions such as poultry vaccines (recently reviewed by Riddle and Guerry, 2016) and the use of specific anti-*Campylobacter* agents or probiotics (Saint-Cyr et al., 2016) on farm have been proposed, but will require identification of appropriate targets and increased knowledge of *C. jejuni* physiology respectively.

The outer membrane (OM) is a structure of the utmost importance in developing such anti-*Campylobacter* strategies. It acts as the interface with the environment and host, and has diverse functions in adhesion, cell signaling, secretion of effectors, host cell damage, and interaction with the immune system (Bos et al., 2007). OM vesicles are also known to be produced by *C. jejuni* (Elmi et al., 2012) and may be a strategy to increase invasion and virulence (Elmi et al., 2012, 2016). Moreover, the OM is an essential permeability barrier (thus affecting antibiotic sensitivity) and a key player in nutrient acquisition, natural competence and biofilm formation. Most of these functions are protein-mediated; in *C. jejuni* the importance of a number of OM proteins (OMPs) have been determined, including porins such as the Major Outer Membrane Porin (MOMP; PorA), the fibronectin binding protein CadF, other adhesins such as PEB1a, CjaA and JlpA and the autotransporter CapA (Rubinchik et al., 2012; Mahdavi et al., 2014; Wu et al., 2016). Highly antigenic OMPs have already been proposed as vaccine candidates, in both chickens and humans (Tribble et al., 2008). However, the mechanism by which OMPs are localized and inserted into the membrane after synthesis in the cytoplasm is still poorly understood in *C. jejuni*.

In Gram-negative bacteria, beta-barrel proteins destined for the OM are translocated through the Sec system in an unfolded state, bound by chaperones in the periplasm, and then presented to an assembly machinery (the "Bam complex") in the OM itself (Bos et al., 2007). In *Escherichia coli*, two of these periplasmic chaperones that have been well studied are Skp (Seventeen-Kilodalton Protein) and SurA (initially known as a protein required for survival during the stationary phase in *E. coli*) (Bos et al., 2007). Typically, unfolded OMPs bind to the SurA chaperone, but if these substrate proteins fail to interact with SurA, then Skp can bind them (Sklar et al., 2007). The function of these chaperones is to translocate the unfolded OMPs to the OM, where the Bam complex then inserts them. Both *skp* and *surA* mutants are viable; however, a *skp/surA* mutant is synthetically lethal (Rizzitello et al., 2001). This suggests that Skp and SurA are functionally related and they work by similar mechanisms for chaperone activity. Other periplasmic chaperone-like proteins in *E. coli* including PpiA, PpiD and FkpA have been identified, which may bind a wider range of client proteins than just OMPs. PpiD is thought to aid in the early periplasmic folding of a diverse array of newly translocated proteins emerging from the Sec translocon, but may not be specifically involved in the maturation of OMPs (Matern et al., 2010). SurA and PpiD contain domains homologous to the small peptidyl-prolyl *cis/trans* isomerase (PPIase) parvulin; an enzyme required for the *cis/trans* isomerisation of proline residues (Stymest and Klappa, 2008). The role of PPIase domains in such chaperones is not always clear, as the *E. coli* PpiD parvulin-like domain is catalytically inactive (Weininger et al., 2010) as is one of the two parvulin domains in SurA itself. Another potential chaperone, VirK, is a 37 kDa periplasmic protein that may have a role in autotransporter assembly and toxin export in *E. coli* (Tapia-Pastrana et al., 2012). Finally, HtrA (DegP) is a chaperone and proteolytic enzyme that degrades unfolded proteins in the periplasm (Ge et al., 2014).

Campylobacter jejuni possesses a network of both proven and putative periplasmic chaperones, but the roles of only a few of these are clear. A homolog of the HtrA protein has been shown to be important for protecting against heat and oxidative stress and possesses both chaperone and serine protease activities (Brøndsted et al., 2005; Baek et al., 2011). It is also a secreted protein that plays a role in host cell invasion by cleavage of *E*-cadherin (Hoy et al., 2012). A *C. jejuni* VirK homolog has been identified as a virulence factor in mouse colonization and involved in resistance to antimicrobial peptides, but unlike the situation in *E. coli*, seemed to be localized at the cytoplasmic side of the inner membrane (Novik et al., 2009). A highly conserved protein, PEB4 (Cj0596), has weak sequence similarity to SurA and from mutant studies has been implicated in the assembly of several OMPs in *C. jejuni* (Asakura et al., 2007; Rathbun et al., 2009). Asakura et al. (2007) showed that a *peb4* mutant in *C. jejuni* strain NCTC 11168 had reduced biofilm formation, adhered less well to INT407 cells than the wild-type and exhibited a lower level and duration of intestinal colonization of a mouse model. Rathbun et al. (2009) also found that a *peb4* mutant generated in strain 81–176 had a reduced ability to colonize mice. These data therefore support a direct correlation between changes in OM protein assembly and virulence in *C. jejuni*.

Kale et al. (2011) elucidated the crystal structure of PEB4. At a resolution of 2.2 Å, its structure reveals a dimeric organization with SurA-like chaperone and PPIase domains. However, unlike SurA, the overall fold of PEB4 is distinct. A large chaperone domain comprising the *N*- and *C*-terminal regions of the protein is linked to a second domain that has a standard PPIase fold. The chaperone domain is closely related to that of SurA but is different in the way helices from both domains interlock to form a domain-swapped structure (Kale et al., 2011). The PPIase domain in PEB4 is active when assayed using denatured ribonuclease T_1, which showed a significant PEB4-dependent acceleration in proline isomerization-limited refolding and PEB4 also strongly inhibits the aggregation of renaturing model proteins like rhodanese. It was therefore suggested that PEB4 is a holdase-type chaperone

whose function is to inhibit protein folding and aggregation prior to delivery of the client protein to the Bam complex (Kale et al., 2011).

Using the SurA chaperone domain in structure prediction searches, two other related chaperones have been identified in *C. jejuni*; Cj1289 and Cj0694 (Kale et al., 2011). Unlike PEB4, the Cj1289 crystal structure at 2.4 Å did not show a domain-swapped structure, which makes it much more similar to SurA itself. However, it only has one parvulin-like PPIase domain instead of the two found in SurA. This domain was active in increasing the refolding rate of ribonuclease T_1. Nevertheless, purified Cj1289 did not prevent rhodanese refolding and aggregation (Kale et al., 2011), suggesting that Cj1289 may chaperone specific *C. jejuni* substrates. Cj0694 has weak sequence similarity to the *E. coli* PpiD protein discussed above. This, combined with a similar domain organization and predicted N-terminal membrane anchored region suggest a similar role for Cj0694 in *C. jejuni* as for PpiD in *E. coli*, although Kale et al. (2011) could not obtain a soluble form of recombinant Cj0694 for biochemical or structural studies.

In this study, we have constructed isogenic deletion mutants in all of the proven and putative periplasmic chaperones in *C. jejuni* NCTC 11168H and compared the overall impact on OM integrity by determining their phenotypes with respect to growth and OM permeability properties. We have focused on the poorly characterized Cj1289 (which we designate SalC; SurA-like chaperone) and Cj0694 (designated PpiD) by analyzing the OM and periplasmic protein profiles of the cognate mutants and we show that the purified Cj0694 protein possesses both PPIase and chaperone activity.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Campylobacter jejuni NCTC 11168H cultures were maintained on Columbia agar base plus 5% v/v defibrinated horse blood at 42°C in microaerobic conditions [10% (v/v) O_2, 5 % (v/v) CO_2, and 85 % (v/v) N_2] generated in a MACS-VA500 Microaerobic Workstation (Don Whitley Scientific Ltd, Shipley, UK). Liquid cultures were routinely grown at 42°C in Brucella broth base (Sigma) plus 1% w/v tryptone and 20 mM L-serine (BTS broth) under standard microaerobic conditions with continuous orbital shaking at 140 rpm. Overnight starter cultures were allowed to grow from fresh 18 h old cells grown on plates prior to inoculation of larger cultures. Plates and broth cultures used for *C. jejuni* growth routinely contained amphotericin B and vancomycin at 10 $\mu g\ ml^{-1}$, with kanamycin and apramycin at 50 $\mu g\ ml^{-1}$ where appropriate. *E. coli* strains were cultured aerobically at 37°C on LB agar or in LB broth (Melford laboratories, UK) with orbital shaking (225–250 rpm), with selective antibiotics carbenicillin, kanamycin or apramycin at 50 $\mu g\ ml^{-1}$, where appropriate.

Cloning, Mutagenesis and Complementation Vector Construction

Putative chaperone genes were inactivated by deletion and insertion of a kanamycin resistance cassette into the reading frame by double homologous cross-over of the mutant allele from a pGEM-3ZF vector containing the kanamycin resistance cassette flanked by ∼500 bp upstream and downstream of the target gene. Mutant vectors were created using the Gibson isothermal assembly method as described by Gibson et al. (2009). Briefly, gene flanking regions were amplified from *C. jejuni* 11168H genomic DNA using primers with adapters homologous to either the kanamycin cassette amplified from pJMK30, or the ends of HincII linearised pGEM-3ZF (Supplementary Table 1). The isothermal assembly reaction specifically anneals all four fragments together to yield the mutant plasmid. Correct constructs were confirmed by automated DNA sequencing. Wild-type *C. jejuni* 11168H was transformed by electroporation and mutants selected on blood agar for kanamycin resistance. Correct insertion of the kanamycin cassette into the genome was confirmed by PCR. Complementation vectors were based on the pRRA system as described by Cameron and Gaynor (2014). Briefly, *cj0694* and *cj1289* were amplified from *C. jejuni* 11168H genomic DNA, including their promoter region, with MfeI and XbaI restriction site adaptors. *cj0694* and *cj1289* both contained an internal restriction site, requiring partial digestion and gel purification of the desired full length insert. Digested inserts were ligated with similarly digested, rSAP phosphatase treated pRRA and transformed into *E. coli* DH5α under apramycin selection. Putative clones were screened by PCR and purified plasmids further screened to ensure insertion of the full length gene. HΔ*cj0694* and HΔ*cj1289* mutants were transformed by electroporation with their respective complementation vector and clones selected for apramycin resistance. Correct insertion of the expression cassette into the genome was confirmed by PCR. All primers are listed in Supplementary Table 1.

Lysozyme Accessibility Assay

Cells from 12 h old broth cultures were washed twice in 20 mM sodium phosphate buffer, pH 7.4, by repeated centrifugation (12,000 × g, 25°C, 5 min) and resuspension of the cell pellet, then adjusted to an optical density at 600 nm of ∼0.8. Nine hundred and ten microliter of cells were mixed by inversion with 50 µl of freshly prepared 0.1 mg ml^{-1} lysozyme from chicken egg white (Sigma) in phosphate buffer and the absorbance at 600 nm monitored for 20 s to provide a background drift rate. Forty microliter of 62.5 mM sodium deoxycholate in phosphate buffer was added, mixed by inversion and the measurement continued for a further 60 s. The rate of cell lysis was determined by subtracting the drift rate from the rate of decrease in absorbance after the addition of deoxycholate. The *cj0694* and *cj1289* mutants have a very compromised OM and were found to show significant lysis even in the absence of deoxycholate. Therefore, for complementation data with these mutants, the susceptibility to lysozyme was determined by subtracting the drift rate (without lysozyme) from the rate of decrease in absorbance after the addition of lysozyme. All experiments were performed in triplicate in a Shimadzu UV-2401 spectrophotometer at 25°C.

Auto-Agglutination (AAG) Assay

Cells from 12 h old broth cultures were washed twice in 20 mM sodium phosphate buffer, pH 7.4, by repeated centrifugation

(12,000 *g*, 25 C, 5 min) and resuspension of the cell pellet, then adjusted to an optical density at 600 nm of exactly 0.40. Six milliliter of cells were transferred to glass tubes (18 mm diameter) in duplicate and left undisturbed at room temperature. One milliliter samples were carefully taken from the top of each aliquot at various time points and the optical density at 600 nm recorded. The experiments were performed in duplicate.

Cell Surface Hydrophobicity Assay

Cells from 12 h old broth cultures were washed twice in 20 mM sodium phosphate buffer, pH 7.4, by repeated centrifugation (12,000 *g*, 25 C, 5 min) and resuspension of the cell pellet, then adjusted to an optical density at 600 nm of ∼0.8. 3 ml of cells were mixed with 1 ml of *n*-hexadecane by vortexing for 30 s, then left undisturbed for 20 min before 1 ml of the aqueous phase cells was carefully removed and the optical absorbance at 600 nm read. Controls without hexadecane were performed to account for autoagglutination and cell lysis as a result of vortexing. The experiments were performed in triplicate, with H-values determined from optical absorbances at 600 nm by the equation:

((control sample – test sample)/control sample)*100

Motility Assay

Bacterial motility was determined by seeding semi-solid (0.4% w/v agar) brain-heart infusion (BHI) plates with 5 μl of cells set to an optical absorbance at 600 nm of 1.0 in BTS broth and measuring the diameter of the swarm rings after 48 h. Plates contained 150 μM triphenyltetrazolium chloride (TTC) to visualize growth and improve the accuracy of growth diameter measurement.

Biofilm Assay

Static biofilm formation assays were performed in 96 well flat bottom plates containing one strain per row (12 replicates). Cells from 12 h old broth cultures were adjusted to an optical density at 600 nm of exactly 0.1 in BTS broth and 200 μl of each culture added to the appropriate wells. Plates were incubated without shaking under standard microaerobic conditions for 24 h. Planktonic cells were carefully pipetted off and 200 μl of 1% (w/v) crystal violet in 95% (v/v) ethanol added to each well. After 5 min incubation, the crystal violet stain was pipetted off and wells gently washed twice with 400 μl dH_2O. The remaining biofilm-bound dye was resuspended in 200 μl of ethanol:acetone (4:1) by agitation of plates at room temperature for 20 min. The optical absorbance at 600 nm of the crystal violet was measured in a Victor2 1420 Multilabel Counter plate scanner (Perkin Elmer, USA). BTS broth controls were used as the blank. All data was divided by the average of the wild-type values to give final data as a ratio of wild-type biofilm formation.

Preparation of *C. jejuni* Periplasm and Outer Membrane Fractions

Campylobacter jejuni strains were grown in 0.5–1.0 L culture volumes until early stationary phase and an OD600 nm of ∼1.0–1.2 (∼16 h) in standard microaerobic conditions. Cells were harvested by centrifugation (7,155 × *g*, 15 min and 4°C), gently resuspended in 20 mL STE buffer [20% (w/v) sucrose, 30 mM Tris-HCl pH 8.0 and 1 mM EDTA] and incubated at room temperature with gentle shaking for 30 min. Then, cells were harvested by centrifugation (11,180 × *g*, 10 min and 4°C) and the supernatant was discarded. The pellets were resuspended in ice-cold 10 mM Tris-HCl pH 8.0 buffer and incubated with gentle shaking at 4°C for 1 h, then centrifuged (15,000 × *g*, 25 min and 4°C). The resulting osmotic shock supernatant containing the periplasmic proteins was stored at – 20°C. The pellet was then used to isolate the OM fraction as follows. The pellets were resuspended in 10 mM HEPES buffer pH 7.4 and sonicated with ice cooling for 6 × 15 s pulses at a frequency of 10 microns using a Soniprep 150 ultrasonic disintegrator (SANYO, Japan). Unbroken cells and cell debris were removed by centrifugation (27,167 × *g*, 30 min and 4°C). The supernatant was transferred to pre-chilled ultra-centrifuge tubes, and the inner and the OMs were pelleted by ultra-centrifugation (100,000 × *g*, 60 min and 4°C). The red pellet containing both inner and OM was resuspended in 2 mL of 10 mM HEPES buffer pH 7.4. The inner membrane was dissolved by addition of 2 mL of 2% sarkosyl (Sodium *N*-lauryl sarcosinate) dissolved in 10 mM HEPES buffer pH 7.4 and incubated at 37°C for 30 min. A further centrifugation (48,297 × *g*, 30 min and 4°C) was carried out to isolate the OM. The supernatant containing the solubilised inner membrane was carefully and fully removed and the OM pellet was washed three times in HEPES buffer prior to being homogenized in 0.5 – 1 mL of 10 mM HEPES buffer pH 7.4 and stored at −20°C.

2D-PAGE and Mass Spectrometry

The OM and periplasmic fractions from independent replicate cultures were analyzed by 2D-PAGE using the methods described previously (Hitchcock et al., 2010). Samples were solubilised in rehydration lysis buffer (RHB; 7 M urea, 2 M thiourea, 2% CHAPS). The first dimension was run on 18 cm 3-11NL Immobiline DryStrips (GE healthcare, UK). Following overnight rehydration, IEF was performed for 80 kVh at 20°C over 24 h using the pHaser system (Genomic Solutions, UK). The focussed strips were treated as described previously (Leon-Kempis Mdel et al., 2006). The second dimension used 4-12% Novex precast gels (Thermo Fisher). Proteins were stained by SYPRO-Ruby (Bio-Rad, UK) and the gels imaged using a Pharos FX+ Molecular Imager with Quantity One imaging software (BioRad, UK). For comparisons, the digital images were differentially colored and overlayed. For protein identification, selected spots were excised from the gel using the ProPick excision robot (Genomic Solutions, UK) and trypsin digested. Tryptic digests were analyzed using a Reflex III MALDI-TOF instrument (Bruker, UK). Proteins were identified by the Protein Mass Fingerprint technique using the MASCOT search tool (Matrix Science[1]).

Immunoblotting

To assess periplasmic contamination of OM fractions, immunoblotting to detect the periplasmic protein MfrA

[1]http://matrixscience.com

(Guccione et al., 2010) was performed. Protein samples were separated on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad, USA). The transfer of protein was carried out at a constant current of 400 mA for 60 min at 4°C in ice cold transfer buffer [25 mM Tris, 190 mM glycine, 20% *(v/v)* methanol]. All transfers were performed at RT with constant stirring. 1X TBS-T buffer [25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% *(v/v)* Tween 20] was used as a base for washing the nitrocellulose membrane (20, 10, and 5 min). 1X PBS-T was used as a base for blocking the membrane [standard phosphate buffered saline plus 0.1% *(v/v)* Tween 20 plus 5% *(w/v)* dried skimmed milk incubated at least 1 h with gentle shaking at RT or overnight at 4°C]. After blocking, the membrane was washed with 1X TBS-T buffer with gentle shaking for 20 min and twice for 5 min. Primary anti-MfrA polyclonal antibody raised in rat (Guccione et al., 2010) was diluted in 1X TBS-T buffer (1:2000) and incubated with the membrane for 1 h with gentle shaking. The membrane was washed with 1X TBS-T for 10 min and twice for 5 min. Secondary antibody (peroxidase-linked Anti-Rat IgG [whole molecule], Sigma) was diluted (1:2000) in TBS-T and incubated with the membrane for another hour. Washing of the membrane in 1X TBS-T was for 10 min and three times for 5 min. Visualization of antibody binding was performed by enhanced chemi-luminescence (ECL Kit, GE Healthcare) and exposure to x-ray film.

Overproduction and Purification of His-tagged Cj0694

Over-production of Cj0694 was performed by cloning the *cj0694* coding sequence minus the first 102 bp encoding the predicted transmembrane anchor (residues 1–34) into the l-arabinose inducible pBAD/His B vector (Invitrogen), which fuses a 6xHis tag to the *N*-terminal end of the protein. The primers 0694-OEF-pBAD and 0694-OER-pBAD (Supplementary Table 1) were used with *C. jejuni* genomic DNA as template and the ∼1.4 kb amplicon cloned into the *Xho*I and *Eco*RI sites of the vector to give pBAD0694, which was transformed into *E. coli* TOP10 cells. *E. coli* TOP10 (pBAD0694) was grown in LB broth containing 50 μg mL^{-1} carbenicillin at 37°C until OD_{600} ∼0.6. Over-expression was induced by addition of 0.02% w/v l-arabinose and cultures were incubated at 37°C with shaking at 250 rpm for 24 h. Cells were harvested by centrifugation and cell-free extracts prepared by sonication. Ice-cold cell-free extracts were fractionated on a 5 ml HisTrap™ column (GE Healthcare, UK). Proteins were bound to the column in binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole) and eluted from the resin over 10 column volumes with a linear gradient of 20–500 mM imidazole in the same buffer. Protein-containing fractions were then pooled, concentrated using a Vivaspin 20 column to a final volume of 2–5 mL, and buffer exchanged into 50 mM Tris-HCl pH 8.0 before SDS-PAGE analysis. These samples were further purified by loading onto a 5 ml HiTrap™ DEAE FF column (GE Healthcare, UK) and proteins were eluted in the same buffer over 10 column volumes with a linear gradient of 0–1 M NaCl. Protein-containing fractions were analyzed by SDS-PAGE. *N*-terminal sequencing, by automated Edman degradation, was performed by Dr. Arthur Moir (Department of Molecular Biology and Biotechnology, University of Sheffield).

PPIase Assay

The wild-type ribonuclease T_1 from *Aspergillus oryzae* (Sigma) was used to evaluate proline isomerisation-limited protein folding as described by Scholz et al. (1997). Disulphide reduced and *S*-carboxymethylated RNase T_1 (RCM-RNase T_1) was prepared following the method developed by Mücke and Schmid (1992). Firstly, 0.036 μmol of RNase T_1 was incubated in 275 μL denaturation buffer (6 M guanidine-hydrochloride [GdnHCl] and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7) for 2 h at 25°C. Then, 30 μL of the reducing buffer (20 mM dithiothreitol [DTT], 6 M GdnHCl, and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7) was added and protein reduction under argon was carried out at 25°C for 2 h. Next, 60 μL of the carboxymethylation buffer (0.6 M iodoacetate in 0.2 M Tris-HCl, pH 7.5) was added and the sample was incubated in the dark for 5 min at 25°C. This step was essential to 'cap' the cysteine residues and prevent the formation of two disulphide bonds during the denaturation process. Finally, 100 μL of 0.5 M reduced glutathione in 0.2 M Tris-HCl, pH 7.5, was added to stop the reaction. The RCM-RNase T_1 was separated from the reagents by dialysis against 10 mM Tris-HCl pH 8.0 at 4°C overnight. The refolding of wild-type RCM-RNase T_1 (which is rate-limited by the prolyl *cis-trans* isomerisation of Pro39 and Pro55 (Mücke and Schmid, 1992), was followed by monitoring the changes in the intrinsic tryptophan fluorescence. Refolding was initiated by a 50-fold dilution of the unfolded protein (stored in the absence of NaCl) to a final concentration of 1.2 μM in a buffer containing 0.1 M sodium acetate, pH 5.0, and 4 M NaCl. Changes in the steady-state Trp59 fluorescence were measured at 320 nm (10 nm bandwidth) with excitation at 268 nm (2.5 nm bandwidth) using a Varian Cary Eclipse spectrofluorimeter with the temperature maintained at 15°C. PEB4 (0.5 μM final concentration) or Cj0694 (0.25 or 0.5 μM final concentration) were added to the RCM-RNase T_1 (final concentration of 0.5 μM). Immediately following dilution into the high salt buffer, the fluorimeter was zeroed and the increase in fluorescence intensity recorded. PEB4 was over-produced and purified for comparative assays as described previously (Kale et al., 2011).

Cj0694 Chaperone Assay

Chaperone activity was demonstrated by measuring the effect of Cj0694 on the aggregation of the model proteins lysozyme and rhodanese during renaturation, following their denaturation with guanidine-HCl. Unfolding and refolding of rhodanese and lysozyme was carried out as previously described (Ideno et al., 2000). Typically, 30 μM pure rhodanese or lysozyme (Sigma) was first denatured for 2 h at 25°C in 50 mM Tris-HCl, pH 7.8, containing 6 M guanidine-HCl and 20 mM DTT. Renaturation was initiated by a 60-fold dilution in 50 mM Tris-HCl, pH 7.8, to reach a final concentration of 1.0 μM of rhodanese or

lysozyme, in the absence or presence of Cj0694 (1.0–5.0 μM) or bovine serum albumin (1.0 μM) as a negative control. The reactions were maintained at 25°C. The light scattering resulting from the formation of protein aggregates was measured by increase in the absorbance at 320 nm in a Shimadzu UV-2401PC spectrophotometer.

RESULTS

Physiological Phenotypes of Mutants in Candidate Periplasmic Chaperone Genes

The effects of removal of Cj0596 (PEB4), Cj1069 (VirK) and Cj1228 (HtrA) have been previously studied in mutants made in different parental wild-type backgrounds (Novik et al., 2009; Rathbun et al., 2009; Boehm et al., 2015). Here, we sought to compare the phenotypes of these mutants with those in genes encoding the putative chaperones Cj0694 and Cj1289, which have not previously been characterized, in a single parental strain background. We therefore constructed a set of isogenic deletion-insertion mutants in *cj0596*, *cj0694*, *cj1069*, *cj1228c,* and *cj1289* in *C. jejuni* NCTC 11168H, a well characterized motile variant of the NCTC 11168 reference strain (Karlyshev et al., 2002), so that we could determine the physiological phenotypes of all five mutants using a range of assays relating to growth, cell surface properties and OM integrity (**Figures 1A–F**).

Deletion of either *cj0694* or *cj1289* was not lethal, but these and the other mutants showed a pronounced growth defect under the conditions tested (microaerobic conditions in complex media), particularly HΔ*cj0596* (*peb4*), consistent with a pleotropic cell envelope defect (**Figure 1A**). Motility was enhanced in all mutants except *htrA* (**Figure 1B**) and biofilm formation was increased in all mutants (**Figure 1C**). These data are consistent with results reported previously for a *peb4* mutant in strain 81–176, where motility and biofilm formation were both enhanced (Rathbun and Thompson, 2009; Rathbun et al., 2009).

Cell surface characteristics were assayed by autoagglutination ability (**Figure 1D**) and cell surface hydrophobicity (**Figure 1E**). All mutants showed increased autoagglutination, but to widely varying degrees; this was most pronounced for the *peb4, cj0694* and *htrA* mutants. Interestingly, the *peb4* and *virK* mutants showed a strongly increased cell surface hydrophobicity, whereas the *cj0694* mutant was unchanged and the *cj1289* mutant showed only a mildly increased hydrophobicity compared to the wild-type. There was no apparent link between autoagglutination and hydrophobicity, suggesting specific OM and/or secreted proteins that are absent, or present at a reduced level, in some mutants may be important in these processes.

Finally, OM integrity was assayed by the susceptibility of cells to lysis by lysozyme. Lysozyme can lyse cells by digestion of the peptidoglycan layer, but this protein (14 kDa) can only access the periplasm when the OM is compromised. Therefore, the rate of cell lysis by lysozyme, especially in the presence of a membrane

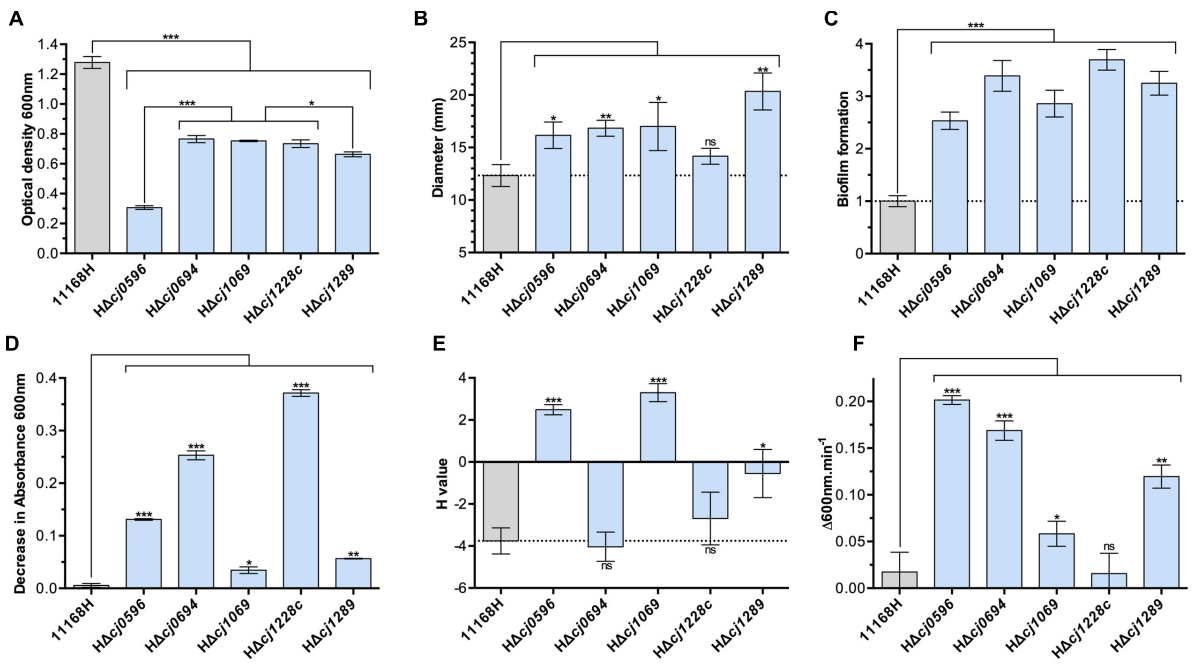

FIGURE 1 | Physiological phenotypes of chaperone mutants (blue bars) and isogenic parent strain *C. jejuni* 11168H (gray bars). (A) Growth measured as cell density at 600 nm at 12 h post-inoculation in BTS broth under standard microaerobic conditions. **(B)** Motility of strains determined by point inoculation of semi-solid agar plates and measuring the diameter of growth after 48 h. **(C)** Biofilm formation in BTS broth in 96-well plates after 24 h determined by crystal violet staining. **(D)** Autoagglutination (AAG) activity determined by the decrease in absorbance of cells in the aqueous phase of static cultures. **(E)** Cell surface hydrophobicity determined by the change in cell absorbance in the aqueous phase after mixing cells with the alkane hydrocarbon hexadecane. **(F)** Susceptibility to cell lysis by lysozyme digestion in the presence of 2.5 mM deoxycholate. Student *t*-test *P*-values are displayed as * < 0.05, ** < 0.01, *** < 0.001.

perturbing compound, (we used the major physiological bile salt sodium deoxycholate as an enhancer), can be interpreted as a function of OM integrity, independent of the inner membrane (**Figure 1F**). The chaperone roles of HtrA and VirK are not thought to be specifically related to OM proteins and so it was expected that their removal from the cells should not lead to an OM integrity defect. In keeping with this, the *htrA* mutant showed no increased susceptibility to lysis compared to wild-type, and the *virK* mutant displayed only a weak phenotype. However, each of the *peb4*, HΔ*cj0694* and HΔ*cj1289* mutants displayed evidence of highly compromised OM integrity (**Figure 1F**), demonstrating the importance of these putative chaperones in OM structure.

Complementation of HΔ*cj0694* and HΔ*cj1289* Restores OM Integrity

Complemented strains of HΔ*cj0694* and HΔ*cj1289* were made using the pRRA vector system, as described in Section "Materials and Methods," with the respective genes expressed from their native promoter (Cameron and Gaynor, 2014). Complementation vectors were transformed into their respective mutant in the 11168H background by electroporation and clones selected for dual kanamycin and apramycin resistance. Genomic DNA was screened by PCR to confirm correct genomic insertion of the target gene into the 16S/28S rRNA locus. Complemented strains showed significant restoration of their growth defect, as measured by increased cell viability under standard microaerobic conditions, and partial to complete restoration of OM integrity measured by lysozyme sensitivity (**Figure 2**). This confirms the phenotypes described for HΔ*cj0694* and HΔ*cj1289* strains are specifically due to their deletion, and supports their role as periplasmic chaperones that, when deleted, significantly alter the structure and integrity of the OM in *C. jejuni*.

Changes in OM and Periplasmic Proteins in *cj0694* and *cj1289* Mutants

Outer membrane and periplasmic fractions of the wild-type and isogenic *cj1289* and *cj0694* mutants were obtained as described in Section "Materials and Methods," in order to identify global changes in protein abundance and potentially identify any client OMPs that are dependent on Cj1289 or Cj0694 for their maturation and assembly in the OM. An analysis by 2D-PAGE was carried out, with the gels stained with SYPRO-Ruby. The images of the WT and mutant periplasm and OM fractions were digitally overlaid using either orange or blue coloring of the protein spots so that differences in protein abundance could be more easily observed; proteins with the same abundance appear black in such overlays (**Figure 3**). Overlaying the 2D-gels of the OMs (**Figures 3A,C**) of the wild-type (stained in orange) and the Δ*cj1289* or *cj0694* mutants (stained in blue) suggested an overall reduction in OMP abundance in both of these mutants compared to wild-type, as many of the spots detected showed up as more orange in the overlays (i.e., more abundant in WT). The prominent major-outer membrane porin (MOMP) appeared black, suggesting it was similar in abundance in wild-type and both mutants. However, two proteins stand out as blue in the overlay of the Δ*cj1289* mutant OM but orange in the Δ*cj0694* overlay. These were identified as Cj0112 (TolB) a periplasmic component of the OM Tol transport system and Cj0964, a putative periplasmic protein, which may therefore not be *bona fide* OM-associated proteins. The purity of the OM fractions was therefore assessed by immunoblotting using anti-MfrA, raised against the very abundant periplasmic subunit of the methylmenaquinol:fumarate reductase (MfrA) in *C. jejuni* (Guccione et al., 2010) (Supplementary Figure 1). This showed that the ∼65 kDa MfrA protein is present exclusively in the periplasmic but not in the OM fractions of the wild-type and *cj0694* mutant, but with evidence of a faint band of the same size present in the *cj1289* OM fraction. Thus, Cj0112 and Cj0964 are most likely to be contaminating periplasmic proteins especially in the Δ*cj1289* OM fraction. Overlaying the 2D-gels of the periplasmic fractions of the wild-type (stained in orange) and the Δ*cj1289* mutant (stained in blue) showed very little alteration in the protein profiles or abundances, with most of the proteins appearing black (**Figure 3B**). Comparison of the 2D-gels of the periplasms of the wild-type and the Δ*cj0694* mutant showed there were more bluish spots and thus more accumulation of proteins in the periplasm of this mutant (**Figure 3D**). This might be expected if some client OM proteins

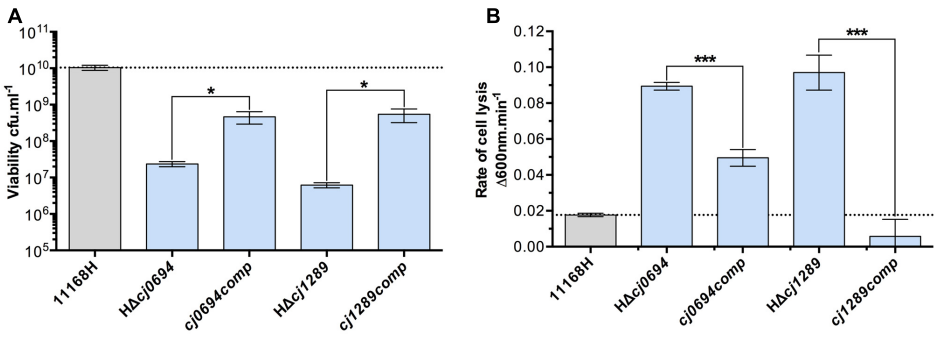

FIGURE 2 | Genetic complementation of HΔ*cj0694* and HΔ*cj1289* strains. (A) Viability of mid-log BTS broth cultures diluted to an optical density at 600 nm of exactly 0.1, as determined by serial dilution and colony counts. **(B)** OM integrity as determined by susceptibility to cell lysis by lysozyme digestion. Student *t*-test *P*-values are displayed as * < 0.05, *** < 0.001.

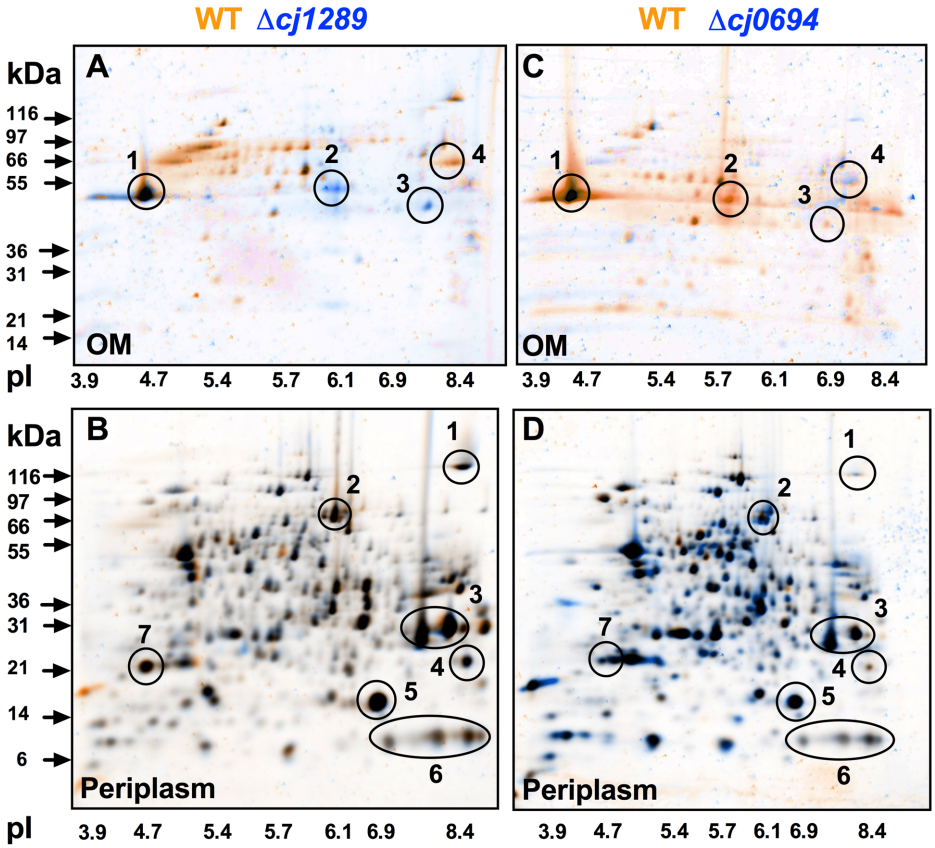

FIGURE 3 | 2D-PAGE analysis of the outer membrane and periplasmic proteins of the wild-type (colored orange) and *cj0694* and *cj1289* mutants (colored blue). Protein samples were prepared as described in Section "Materials and Methods" and separately resolved by 2D-PAGE. Orange spots represent proteins found in the wild-type fractions only, and absent in the mutant fractions. Blue spots represent proteins found in mutant fractions only, and absent in the wild-type fractions. Black spots represent proteins found in both the wild-type and mutant fractions. In **(A,C)**, the circled numbered protein spots in the 2D-gels of the OMs were identified by mass spectrometry analysis. These are: 1, MOMP (PorA); 2, Cj0964 (Mascot score 88); 3, Cj0112 (TolB, Mascot score 2115); 4, Cj1228 (HtrA, Mascot score 2056). In **(B,D)**, the circled numbered protein spots in the periplasms were correlated with those published in our previous study (Hitchcock et al., 2010). These are: 1, TorA; 2, MfrA; 3, Peb1A; 4, Cj0998; 5, Cj0715; 6, Cj1153; 7, Cj1659 (p19 protein).

of Cj0694 are now mislocalised to the periplasm. Overall, the general reduction in OMPs in the OM fractions of these mutants is consistent with a role for both Cj0694 and Cj1289 in OMP biogenesis.

Over-Production and Purification of Cj0694

Although Kale et al. (2011) investigated the PPIase and chaperone properties of Cj1289, these activities could not be determined for Cj0694 due to problems with heterologous expression of the protein in a pET vector system. Here, the *cj0694* gene was cloned and successfully over-expressed in the pBAD vector system as described in Section "Materials and Methods." The over-produced Cj0694 recombinant protein lacking the N-terminal membrane anchor and containing a hexahistidine tag was initially purified by Ni-NTA affinity chromatography. This resulted in a considerable enrichment, although the protein was not pure (**Figure 4A**). Anion-exchange chromatography using a DEAE-sepharose column resulted in significant further purification, as judged by Coomassie blue staining (**Figure 4B**; note that the presence of salt in the column elution buffer slows the migration of the protein so it appears larger than in **Figure 4A**). The purified protein was stable for at least a week at 4°C. *N*-terminal sequencing confirmed the expected sequence MGGSHHHH. The protein ran as a monomer on a calibrated gel filtration column, with an estimated native molecular weight of 54.8 kDa.

Cj0694 has PPIase Activity and Accelerates the Refolding of RCM-RNase T_1

Our previous bioinformatics analysis revealed that Cj0694 is a homolog of PpiD in *E. coli* (Kale et al., 2011), which has a parvulin-like PPIase domain from residues 227 to 357 (Dartigalongue and Raina, 1998). In order to gain evidence for potential PPIase activity for Cj0694, the ability of the protein to accelerate the rate of the proline isomerisation-limited refolding of RCM-RNase T_1 was examined (Rudd et al., 1995).

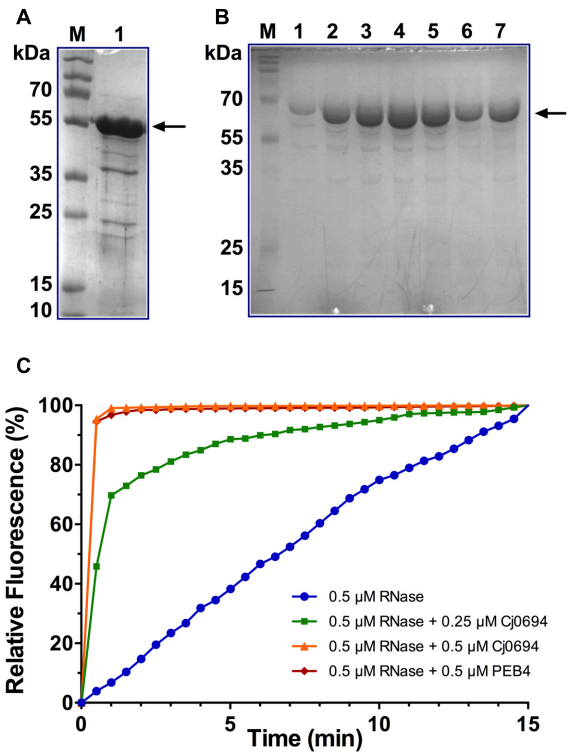

FIGURE 4 | Overproduction, purification and PPIase activity of Cj0694. **(A)** Lane 1; Cj0694 lacking the N-terminal membrane anchor and with a C-terminal his-tag initially purified by Ni-NTA affinity chromatography (monomer molecular weight 54.8 kDa). Lane M; molecular weight markers. **(B)** Further purification of Cj694 by DEAE anion-exchange chromatography with elution from 0 to 1 M NaCl. Lanes 1–7 are samples taken across the UV-absorbing peak eluted from the column and show a single major band on SDS-PAGE after staining with Coomassie Blue. Note that the migration of the protein is affected by the salt present in the elution buffer and the apparent molecular weight is higher than in **(A)**. Lane M; molecular weight markers. **(C)** PPIase activity of Cj0694 demonstrated by refolding of RCM-RNaseT$_1$ in the presence of 4 M NaCl (see Materials and Methods) either without or with the addition of purified Cj0694 as shown. The purified periplasmic chaperone PEB4 was used as a positive control. The fluorimeter was set to zero at the time of dilution, so that the increase in fluorescence results from the uncatalyzed (blue progress curve) or chaperone catalyzed (green, orange, and red progress curves) refolding process. Results shown are a single representative experiment.

The refolding of RCM-RNase T$_1$ is rate-limited by the prolyl *cis-trans* isomerisation of Pro39 and Pro55, and can be followed by tryptophan fluorescence spectroscopy (Mücke and Schmid, 1992). The two disulphide bonds in RNase T$_1$ (Cys2-Cys10 and Cys6-Cys103) are essential in maintaining its conformational stability. Therefore, breaking these bonds results in unfolding of the protein under native conditions. The RCM-RNaseT$_1$, like the native RNaseT$_1$, becomes catalytically active in the presence of 2 M NaCl (Pace et al., 1988). Thus, re-folding of the protein can be enhanced, by increasing the concentration of NaCl. RNase T$_1$ has a single tryptophan (Trp59) which is located in a hydrophobic environment in the folded protein (Moors et al., 2009). Refolding of the RCM-RNase T$_1$ results in an increase of Trp fluorescence.

The PPIase activity of Cj0694 was demonstrated by monitoring the tryptophan fluorescence of RCM-RNaseT$_1$ in the presence of 4 M NaCl. The purified periplasmic chaperone PEB4 was used as a positive control for PPIase activity (Kale et al., 2011). Refolding of RCM-RNaseT$_1$ was initiated by a 50-fold dilution of the unfolded protein (stored in the absence of NaCl). Cj0694 or PEB4 were added to the RCM-RNase T$_1$ prior to the dilution. As shown in **Figure 4C**, the rate of refolding, as reported by the increase in the steady-state Trp59 fluorescence intensity, is slow in the absence of a PPIase. However, a marked acceleration of the RCM-RNase T$_1$ refolding rate was clearly seen in the presence of Cj0694. The activity was dependent on the concentration of Cj0694 and comparable to that determined for PEB4 as a control protein. The data clearly show that Cj0694 has PPIase activity, similar to that of PEB4.

Cj0694 Has Chaperone Activity With Model Proteins

In order examine the chaperone activity of Cj0694, the ability of the protein to inhibit the aggregation of renaturing substrate proteins, measured spectrophotometrically by light scattering, was determined. Two unrelated commercially available model substrate proteins were used; rhodanese and lysozyme. Unfolding of these proteins was carried out as previously described Ideno et al. (2000; see Materials and Methods). Renaturation was initiated by a large dilution of the denatured protein into buffer, to give a final concentration of 1.0 μM, with incubation at 25°C in the absence or presence of Cj0694, with BSA as a negative control. In the absence of Cj0694, the renaturation of either protein resulted in progressive protein aggregation as indicated by an increase in light scattering at 320 nm (**Figure 5**). However, adding Cj0694 in increasing concentrations progressively inhibited the aggregation of both rhodanese and lysosyme as measured by a clear decrease in the light scattering kinetics (**Figure 5**). The control protein BSA added in place of Cj0694 did not inhibit protein aggregation. These results suggest that Cj0694 has chaperone activity that prevents protein aggregation, a role consistent with binding client proteins maintained in only a partially folded state before transfer to the BAM complex for insertion in the OM.

DISCUSSION

The functioning of the OM requires the correct localization of OMPs catalyzed by the BAM complex and a network of periplasmic chaperone proteins. From previous studies, homology searches and structural comparisons to *E. coli* chaperones, there appear to be five chaperone-like proteins that could play a role in OMP biogenesis in *C. jejuni*: Cj0596 (PEB4), Cj1069 (VirK-like), Cj1228 (HtrA), Cj0694 (PpiD-like) and Cj1289 (SurA-like). We have previously solved the structure of Cj1289 and showed it is indeed a SurA-like enzyme but with only one parvulin domain, while Cj0694 was identified as a likely PpiD homolog but was not further characterized (Kale et al., 2011). In this study, we successfully obtained mutants in *cj0694*

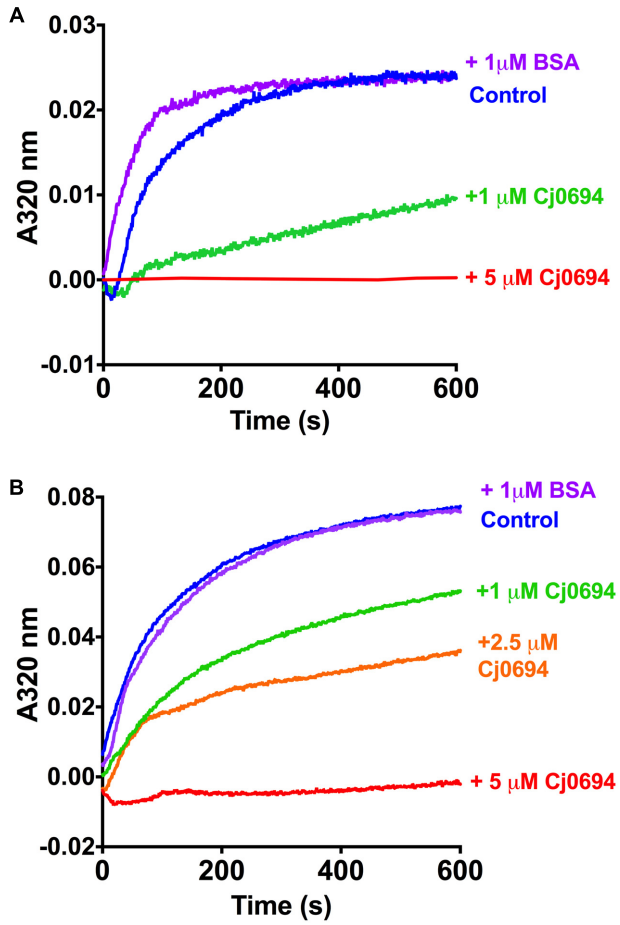

FIGURE 5 | Inhibition of protein aggregation by Cj0694. Model proteins Lysozyme **(A)** or Rhodanese **(B)** were unfolded using guanidine-HCl and their aggregation due to renaturation at 25°C monitored in the absence (control) or presence of Cj0694 by light scattering at 320 nm. An additional control contained 1 μM bovine serum albumen (BSA) to ensure that the observed inhibition of aggregation was specifically catalyzed by Cj0694. The traces shown are typical of several experiments performed.

and *cj1289* and by comparing their phenotype to *peb4*, *virK* and *htrA* mutants in an isogenic background, we have obtained evidence for their involvement in OM integrity.

Novik et al. (2009) reported the reduced virulence of a *virK* mutant in *C. jejuni* 81–176 in epithelial cells lines and a mouse model, demonstrating its importance as a virulence factor. In *E. coli* VirK is thought to be a periplasmic chaperone for the plasmid-encoded toxin (Pet), an autotransporter produced by enteroaggregative *E. coli* (Tapia-Pastrana et al., 2012). While VirK in *E. coli* is periplasmic, Novik et al. (2009) showed that the *C. jejuni* VirK homolog is associated with the inner membrane on the cytoplasmic face, and so may act as a chaperone prior to Sec-mediated export. We have shown that a *virK* mutant in *C. jejuni* NCTC 11168 displays decreased growth, enhanced motility and biofilm formation, and a strongly hydrophobic cell surface – the latter phenotype shared only with the *peb4* mutant (**Figures 1A–C,E**). This supports the hypothesis that VirK in *C. jejuni* may play a more general role in OM or cell surface biogenesis than reported in *E. coli*, where VirK is necessary, and potentially specific, for Pet toxin secretion (Tapia-Pastrana et al., 2012), a system absent in *C. jejuni*.

Cj1228 in *C. jejuni* is homologous to the *E. coli* high temperature required protein HtrA, formerly DegP, a serine protease with chaperone activity. HtrA has been shown to be essential for *E. coli* survival at high temperatures, and this phenotype has been confirmed in *C. jejuni* (Lipinska et al., 1989; Brøndsted et al., 2005; Boehm et al., 2015). It is known that HtrA is secreted by *C. jejuni* and *H. pylori* in the gut to digest the host cell adhesion protein *E*-cadherin, and recently this was shown to be mediated by OM vesicles (Elmi et al., 2016). However, the reduced viability of *C. jejuni* at high temperatures in the absence of HtrA *in vitro* suggests it plays a role in the cell envelope unrelated to pathogenesis. It has been suggested that HtrA in *E. coli* may function to rescue OMPs that dissociate from the SurA pathway, preventing their aggregation in the periplasm (Sklar et al., 2007). In our work, the *htrA* mutant had a similar growth defect to the other chaperone mutants, but displayed no change in motility, cell surface hydrophobicity or OM integrity compared to wild-type (**Figures 1B,E,F**). However, the *htrA* mutant did show increased biofilm formation and the highest autoagglutination rate of all mutants tested, which could be consistent with a lack of extracellular protease activity.

The remaining chaperones are all related to the *E. coli* SurA protein (Kale et al., 2011). Asakura et al. (2007) and Rathbun et al. (2009) reported a growth defect in a *peb4* mutants made in *C. jejuni* NCTC 11168 and 81-176 respectively, however, the growth defect we found here is much more severe. This may be attributed to the difference in parental strains or growth conditions (42°C in our study vs. 37°C, different microaerobic atmospheres). However, our data do show that a Δ*cj0596* (*peb4*) mutant in NCTC 11168H displays enhanced autoagglutination, motility and biofilm formation (**Figures 1B–D**), similar to the phenotypes found previously for a 81–176 *peb4* mutant (Rathbun et al., 2009; Rathbun and Thompson, 2009), although Asakura et al. (2007) reported lower biofilm forming ability of an NCTC 11168 *peb4* mutant. Of all the mutants, HΔ*cj0596* displayed the greatest growth defect and strongest deficiency in OM integrity (**Figures 1A,F**), supporting a key role for PEB4 in OM biogenesis in *C. jejuni*.

Structurally, Cj1289 more closely resembles SurA than does PEB4 (Kale et al., 2011) and we propose to designate it SalC (SurA-like chaperone). SurA is considered the major periplasmic chaperone in *E. coli*, and it has been shown by differential proteomics that inactivation of *surA* in a *skp* minus background results in diminished levels of nearly all OM β-barrel proteins (Denoncin et al., 2012). The HΔ*cj1289* mutant displayed generally similar phenotypes to the *peb4* mutant, with the exception of a less severe growth defect and a less hydrophobic cell surface (**Figures 1A–F**). If PEB4 and Cj1289 were simply redundant then neither single mutant would be expected to show a strong phenotype unless both were deleted in the same background, as is the case for *skp* and *surA* in *E. coli*, where deletion of both is synthetically lethal (Rizzitello et al., 2001).

Given both single mutants showed strong phenotypes relating to OM structure and function, and purified PEB4 and Cj1289 had different folding activities *in vitro* (Kale et al., 2011), we suggest PEB4 and Cj1289 represent the two major periplasmic chaperones in *C. jejuni* that operate as non-redundant pathways for specific client proteins. Further work on the OM protein profile of *peb4* and *cj1289* mutants using a proteomics approach as used by Denoncin et al. (2012) is needed to confirm this hypothesis and to identify specific client proteins.

Cj0694 is predicted to be a periplasmic facing, inner membrane anchored protein most closely resembling PpiD from *E. coli* (Kale et al., 2011). Overexpression of PpiD was able to rescue a lethal *surA skp* double mutant in *E. coli*, and deletion of *ppiD* confers a conditional phenotype on a *htrA* mutant background, suggesting cooperation between *ppiD* and *htrA* as general foldases (Matern et al., 2010). The *cj0694* mutant we constructed had a strong OM integrity phenotype, comparable to that of the *peb4* and *cj1289* mutants (**Figure 1F**), consistent with Cj0694 acting as a chaperone having a significant effect on OM composition. There is clear evidence that in *E. coli*, PpiD interacts with the Sec complex in the inner membrane and participates in folding of newly emerging OM and periplasmic proteins (Sachelaru et al., 2014; Wang et al., 2016). Given the topology, bioinformatics, the pleiotropic phenotype of *cj0694* deletion and Cj0694's broad substrate range *in vitro* (see below), we suggest Cj0694 is the *C. jejuni* equivalent of PpiD.

Overall, the HΔ*cj0694* and HΔ*cj1289* strains displayed a range of similar physiological phenotypes (except autoagglutination) which indicated defects affecting OM structure and function. The highly compromised OM integrity phenotype of HΔ*cj0694* and HΔ*cj1289* was significantly restored by genetic complementation; an incomplete phenotype presumably results from decreased gene expression at the integration locus, even though the native gene promoters were used. Nevertheless, taken together, the mutant and complementation data demonstrate the importance of PpiD and SalC in OM structure and reinforces their role as periplasmic chaperones in *C. jejuni* (**Figure 6**). The 2D-gel results obtained here suggested that a general reduction in OM protein abundance occurred in both the *cj1289* and *cj0694* mutants but we were unable to definitively identify client proteins of the cognate chaperones. Previous 2D-gel studies with *peb4* mutants have identified changes in the expression levels of several proteins compared to the wild-type strain, with decreases in abundance of several OM and periplasmic proteins, including the major outer membrane protein (MOMP), porins (OmpA,

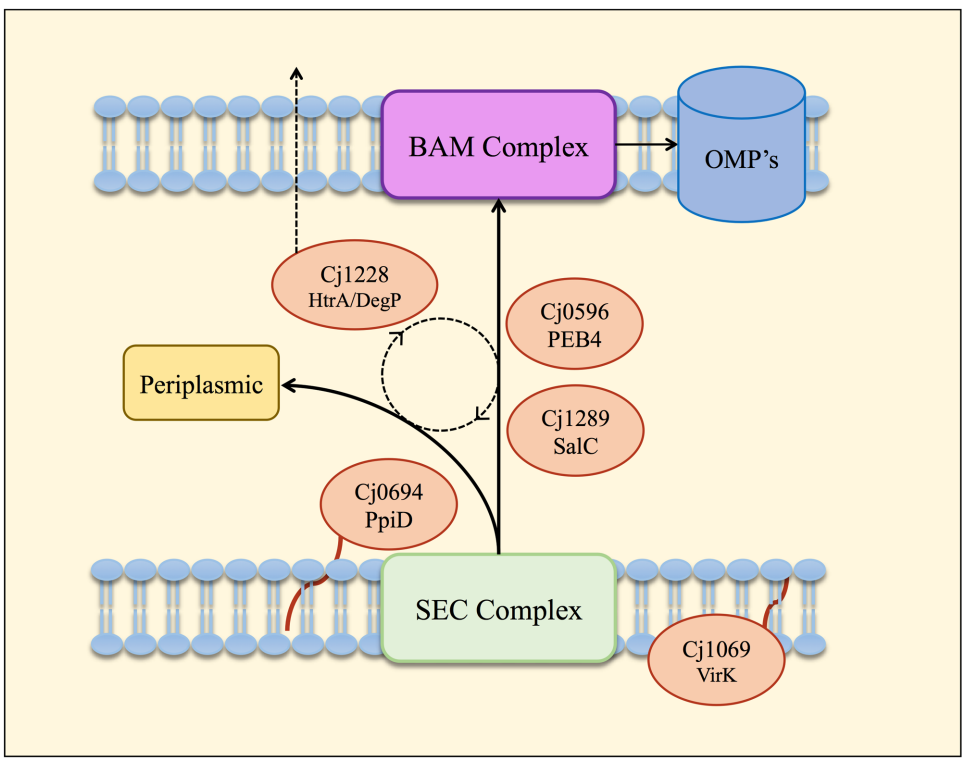

FIGURE 6 | Model of the periplasmic chaperone network of *C. jejuni*. OM and periplasmic proteins emerging from the Sec export complex interact with PpiD (Cj0694) for initial folding and translocation to the SalC (Cj1289) or PEB4 (Cj0596) pathway. VirK (Cj1069) may interact with certain substrates in the cytoplasm prior to their entrance into the SEC complex, based on the localization of this protein determined by Novik et al. (2009). SalC and PEB4 are proposed to translocate partially folded OMPs across the periplasm to the BAM complex, where they are inserted into the OM. HtrA (Cj1228) may participate in folding various periplasmic proteins or possibly to rescue OMPs that dissociate from SurA or PEB4 before reaching the OM. HtrA is also secreted from the cell (Hoy et al., 2012) probably mediated by OM vesicles (Elmi et al., 2016).

Omp50), the haemin OM receptor (CirA), the cysteine binding protein (Cj0982) and the iron receptor (FepA) (Asakura et al., 2007). In addition, Rathbun et al. (2009) found a decrease in the level of three OMPs, the MOMP, the fibronectin binding protein (CadF) and the Omp50 protein (Rathbun et al., 2009; Rathbun and Thompson, 2009). However, a problem with analyzing OM samples of *C. jejuni* is that because the MOMP is such an abundant protein it can make observing much less abundant OMPs very difficult unless the gels are overloaded, which leads to resolution problems. This, combined with the simple protein staining based method used here (and in most other studies) does not allow subtle variations in individual protein abundance to be reliably quantified. Ideally, a method such as SILAC should be applied in future work, where differentially isotopically labeled wild-type and mutant cells can be mixed and processed as a single sample, with mass spectrometry of the proteins allowing accurate abundance ratios to be determined.

The clear phenotypic changes in the *cj0694* mutant discussed above prompted us to examine the biochemical properties of the Cj0694 protein, which we successfully purified in recombinant form in this study. Our results revealed that Cj0694 has an easily demonstrable catalytic activity as a PPIase. Interestingly, despite the similarity between Cj0694 and *E. coli* PpiD discussed above, the latter protein was shown to be devoid of PPIase catalytic activity (Matern et al., 2010; Weininger et al., 2010), and this was also found to be the case for *Yersinia pseudotuberculosis* PpiD (Obi et al., 2011). *E. coli*, PpiD consists of an α-helical transmembrane domain and three periplasmic domains. The first and third domains are proposed to be chaperone domains and the second domain (residues 227 – 357) was identified as a parvulin-like PPIase domain (Dartigalongue and Raina, 1998), which was structurally confirmed by NMR spectroscopy (Weininger et al., 2010). This domain was shown to have high structural similarities to the first parvulin domain of SurA (Weininger et al., 2010) which is known to be inactive as a PPIase (Behrens et al., 2001). The molecular basis of the intriguing difference in the PPIase activity of Cj0694 and PpiD must await structural studies of Cj0694; we have thus far been unsuccessful in obtaining diffracting crystals of this protein. The ability of Cj0694 to act as a chaperone was tested by refolding assays, using the unrelated model proteins lysozyme and rhodanese. It was found that Cj0694 was active in preventing aggregation of both these proteins, in a concentration-dependent manner. This would support the conclusion that Cj0694 has a rather general role in the periplasm as a low specificity chaperone for both periplasmic and OM proteins, which is consistent with work which indicates that *E. coli* PpiD has much lower substrate specificity than SurA (Stymest and Klappa, 2008).

CONCLUSION

We have obtained functional and biochemical evidence for a key role for Cj0694 and Cj1289 as periplasmic chaperones acting alongside PEB4 and possibly HtrA in a network (**Figure 6**) that ensures correct OM biogenesis and integrity, properties essential for *C. jejuni* survival and pathogenesis.

AUTHOR CONTRIBUTIONS

AT, SZ, and DK designed and executed experiments and analyzed the data. AT and DK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00531/full#supplementary-material

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An Improved Culture Method for Selective Isolation of *Campylobacter jejuni* from Wastewater

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Campylobacter jejuni is one of the leading foodborne pathogens worldwide. *C. jejuni* is isolated from a wide range of foods, domestic animals, wildlife, and environmental sources. The currently available culture-based isolation methods are not highly effective for wastewater samples due to the low number of *C. jejuni* in the midst of competing bacteria. To detect and isolate *C. jejuni* from wastewater samples, in this study, we evaluated a few different enrichment conditions using five different antibiotics (i.e., cefoperazone, vancomycin, trimethoprim, polymyxin B, and rifampicin), to which *C. jejuni* is intrinsically resistant. The selectivity of each enrichment condition was measured with C_t value using quantitative real-time PCR, and multiplex PCR to determine *Campylobacter* species. In addition, the efficacy of *Campylobacter* isolation on different culture media after selective enrichment was examined by growing on Bolton and Preston agar plates. The addition of polymyxin B, rifampicin, or both to the Bolton selective supplements enhanced the selective isolation of *C. jejuni*. The results of 16S rDNA sequencing also revealed that *Enterococcus* spp. and *Pseudomonas aeruginosa* are major competing bacteria in the enrichment conditions. Although it is known to be difficult to isolate *Campylobacter* from samples with heavy contamination, this study well exhibited that the manipulation of antibiotic selective pressure improves the isolation efficiency of fastidious *Campylobacter* from wastewater.

Keywords: *Campylobacter jejuni*, wastewater, isolation, antibiotics, qRT-PCR

INTRODUCTION

Campylobacter is the major bacterial cause of foodborne infection, annually accounting for approximately 166 million foodborne illnesses around the world (Kirk et al., 2015). In addition to the clinical symptoms of gastroenteritis, *Campylobacter* is the major risk factor of Guillain–Barré syndrome (GBS), a neurological disorder causing muscular paralysis, as a post-infection

complication (Hughes and Cornblath, 2005). Among pathogenic *Campylobacter* species, *C. jejuni* and *C. coli* are most frequently associated with human infection (Kaakoush et al., 2015). Thus far, the consumption of contaminated poultry is the primary cause of developing human campylobacteriosis (Whiley et al., 2013).

Despite the well-known fastidious nature of *Campylobacter* (Silva et al., 2011), *Campylobacter* is isolated from environmental sources, such as lake, river, sea, and sewage, suggesting that environmental water is a possible vehicle that transmits *Campylobacter* to humans (Jones, 2001). *C. jejuni* is the pathogenic species that is mainly related to water-borne campylobacteriosis worldwide (Pitkanen, 2013). In Canada, *Campylobacter* outbreaks caused by cross contamination related with meltwater and heavy rainfall are problematic to public health (Millson et al., 1991; Clark et al., 2003). However, the isolation of *Campylobacter* implicated in water-borne outbreak appear to be challenging, not only due to rapid loss in culturability of isolates from the environment (Wingender and Flemming, 2011) and from stool samples (Bullman et al., 2012), but also due to the time gap between the initial infection and outbreak investigation (Hanninen et al., 2003; Jakopanec et al., 2008). Therefore, regular monitoring system of water resources by using culture-based methods is likely to underestimate the prevalence of *Campylobacter* spp. in the environment. This might mislead our understanding of the role played by the environmental sources in human infection and possibly the contamination of food chain by *Campylobacter,* even though *Campylobacter* is most frequently detected in animal fecal samples (29.7%), untreated human sewage (25.6%), and surface water (26.6%), according to a study in Alberta, Canada, among the three major foodborne pathogens, including *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7 (Jokinen et al., 2011).

Various culture supplements have been examined to improve selective isolation of *Campylobacter* spp. (Corry et al., 1995). For example, ISO method 2005 has been applied for the detection of thermo-tolerant campylobacters from water, and alternative culture-based methods in combination with molecular end-point confirmation (Hokajarvi et al., 2013; Pitkanen, 2013). Sample volume, incubation time, enrichment volume, passage of enrichment, and PCR-primer specificity all play an important role (Levesque et al., 2011) and enrichment procedures as well (Rosef et al., 2001). Khan et al. (2009, 2013) compared two methods (i.e., centrifugation vs. membrane filtration) for the isolation and detection of *Campylobacter* from agriculture watersheds, and reported the effect of incubation temperature on the detection rates and the type of dominant *Campylobacter* species detected from water samples. However, wastewater samples are even more challenging than samples from agricultural watersheds due to the relatively low number of *Campylobacter* in comparison with the high levels of microbial competitors and PCR inhibitors in wastewater (Koenraad et al., 1997; Abulreesh et al., 2005; Schrader et al., 2012).

To overcome the limitations in traditional culture-based methods for the detection of foodborne pathogens, molecular methods, such as PCR, have become practical and widely used due to the speed and reproducibility (Law et al., 2015). Recently, direct quantitative PCR was applied to the detection of *Campylobacter* in river water and showed the possibility of an alternative method for *Campylobacter* detection (Van Dyke et al., 2010). Nevertheless, the detection validation of *Campylobacter* with culture-based methods would still be necessary to reveal the direct correlation between a clinical illness and its etiological agents.

In this study, we present amendments to existing culture methods to improve the enrichment and isolation of *Campylobacter* spp. from wastewater. We used raw sewage influent samples since they are contaminated more heavily than effluent samples. By adopting different incubation temperatures and several antibiotics, to which *C. jejuni* is intrinsically resistant, we developed an improved enrichment method to recover culturable *C. jejuni* from wastewater samples. The efficiency of *Campylobacter* isolation was evaluated using quantitative real-time PCR (qRT-PCR) targeting genus-specific 16S rDNA primers, and a second end-point multiplexed PCR with species-specific primers. By using 16S rDNA amplicon sequencing, in addition, we identified the major bacteria in wastewater that compete with *Campylobacter* under the selective enrichment conditions.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Primers

Campylobacter jejuni ATCC 33560 and NCTC 11168 were routinely cultured in Mueller Hinton (MH) media at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). The primers used in the study are described in **Table 1**.

Enrichment Conditions for Post Grit Samples from Wastewater Treatment Facilities

Raw sewage samples (post grit influent; PG) were collected from two different wastewater treatment facilities (Pine Creek and Bonnybrook) in Calgary, Alberta, in November and December, 2014. The samples were stored at 4–8°C and processed within 12 h after arrival. The wastewater samples (100 ml) were concentrated by centrifugation at 9000 rpm for 20 min at 4°C (Sorvall RC-5B), and pellets were resuspended in 4 ml of Bolton broth (Oxoid) for further enrichment process as described by Chenu et al. (2013) with minor modifications. Briefly, four different kinds of Bolton Broth (Oxoid) enrichment broth were prepared: (1) Bolton's with *Campylobacter*-selective supplements [BN; cefoperazone 20 μg/ml, vancomycin 20 μg/ml, trimethoprim 20 μg/ml, and cycloheximide 50 μg/ml, Dalynn], (2) BN plus 10 μg/ml rifampicin [BNR], (3) BN plus 5 IU/ml polymyxin B [BNP], and (4) BN with both rifampicin and polymyxin B [BNRP]. Independently, 1 ml of pellet suspension was transferred to three wells in a 96-well plate and serially

TABLE 1 | Primers used in this study.

Primer	Sequence (5′→ 3′)	Reference
*CampyLvl-16S-F	CCT GAM GCA GCA ACG CC	de Boer et al., 2013
*CampyLvl-16S-R	CGG AGT TAG CCG GTG CTT ATT	
*CampyLvl-16S-P	CTC CGA AAA GTG TCA TCC T	
**CampyYM-16S-F	GGA TGA CAC TTT TCG GAG C	Yamazaki-Matsune et al., 2007
**CampyYM-16S-R	CAT TGT AGC ACG TGT GTC	
***C. hyointest*-23S-F	ATA ATC TAG GTG AGA ATC CTA G	
***C. hyointest*-23S-R	GCT TCG CAT AGC TAA CAT	
***C. coli*-ask-F	GGT ATG ATT TCT ACA AAG CGA G	
***C. coli*-ask-R	ATA AAA GAC TAT CGT CGC GTG	
***C. fetus*-cstA-F	GGT AGC CGC AGC TGC TAA GAT	
***C. fetus*-cstA-R	AGC CAG TAA CGC ATA TTA TAG TAG	
***C. lari*-glyA-F	TAG AGA GAT AGC AAA AGA GA	
***C. lari*-glyA-R	TAC ACA TAA TAA TCC CAC CC	
***C. jejuni*-cj0414-F	CAA ATA AAG TTA GAG GTA GAA TGT	
***C. jejuni*-cj0414-R	CCA TAA GCA CTA GCT AGC TGA T	
***C. upsal*-lpxA-F	CGA TGA TGT GCA AAT TGA AGC	
***C. upsal*-lpxA-R	TTC TAG CCC CTT GCT TGA TG	
***IAC-F	CTA ACC TTC GTG ATG AGC AAT CG	Deer et al., 2010
***IAC- R	GAT CAG CTA CGT GAG GTC CTA C	
***IAC-P	AGC TAG TCG ATG CAC TCC AGT CCT CCT	
27F	AGA GTT TGA TCM TGG CTC AG	Weisburg et al., 1991
1492R	TAC GGY TAC CTT GTT ACG ACT T	

*Primers used in qRT- PCR to detect Campylobacter genus, **Primers for multiplex PCR to detect Campylobacter species, ***Primer used as Internal Control template (IAC).

diluted to determine most probable number (MPN). For the 1st enrichment procedure, the plates were incubated at 37 or 42°C for 40–48 h under microaerobic conditions. Then, the culture broths were transferred to a 2nd enrichment medium consisting of the same antimicrobial supplements with 150 μg/ml 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma) and incubated for 24 h. TTC is a color indicator to show metabolic activity, and the inclusion of the dye in the assay aids in detection of levels of growth (Gabrielson et al., 2002). The cultures were subject to qRT-PCR and multiplex PCR.

Validation of *C. jejuni* Growth with Antibiotic Supplements

C. jejuni ATCC 33560, which is a quality control (QC) strain for antimicrobial susceptibility testing of *C. jejuni* (Clinical and Laboratory Standards Institute [CLSI], 2010), and NCTC 11168 were employed to evaluate the growth capability of *C. jejuni* under different enrichment conditions. Four different kinds of enrichment broth were prepared as described above. *C. jejuni* ATCC 33560 and NCTC 11168 were cultured on MH agar plates at 42°C for 24 h and harvested with fresh MH broth. The bacterial suspension was adjusted to an OD_{600} of 0.07 and incubated at 42°C with shaking at 200 rpm under microaerobic conditions. To determine the growth of *C. jejuni* strains, the samples were taken at 0, 3, 6, 12, and 24 h, and CFU and OD_{600} values were measured.

Confirmation of *Campylobacter* Growth using qRT-PCR

To confirm if *Campylobacter* was successfully enriched, 50 μl of culture broth was transferred to 96 well PCR plates and heated to 95°C for 10 min to extract DNA. Quantitative PCR was performed using an ABI 7500 (Applied Biosystems) system with *Campylobacter* genus-specific 16S rDNA primers (de Boer et al., 2013). The internal control template (IAC) and primers were included in reaction mixtures to measure inhibitory effects in enrichment samples (Deer et al., 2010). Amplification was carried out with following conditions: 50°C for 2 min and 95°C for 30 s; 40 cycles at 95°C for 3 s and 60°C for 30 s. C_t values were evaluated to determine the growth of *Campylobacter* and 3-tube MPN estimates.

Confirmation of *Campylobacter* spp. using Multiplex PCR

To identify *Campylobacter* spp. in the enrichment broths, multiplex PCR was performed for 42°C enrichment broths as described elsewhere with primer sets for 16S rDNA and six species-specific primers (Yamazaki-Matsune et al., 2007). Same templates used in qRT-PCR were also employed for multiplex PCR. The amplification reaction was performed following conditions: 95°C for 15 min; 40 cycles at 95°C for 30 s, 58°C for 1 min and 30 s, 72°C for 1 min; 72°C for 7 min.

Isolation of *Campylobacter* spp. and Identification of Non-*Campylobacter* Isolates by 16S rRNA Sequencing from Wastewater

To isolate *Campylobacter* spp. from enrichment cultures, wells showing the lowest C_t value in qRT-PCR results in the 2nd enrichment plate were selected. The cultures were prepared with 10-fold serial dilutions and sub-cultured on Bolton agar plates with Bolton supplement (BB, Dalynn) or Bolton agar plates with Preston supplement (BP, Oxoid). Following 2–3 days incubation at 42°C under microaerobic conditions, several colonies showing different shape, color, and transparency were randomly picked and transferred to the same fresh broth. After 2 days incubation, 50 μl of the cultures was harvested and boiled at 95°C for 10 min. Genus-specific 16S rDNA PCR amplification was carried out to distinguish between *Campylobacter* and non-*Campylobacter* (Linton et al., 1996). Amplification was performed following conditions: 94°C for 1 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; 72°C for 5 min. PCR amplicons were visualized using 2% agarose gel with SYBR safe DNA gel stain solution (Invitrogen). To identify non-*Campylobacter* competitors growing in the selective enrichment conditions, 16S rDNA was amplified with universal bacterial domain primers (27F and 1492R) for 100 *Campylobacter* genus-specific 16S rDNA negative isolates (Weisburg et al., 1991). Amplification was conducted following conditions: 94°C for 1 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, 72 °C for 1 min and 30 s; 72°C for 5 min. The amplified PCR products were purified and commercially

sequenced by Sanger sequencing method (Macrogen, Inc., South Korea), and the results were analyzed by using Blastn[1].

RESULTS

Campylobacter jejuni Growth in the Presence of Additional Antibiotic Supplements

To improve the frequency of *C. jejuni* isolation from wastewater samples that are heavily contaminated with various microorganisms, we decided to increase antibiotic selective pressure by using different combinations of multiple antibiotics to which *C. jejuni* is intrinsically resistant (Taylor and Courvalin, 1988; Corry et al., 1995). For the growth testing, we used *C. jejuni* ATCC 33560, a QC strain for antibiotic susceptibility testing (Clinical and Laboratory Standards Institute [CLSI], 2010), and *C. jejuni* NCTC 11168, the first genome-sequenced *Campylobacter* strain (Parkhill et al., 2000). Whereas the Bolton selective supplement (BN) consists of three antibiotics, including cefoperazone, vancomycin, and trimethoprim, the Preston *Campylobacter*-selective supplement contains polymyxin B, rifampicin, and trimethoprim. The two selective supplements for *Campylobacter* isolation commonly contain trimethoprim. In the experiment, BN was used as basic antimicrobial supplements, and polymyxin B and/or rifampicin were added to BN to increase antibiotic selective pressure. The addition of either polymyxin B or rifampicin to BN did not affect the growth. The supplementation with both rifampicin and polymyxin B slightly reduced the OD_{600} at 12 h; however, there was no significant difference in growth in the four different enrichment conditions (**Figure 1**). The results indicate that *C. jejuni* can grow in the presence of combinations of the multiple antibiotics to which *C. jejuni* is naturally resistant.

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi

C_t Values of qRT-PCR in *Campylobacter* Detection under different Enrichment Conditions

The C_t values of qRT-PCR for the detection of *Campylobacter* varied depending on the antimicrobial enrichment. The addition of one of the antibiotics (i.e., either rifampicin or

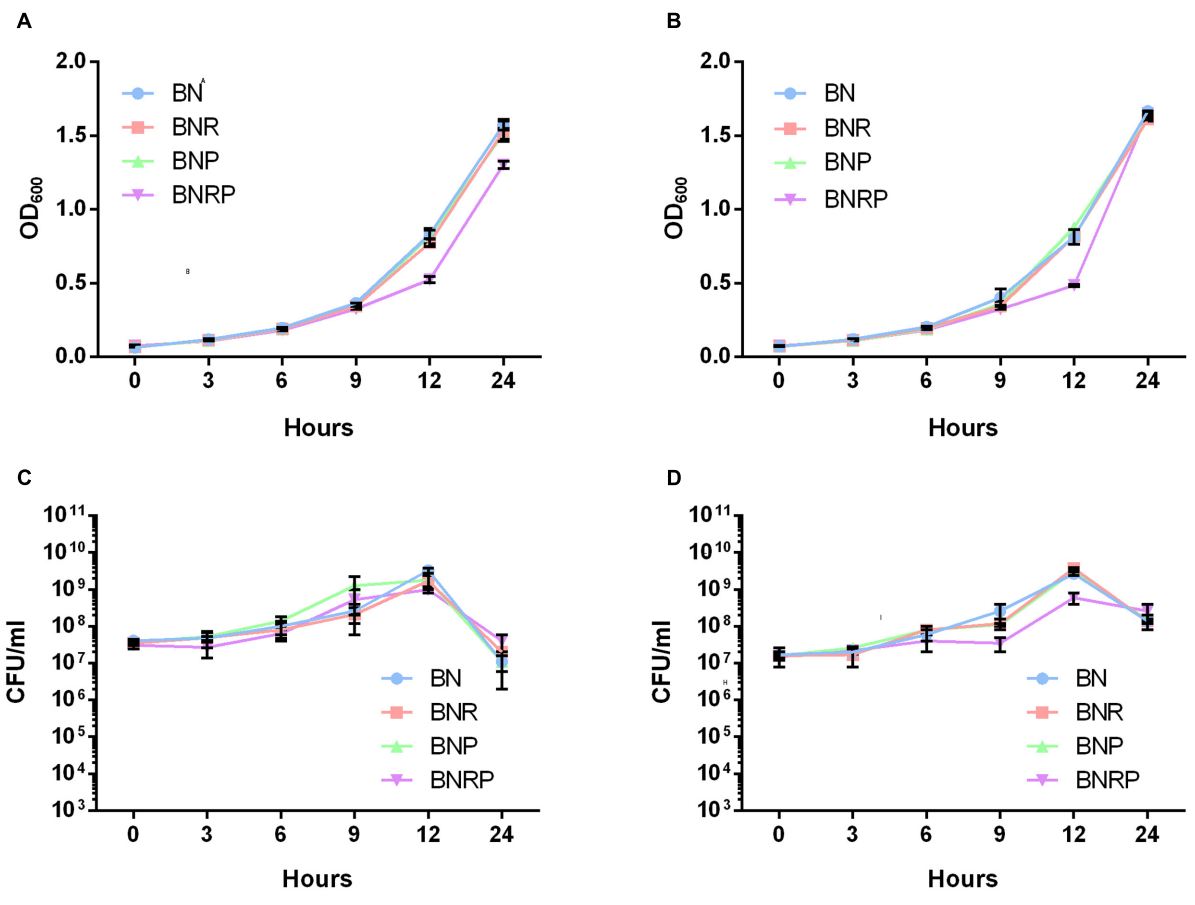

FIGURE 1 | Growth of *Campylobacter jejuni* ATCC 33560 and NCTC 11168 in four different antimicrobial enrichment conditions at 42°C. Measurement of OD_{600} and CFU counting in *C. jejuni* ATCC 33560 **(A,C)** and *C. jejuni* NCTC 11168 **(B,D)**. **BN**, Bolton broth with Bolton *Campylobacter*-selective supplement; **BNR**, BN supplemented with rifampicin; **BNP**, BN supplemented with polymyxin B; **BNRP**, BN supplemented with and rifampicin and polymyxin B.

polymyxin B) significantly decreased the C_t value, meaning that *Campylobacter* population was increased by the selective enrichment. Furthermore, supplementation of both antibiotics showed the lowest C_t value compared to the other enrichment conditions (**Figure 2**), indicating that the increased antibiotic selective pressure enhanced the enrichment of *Campylobacter* in raw sewage samples. Positive samples were more frequently detected at 42°C than 37°C, and non-interpretable results, where C_t values could not be determined, were sometimes observed at 37°C (data not shown). This suggests that contaminating bacteria cannot be effectively inhibited at 37°C.

Multiplex PCR Detection of *Campylobacter* spp. under different Enrichment Conditions

In addition to qRT-PCR detection, multiplex PCR was performed to determine the species of *Campylobacter* isolates. The results of multiplex PCR demonstrated that the primary *Campylobacter* spp. were *C. jejuni* and *C. coli* (**Table 2**). *C. jejuni* and *C. coli* were more frequently detected by the addition of rifampicin compared to polymyxin B. In many cases, positive results were discrepant between qRT-PCR and multiplex PCR (54% in qRT-PCR in comparison with multiplex PCR). For example, the same sample that was *Campylobacter*-negative based on qRT-PCR was shown to be positive by multiplex PCR (data not shown).

Enhanced *Campylobacter* Isolation from Raw Sewage by Increased Antibiotic Selective Pressure

The frequency of *Campylobacter* isolation from raw sewage was determined under the four different antibiotic enrichment conditions. To examine the effect of agar media on the *Campylobacter* isolation, we plated the enrichment cultures on Bolton and Preston agars, common culture media for *Campylobacter*. Consistent with the qRT-PCR results, the addition of rifampicin, polymyxin B, and both antibiotics significantly increased the isolation frequency for *Campylobacter* and decreased the isolation frequency of non-*Campylobacter* (**Figure 3**). In particular, BNRP showed the highest isolation rate of *Campylobacter*, whereas BN did not recover any *Campylobacter* spp. (**Figure 3**). Whereas the antibiotic enrichment significantly affected the isolation frequency, Bolton and Preston agar media did not make any differences in the isolation frequency (**Figure 3**). Morphologically, small pinkish or transparent colonies usually turned out to be *Campylobacter* (data not shown). To identify the major non-*Campylobacter* populations growing on the selective enrichment media, we randomly selected 100 colonies based on colony morphologies and performed 16S rDNA amplicon sequencing. The major non-*Campylobacter* spp. included *Enterococcus*, *E. coli*, *Klebsiella*, *Proteus*, and *Pseudomonas* (**Table 3**). The supplementation of additional antibiotics, either single (i.e., BNR and BNP) or both (i.e., BNPR), suppressed the growth of other bacterial populations. However, *Enterococcus* spp., such as *Enterococcus durans* and *Enterococcus faecium*, were still isolated in BNPR (**Table 3**). Importantly, increased antibiotic selective pressure improved the frequencies of isolating *Campylobacter* from wastewater (**Table 3**).

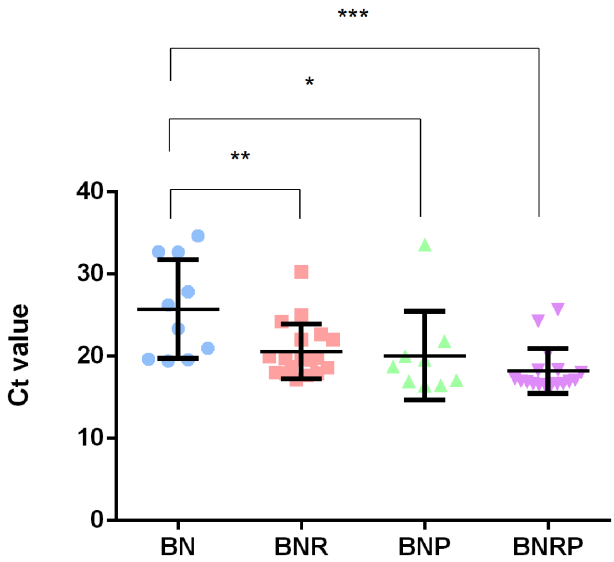

FIGURE 2 | Distribution of C_t values in four different enrichment conditions at 42°C. Bolton broth with Bolton supplement (BN), BN with rifampicin (BNR), BN with polymyxin B (BNP), and BN with rifampicin and polymyxin B (BNRP). Statistical analysis was conducted with one-way analysis of variance (ANOVA) using GraphPad Prism 6 (GraphPad Software Inc., USA). $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

TABLE 2 | The number of positive detection of *Campylobacter* 16S rDNA, *C. jejuni*, *C. coli*, and *C. lari* with multiplex PCR in four different enrichment conditions at 42°C.

Detection with multiplex PCR	BN	BNR	BNP	BNRP
16S rDNA only*	10 (40%)	3 (12%)	9 (33%)	2 (9%)
16S rDNA + *C. jejuni*	7 (28%)	9 (36%)	11 (41%)	9 (39%)
16S rDNA + *C. coli*	4 (16%)	6 (24%)	4 (15%)	7 (30%)
16S rDNA + *C. jejuni* + *C. coli*	3 (12%)	7 (28%)	2 (7%)	5 (22%)
16S rDNA + *C. lari*	1 (4%)	0	1 (4%)	0
Total 16s rDNA* positive	25 (100%)	25 (100%)	27 (100%)	23 (100%)

**Positive in multiplex PCR detection with Campylobacter 16S rDNA primers but negative for species-specific detection.*

DISCUSSION

In this study, we improved the efficacy of *C. jejuni* isolation from wastewater by increasing antibiotic selective pressure in the enrichment step. The addition of rifampicin, polymyxin B, or both to the enrichment media affected the C_t values of qRT-PCR results (**Figure 2**). According to the distribution of C_t values, the addition of the antibiotic(s) decreased C_t values, meaning that antibiotic supplements improved the growth of *Campylobacter*. In particular, rifampicin significantly reduced C_t values (**Figure 2**). A few studies have thus far reported that increased selective pressure enhances *Campylobacter* isolation from food. Yoo et al. (2014) reported that the addition of

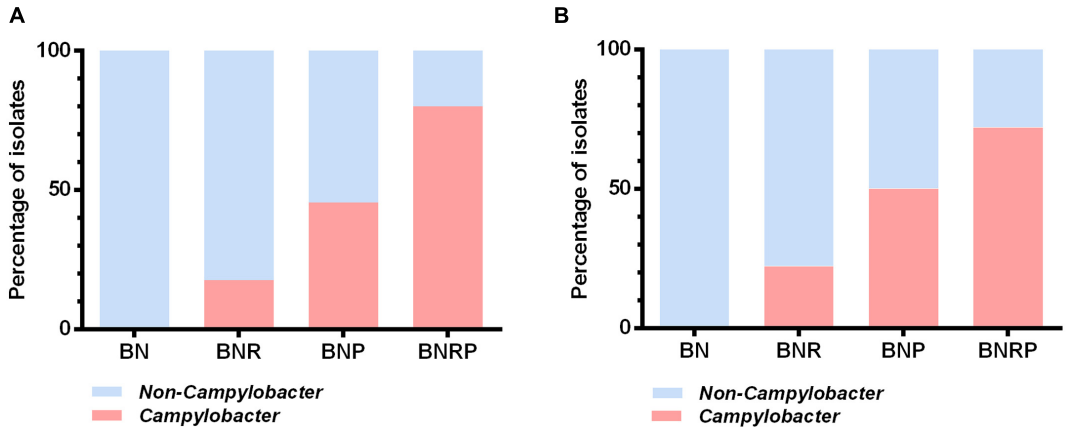

FIGURE 3 | Percentage distribution of *Campylobacter* and non-*Campylobacter* isolates in four different enrichment conditions at 42°C. After antimicrobial enrichment, strains were isolated by growing on Bolton agar plates supplemented with Bolton selective supplement (BB; **A**) and Bolton agar plates supplemented with Preston selective supplement (BP; **B**). The results are based on PCR detection with primers for *Campylobacter* 16S rDNA. The number of isolates in BB is as follows; BN 18, BNR 17, BNP 22, and BNRP 25. The number of isolates in BP is as follows; BN 18 BNR 18, BNP 24, and BNRP 25.

rifampicin (10 μg/ml) or polymyxin B (5 IU/ml) to Bolton agar (Bolton agar with Bolton supplement) restrained the growth of non-*Campylobacter* without any inhibition of *C. jejuni* and *C. coli* in fresh produce foods. Chon et al. (2013) demonstrated that the addition of high concentrations of polymyxin B to the mBolton supplement in enrichment procedure improved the efficiency of *C. jejuni* and *C. coli* recovery and suppressed background competing bacteria. Consistently, our results showed that the supplementation with additional antibiotics improved the efficacy of *C. jejuni* isolation even from heavily contaminated wastewater samples. In addition, we also identified bacterial populations that compete with *Campylobacter* under the four different selective enrichment conditions. The inputs of *Campylobacter* entering the influent of wastewater treatment facilities in this study would be primarily from sewage effluent in Calgary and also possibly from wildlife, such as migrating birds (Cody et al., 2015). Depending on the treatment procedure, the incidence rate of *Campylobacter* in sewage effluent can be altered, and cross contamination between water resources and sewage is associated with water-borne *Campylobacter* outbreaks (Jones, 2001; Pitkanen, 2013).

The 16S rDNA amplicon sequencing analysis of individual colonies from the enrichment plates revealed that *Escherichia*, *Pseudomonas, Klebsiella*, and *Enterococcus* were the major competing bacteria in *C. jejuni* isolation from wastewater (**Table 3**). Baylis et al. (2000) identified competitor organisms in foods by using Preston and Bolton selective supplement media, showing that *Yersinia, Enterobacter*, *Escherichia*, *Enterococcus*, *Pseudomonas*, and *Klebsiella* are representative competitors. This is quite similar to our results from the BN enrichment conditions. *Escherichia* were frequently isolated in BN (**Table 3**), presumably because extended-spectrum beta-lactamase (ESBL)-producing *E. coli* may reduce the selectivity of Bolton supplement and consequently *E. coli* growth would suppress *Campylobacter* (Moran et al., 2011). Although the supplementation of additional antibiotic(s) suppressed the overgrowth of competing bacteria

TABLE 3 | Distribution of *Campylobacter* and non-*Campylobacter* strains in four different enrichment conditions at 42°C.

Species	BN	BNR	BNP	BNRP
E. coli	9 (25%)	10 (28.6%)	1 (2.2%)	0
E. fergusonii	3 (8.3%)	4 (11.4%)	0	0
E. durans	0	5 (14.3%)	7 (15.2%)	8 (16%)
E. faecium	4 (11.1%)	6 (17.1%)	6 (13%)	4 (8%)
P. aeruginosa	5 (13.9%)	3 (8.6%)	5 (10.9%)	0
P. penneri	0	0	1 (2.2%)	0
P. mirabilis	0	0	4 (8.7%)	0
K. pneumoniae	15 (41.7%)	0	0	0
Campylobacter	0	7 (20%)	22 (47.8%)	38 (76%)
Total number of isolates	36 (100%)	35 (100%)	46 (100%)	50 (100%)

and enriched *Campylobacter*, *Enterococcus* survived well in the presence of five different antibiotics as it was frequently isolated with *Campylobacter* (**Table 3**). The survival of *Enterococcus* in the presence of vancomycin (20 μg/mL) in Bolton supplement indicated that *Enterococcus* isolated from the enrichment broth is vancomycin-resistant enterococci (VRE), a drug-resistant strain of serious public health concern (Cetinkaya et al., 2000). This study aimed at developing an improved culture method to isolate *Campylobacter* from wastewater, and we used the influent samples, not the effluent, since the influent is more contaminated than the effluent. Therefore, the results do not provide the information about the level of *Campylobacter* contamination in the effluent that may have a direct impact on public health compared to the influent data.

In this study, we demonstrated that antibiotic selective pressure and culture temperature are the critical factors for *C. jejuni* isolation from raw sewage. The BN, BNR, BNP, and BNRP conditions showed similar MPN values at 42°C; however, only BNRP showed reasonable MPN values and BNR and BNP showed relatively lower MPN numbers at 37°C compared to those at 42°C (data not shown). The results exhibited that

culture temperature also plays an important role in the selective enrichment of *C. jejuni*. Humphrey et al. showed the effect of antibiotics and temperature on the recovery rate in cold-damaged *C. jejuni*. The sub-lethally injured cells are more sensitive to antibiotics in 43°C than 37°C, affecting the restoration of *C. jejuni* (Humphrey, 1986). In previous studies, Humphrey et al. also suggested that pre-incubation at 37°C for 4–18 h followed 42 or 37°C incubation for 48 h would be beneficial to the recovery of *Campylobacter* in comparison with 42°C (Humphrey, 1989; Humphrey and Muscat, 1989). Whereas Khan et al. (2013) demonstrated that the detection frequency of *Campylobacter* spp. was higher at 37°C in BN than 42°C, *C. jejuni* was detected more frequently at 42°C than at 37°C. Consistently, our results suggested that 42°C seems to enhance *C. jejuni* growth in raw sewage samples.

The additional antibiotic(s) plus an increased incubation temperature (i.e., 42°C) improved the isolation rates of *C. jejuni* and *C. coli* from heavily contaminated raw sewage samples. The addition of rifampicin and polymyxin B specifies the selective enrichment of thermo-tolerant *Campylobacter* spp., such as *C. jejuni* and *C. coli*, the major human pathogenic species (Kaakoush et al., 2015). Based on our findings, increased antibiotic selective pressure and culture temperature are the key parameters impacting the success in *C. jejuni* isolation from heavily contaminated wastewater samples. Additionally, rifampicin appears to be effective in improving the selectivity of *Campylobacter* enrichment for PCR-based quantitative methods, whereas both rifampicin and polymyxin B are required to suppress competing bacterial growth and improve the selectivity of *C. jejuni* isolation with culture-based methods.

AUTHOR CONTRIBUTIONS

Design of the project: JK, NA, NN, and BJ; Performance of the experiments: JK, EO, GB, and SB; Data analysis: JK, EO, GB, SB, LC, NA, NN, and BJ; Writing of the manuscript: JK, NA, and BJ.

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Age-Related Differences in the Luminal and Mucosa-Associated Gut Microbiome of Broiler Chickens and Shifts Associated with *Campylobacter jejuni* Infection

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Despite the importance of gut microbiota for broiler performance and health little is known about the composition of this ecosystem, its development and response towards bacterial infections. Therefore, the current study was conducted to address the composition and structure of the microbial community in broiler chickens in a longitudinal study from day 1 to day 28 of age in the gut content and on the mucosa. Additionally, the consequences of a *Campylobacter (C.) jejuni* infection on the microbial community were assessed. The composition of the gut microbiota was analyzed with 16S rRNA gene targeted Illumina MiSeq sequencing. Sequencing of 130 samples yielded 51,825,306 quality-controlled sequences, which clustered into 8285 operational taxonomic units (OTUs; 0.03 distance level) representing 24 phyla. *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria,* and *Tenericutes* were the main components of the gut microbiota, with *Proteobacteria* and *Firmicutes* being the most abundant phyla (between 95.0 and 99.7% of all sequences) at all gut sites. Microbial communities changed in an age-dependent manner. Whereas, young birds had more *Proteobacteria, Firmicutes,* and *Tenericutes* dominated in older birds (>14 days old). In addition, 28 day old birds had more diverse bacterial communities than young birds. Furthermore, numerous significant differences in microbial profiles between the mucosa and luminal content of the small and large intestine were detected, with some species being strongly associated with the mucosa whereas others remained within the luminal content of the gut. Following oral infection of 14 day old broiler chickens with 1×10^8 CFU of *C. jejuni* NCTC 12744, it was found that *C. jejuni* heavily colonized throughout the small and large intestine. Moreover, *C. jejuni* colonization was associated with an alteration of the gut microbiota with infected birds having a significantly lower abundance of *Escherichia (E.) coli* at different gut sites. On the contrary, the level of *Clostridium* spp. was higher in infected birds compared with birds from the negative controls. In conclusion, the obtained results demonstrate how the bacterial microbiome

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composition changed within the early life of broiler chickens in the gut lumen and on the mucosal surface. Furthermore, our findings confirmed that the *Campylobacter* carrier state in chicken is characterized by multiple changes in the intestinal ecology within the host.

Keywords: broiler chickens, microbiota, 16S rRNA gene, age, luminal content, mucosa, *Campylobacter jejuni*, MiSeq sequencing

INTRODUCTION

A diverse microbiota is found throughout the gastrointestinal tract (GIT) of chickens, most predominant in the cecum (Mead, 1997; Videnska et al., 2014). The gut microbiota plays an essential role in nutrition, detoxification of certain compounds, growth performance and protection against pathogenic bacteria. The microbiota is crucial to strengthen the immune system, thereby affecting growth, health, and wellbeing of chicken. Generally, the gut microbiota modulates host responses to limit the colonization of pathogens (Rehman et al., 2007). There is little information about the diversity and function of the gut microbiota in chickens, its impact on the host and the impact of certain pathogens.

Development of the gut microbiota in chickens occurs immediately after hatching and is influenced by both genetic and external factors like diet and environment (Apajalahti et al., 2004). It was reported that disturbances in the intestinal microbiota leads to a delay in growth, weakens the host resistance and increases the susceptibility to various infectious diseases (Lan et al., 2004). Gong et al. (2002) demonstrated that the cecal microbiota protects chickens against bacterial infections, while microbiota in the small intestine contributes significantly to its function, including digestion and nutrient absorption, which significantly determines the growth rate of the bird. Studies on gut microbiota have mostly been performed with chickens older than 1 week of age due to the various influences in day-old birds. However, the composition of gut microbiota at the first day of life in newly hatched chickens is a matter of interest within a longitudinal study. Therefore, the focus of the actual study was to determine the diversity and community structures of the microbiota within the small and large intestine from hatch until 4 weeks of age. Furthermore, differences among the mucosa-associated and luminal content microbiota were determined for the first time.

Campylobacter (C.) jejuni is the most common cause of food-borne bacterial enteritis worldwide (EFSA, 2011). *C. jejuni* infection of chickens had previously not been considered to influence bird health and it was thought that *C. jejuni* is part of the normal microbiota of birds (EFSA, 2011). Understanding how *Campylobacter* species, especially *C. jejuni*, establishes successful colonization in chickens remains a foremost research priority as this gastrointestinal pathogen not only overcomes the host's defense system, but also competes with the microbial community for space and nutrients.

It has been shown that *Campylobacter* requires numerous factors to successfully colonize the host, to translocate and to avoid clearance (Awad et al., 2014, 2015a,b, 2016; Humphrey et al., 2014). In addition, Awad et al. (2016) showed that *Campylobacter* had the ability to reduce butyrate, isobutyrate, valerate, and isovalerate which might be due to the utilization of short-chain fatty acids (SCFAs) as a carbon source (Masanta et al., 2013) or due to the reduction of butyric acid producing bacteria amongst the microbiota. In general, there is a complex interplay between microbiota composition and SCFAs concentration and it was found that the type and level of SCFAs in the gut can affect different members of the microbial community in various ways (Mon et al., 2015).

It is still unknown how *C. jejuni* affects the ecology of the chicken gut, a feature of high importance considering a possible detrimental effect on the health of birds associated with *C. jejuni* colonization. Haag et al. (2012) demonstrated that *C. jejuni* colonization in mice depends on the microbiota of the host and *vice versa* and *Campylobacter* colonization induces a shift of the intestinal microbiota. Thus, it can be hypothesized that *Campylobacter* colonization is associated with an alteration in the intestinal microbiota of chickens as well. Therefore, the second aim of the actual study was to investigate the dynamics of an experimental *Campylobacter jejuni* NCTC 12744 infection in 14–28 days old chickens and the consequences on the alteration of the gut microbiome.

MATERIALS AND METHODS

Ethics Statement

The animal experiment was approved by the institutional ethics committee of the University of Veterinary Medicine and the Ministry of Research and Science under the license number GZ 68.205/0011-11/3b/2013. All husbandry practices were performed with full consideration of animal welfare.

Experimental Design

In this study, a total of 45 1-day-old broiler chickens (males and females) were obtained from a commercial hatchery (Ross-308, Geflügelhof Schulz, Graz, Austria). Five day-old birds were immediately sacrificed for determining the gut microbiota of the jejunal and cecal mucosa. At 7 and 14 days of age, five birds were randomly selected for measuring the development of gut microbiota from gut content and mucosa. The birds were kept as non-infected for the first 2 weeks and were housed on wood shavings with feed and water supplied *ad libitum*. The birds were fed a standard commercial diet for the whole experimental period in order to avoid an influence of the change of diet on the microbial composition.

At the first and 14 days of age birds were confirmed as *Campylobacter*-free by taking cloacal swabs which were

streaked onto modified charcoal-cefaprazone-deoxycholate agar (CM0739, OXOID, Hampshire, UK) and grown for 48 h under microaerophilic conditions at 42°C. At 14 days of age, 15 birds were infected with *Campylobacter jejuni (C. jejuni)* reference strain NCTC 12744 and kept separately from 15 non-infected control birds which were inoculated with PBS only. *C. jejuni* was routinely grown in Lennox L Base broth (LB broth) (Invitrogen, California, USA) at 42°C for 48 h in a shaking incubator. *Campylobacter* colony-forming unit (CFU) was determined from each suspension by serial dilutions in duplicate using LB agar. *Campylobacter* suspensions were stored at −80°C by adding 2 mL of 40% glycerol/10 mL LB broth. For infection, *Campylobacter* suspensions were centrifuged for 5 min at 10,000 × rpm. The pellet was washed 3 times with phosphate-buffered saline (PBS) each time centrifuged at the same conditions as mentioned above. Finally, the pellets were resuspended in PBS and the necessary concentration was adjusted for birds' infection.

The infection was performed orally via feeding tube (gavage) with a dose of 1×10^8 CFU/bird at 14 days of age as described previously (Awad et al., 2015a). At 7 days post infection 5 birds from each group were anesthetized by injection of a single dose of thiopental (20 mg/kg) into the wing vein and slaughtered by bleeding of the jugular vein. The final 10 birds/group were killed at 14 days post infection. At each time point the gastrointestinal content from the jejunum and ceca, as well as jejunal and cecal mucosa from 5 birds/group were taken to determine the gut microbiota. Intestinal segments were disclosed at the mesentery with sterile instruments and the digesta was removed. The luminal site of the intestinal segments was washed with sterile ice-cold PBS until the mucosa was completely cleaned from the digesta. The mucosa was rinsed several times with sterile ice cold PBS, after which the mucosa was collected aseptically by scraping off the mucosa using scalpel blades. All samples were stored at −80°C until further processing.

DNA Extraction, PCR Amplification of the 16S rRNA Gene, and Illumina MISeq Sequencing

DNA from luminal content and gut mucosa samples was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) as described previously (Mann et al., 2014; Yasuda et al., 2015). The same protocol of DNA extraction was applied to luminal content and gut mucosa. From each of the 130 samples a total of 250 mg of gut content or mucosa was used for DNA isolation according to manufacturer's instructions. DNA concentration was determined by a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The V345 hypervariable region of the 16S rRNA genes was amplified with the primers F341 (5′-GTGYCAGCMGCCGCGGTAA-3′) (Zakrzewski et al., 2012) and R909 (5′-CCGYCAATTYMTTTRAGTTT-3′) (Tamaki et al., 2011). An amplicon size of approximately 568 bp was produced.

16S rRNA gene PCRs, library preparation and sequencing were performed by Microsynth (Microsynth AG, Balgach Switzerland). Libraries were constructed by ligating sequencing adapters and indices onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina) and equimolar amounts of each of the libraries were pooled and sequenced on an Illumina MiSeq Sequencing Platform. Sequence data were analyzed with the software package QIIME (Caporaso et al., 2010). Low quality sequences ($q < 20$) were filtered, chimeric sequences were excluded by using the USEARCH 6.1 database (Edgar, 2010) and sequences were clustered into operational taxonomic units (OTUs; 97% similarity) with the QIIME script "pick_open_reference_otus.py." OTUs with less than 10 sequences were removed, resulting in 8285 OTUs, which were used for all downstream analysis. The representative sequences of the 50 most abundant OTUs over all sampling time points were classified against type strains using the Greengenes database (http://greengenes.lbl.gov) (DeSantis et al., 2006).

Microbial Diversity Analysis

Both alpha and beta diversity indices were used to estimate the microbiome diversity within—and between microbial communities. Calculations were done with the "summary.single" command in the software package mothur (http://www.mothur.org/; Kozich et al., 2013). Alpha diversity indices analysis included Chao1 index (richness estimate), abundance-based coverage estimator (ACE, richness estimate), Shannon's diversity, and Simpson's diversity index.

For the Bray-Curtis similarity, the dataset was rarefied to the minimum number of sequences per sample. Rarefaction curve was constructed based on the observed number of OTUs and nearly reached asymptotes for all samples (data not shown).

Principal component analysis was done with JMP® (Version 10.0.0, SAS Institute Inc., Cary, NC). Shared OTUs among gut sites at different age were plotted as Venn diagrams using the R environment (package "VennDiagram," version 1.6.17.) (Chen, 2016). Heatmaps were created using JColorGrid (Joachimiak et al., 2006).

Statistical Analysis

Statistically significant differences in relative abundance with regard to sampling sites and time were calculated using "metastats" in mothur, which is based on the homonymous bioinformatics program (White et al., 2009; Paulson et al., 2011). "Metastats" uses repeated *t* statistics and Fisher's tests on random permutations to handle sparsely-sampled features (White et al., 2009). Results were reported as a mean and standard deviation (*SD*). The significance level was set to $P < 0.05$. The *P*-values were adjusted with the Benjamini and Hochberg false discovery rate correction (FDR, *q*-value), and a $q < 0.25$ was considered significant (Lim et al., 2016). Furthermore, significant differences between the diversity estimators of the two groups were performed using the non-parametric Kruskal-Wallis-test followed by Mann-Whitney-test. PASW statistics 20, SPSS software (Chicago, Il., USA) was used for statistical analyses of diversity estimators.

Accession Numbers

Sequencing data are available in the European Nucleotide Archive (ENA) database under the accession number PRJEB14860.

RESULTS

Sequence Analysis, Phylum and OTU Classification

Sequencing of 130 samples yielded 51,825,306 quality-controlled sequences, clustering into 8285 operational taxonomic units (OTUs; 0.03 distance level). Throughout all gut sites 24 phyla were identified with *Firmicutes, Proteobacteria,* and *Tenericutes* being the most abundant ones. In **Figure S1A**, **Tables S1A–D**, relative abundances of all phyla are delineated with respect to age and groups. The results showed that in the jejunum and cecum, *Firmicutes* and *Proteobacteria* were the dominating luminal and mucosal-associated phyla in all birds investigated (**Tables S2**, **S3**).

At the first day of life *Proteobacteria* were significantly higher in the jejunal ($P = 0.0000$, $q = 0.0000$) and cecal ($P = 0.016$; $q = 0.059$) mucosa of the birds and decreased thereafter, as no significant differences were found between samples from day 14 to day 28 of age ($P = 0.140$; $q = 0.438$ and $P = 0.519$; $q = 0.955$). On the contrary, *Firmicutes* were significantly lower at day 1 and increased thereafter ($P = 0.001$; $q = 0.016$ and $P = 0.006$; $q = 0.055$ in the jejunal and cecal mucosa, respectively).

For infected birds, relative abundances of bacterial phyla at the two sampling time points carried out post infection are represented in **Figure S1B**, **Tables S4A–D**. **Figure 1** shows that the phylum *Proteobacteria* decreased while *Firmicutes* increased at either 21 (7 dpi) or 28 days of age (14 dpi). There was a significant decrease in *Actinobacteria* and *Proteobacteria* in the jejunal mucosa at 14 dpi ($P = 0.006$; $q = 0.100$ and $P = 0.005$; $q = 0.100$), while *Firmicutes* and *Bacteroidetes* were more abundant in the infected birds compared to the controls ($P = 0.005$; $q = 0.100$ and $P = 0.023$; $q = 0.217$, **Table S4A**). However, in the cecal content and cecal mucosa, *Bacteroidetes* ($P = 0.001$; $q = 0.019$) increased at 7 dpi, but decreased ($P = 0.002$; $q = 0.026$ and $P = 0.005$; $q = 0.048$) at 14 dpi in the infected birds compared with the controls, indicating that the *Campylobacter* infection modulates the jejunal and cecal phylum abundances in different ways.

In **Table 1**, the 50 most abundant OTUs from all birds are listed including the internal OTU number, relative abundance together with the reference strain and similarity (compared with strains of the Greengenes database). Relative OTUs abundances at different ages in all birds are shown in **Tables S5A–D**, **S6A–D**. The OTUs and species abundances sorted by age at the four gut sites of the birds are shown in the heatmaps of **Figure S2**. In total, the 50 most abundant OTUs accounted for 73.9% of all sequences and of those 42 OTUs differed significantly in their relative abundances over all gut sites independent of the age (**Tables 2, 3**). At the first day of age, a notable high relative abundance of OTU 1, 25, 27, and 35 (best type strain hits: *Escherichia coli, Enterococcus faecalis, Clostridium paraputrificum*, and *Clostridium sartagoforme*) were found in both jejunal and cecal mucosa (**Tables S5A,C**), whereas OTU 38 (best type strain hit: *Acinetobacter johnsonii*) was only abundant in the jejunal mucosa and OTU 42 (best type strain hit: *C. paraputrificum*) was only abundant in the cecal mucosa. All these abundant OTUs decreased by age. In the jejunal mucosa, OTU 1 was the most abundant (57.9%), followed by the other four OTUs which ranged between 2.6 and 7.9%. Similarly, in the mucosa of the cecum, OTU 1 was highly abundant (65.9%), followed by OTUs 27, 25, 35, and 42 which ranged between 7.8 and 3.3%.

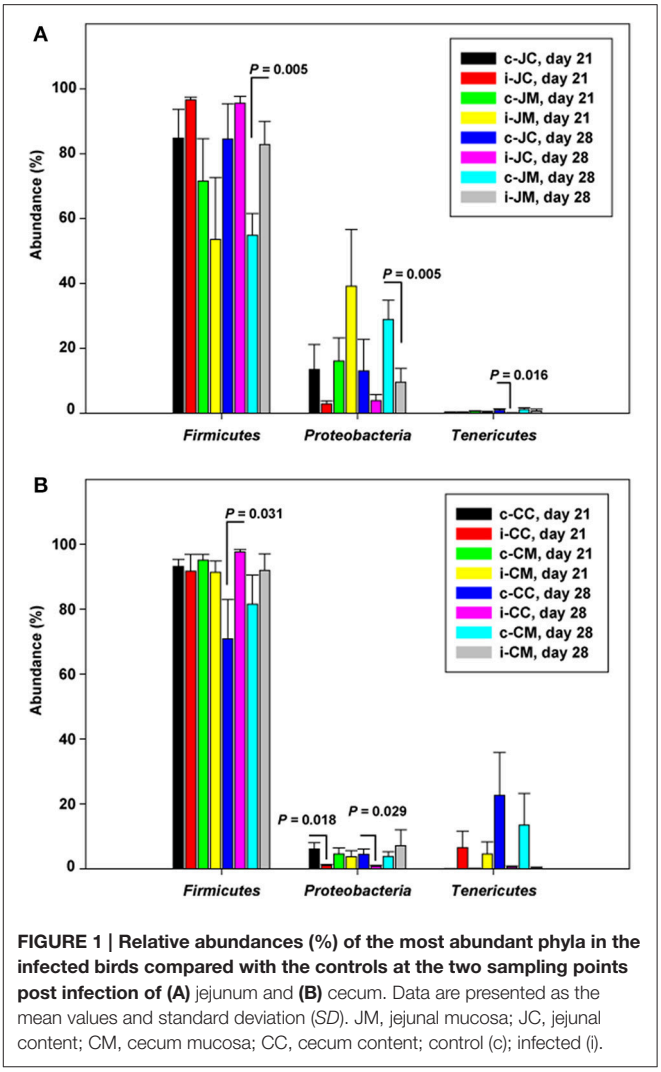

FIGURE 1 | Relative abundances (%) of the most abundant phyla in the infected birds compared with the controls at the two sampling points post infection of (A) jejunum and **(B)** cecum. Data are presented as the mean values and standard deviation (*SD*). JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content; control (c); infected (i).

The OTUs and species abundances sorted by gut sites of the infected birds compared with the control birds are shown in the heatmaps (**Figure 2**). Interestingly, in the infected birds, the abundance of *E. coli* and *Eubacterium desmolans* (best type strain hits) were lower at different gut sites (**Figure 3A**). On the contrary, *Clostridium* spp. abundance was higher in the infected birds compared with the negative controls (**Figure 3B**).

Assessment of the Microbial Community Diversity

Diversity indices estimating species richness and evenness for birds are shown in **Figure 4**. Diversity indices indicated that microbial richness and diversity increased with age. Interestingly, diversity indices were not different comparing samples from days 1 and 7. However, older chickens (14–28 days of age) had a significantly more diverse microbial community structure as indicated by the number of OTUs observed (Sobs), Chao1, ACE, Shannon's index, and Simpson index ($P < 0.01$). In addition, the

TABLE 1 | The 50 most abundant OTUs retrieved from different gut sites from all birds independent of the infection status.

OTUs	No. of sequences	Relative abundance (%)	Best type strain hit[a]	Similarity (%)
OTU 1	642068	10.64	*Escherichia coli* (GU968184.1)	100
OTU 2	341923	5.66	*Lactobacillus johnsonii* (HM772969.1)	100
OTU 3	339288	5.62	*Lactobacillus salivarius* (NZ_AEBA01000145.1)	100
OTU 4	310060	5.14	*Clostridium* spp.(FJ808599.1)	87
OTU 5	273783	4.54	*Enterococcus durans* (FJ917726.1)	100
OTU 6	209121	3.46	*Anaerotruncus colihominis* (NR_027558.1)	100
OTU 7	208070	3.45	*Eubacterium desmolans* (L34618.1)	98
OTU 8	161864	2.68	*Clostridium cellulolyticum* (X71847.1)	87
OTU 9	157466	2.61	*Spiroplasma lampyridicola* (AY189134.1)	82
OTU 10	151864	2.52	*Clostridium leptum* (AJ305238.1)	97
OTU 11	130587	2.16	*Eubacterium desmolans* (L34618.1)	99
OTU 12	127446	2.11	*Eubacterium desmolans* (L34618.1)	98
OTU 13	120732	2.00	*Clostridium papyrosolvens* (NR_026102.1)	87
OTU 14	113436	1.88	*Eubacterium coprostanoligenes* (HM037995.1)	92
OTU 15	98491	1.63	*Clostridium straminisolvens* (NR_024829.1); *Clostridium thermocellum* (AB558166.1)	87
OTU 16	87863	1.46	*Clostridium cellulolyticum* (NC_011898.1)	87
OTU 17	70842	1.17	*Bacillus subtilis* (FJ608705.1)	94
OTU 18	67038	1.11	*Lactobacillus reuteri* (EU547311.1)	99
OTU 19	66212	1.10	*Ruminococcus bromii* (NR_025930.1)	94
OTU 20	65592	1.09	*Eubacterium desmolans* (L34618.1)	95
OTU 21	53133	0.88	*Clostridium cellulolyticum* (NC_011898.1); *Clostridium papyrosolvens* (NR_026102.1)	87
OTU 22	49736	0.82	*Clostridium thermocellum* (AB558166.1)	85
OTU 23	47570	0.79	*Clostridium thermosuccinogenes (*Y18180.1); *Clostridium thermocellum* (AB558166.1)	86
OTU 24	40373	0.67	*Clostridium leptum* (AJ305238.1)	95
OTU 25	36925	0.61	*Enterococcus faecalis* (FJ607291.1)	100
OTU 26	35311	0.58	*Proteus mirabilis* (GU477712.1)	100
OTU 27	31154	0.52	*Clostridium paraputrificum* (AY442815.1)	100
OTU 28	29186	0.48	*Variovorax paradoxus* (HQ005421.1)	100
OTU 29	28876	0.48	*Campylobacter* subsp. *jejuni* (NC_008787.1)	100
OTU 30	27988	0.46	*Clostridium leptum* (AJ305238.1)	93
OTU 31	23587	0.39	*Anaerotruncus colihominis* (NR_027558.1)	93
OTU 32	23559	0.39	*Lactobacillus crispatus* (FN692037.1)	100
OTU 33	22863	0.38	*Phyllobacterium myrsinacearum* (AY785330.1)	100
OTU 34	21547	0.36	*Catabacter hongkongensis* (AY574991.1); *Clostridium thermosuccinogenes* (Y18180.1)	86
OTU 35	20378	0.34	*Clostridium sartagoforme* (FJ384380.1)	100
OTU 36	18849	0.31	*Acetivibrio cellulolyticus* (L35515.1)	95
OTU 37	18055	0.30	*Clostridium papyrosolvens* (NR_026102.1)	86
OTU 38	17890	0.30	*Acinetobacter johnsonii* (EU977694.1)	100
OTU 39	15658	0.26	*Clostridium papyrosolvens* (NR_026102.1)	87
OTU 40	15361	0.25	*Lactobacillus johnsonii* (EU381128.1)	96
OTU 41	15143	0.25	*Clostridium thermosuccinogenes* (Y18180.1)	87
OTU 42	14985	0.25	*Clostridium paraputrificum* (AY442815.1)	100
OTU 43	14682	0.24	*Marinobacter* sp. (FJ889664.1)	99
OTU 44	14652	0.24	*Clostridium papyrosolvens* (NR_026102.1)	87
OTU 45	14304	0.24	*Shigella flexneri* (CP000266.1)	99
OTU 46	13619	0.23	*Clostridium cellulolyticum* (NC_011898.1)	86
OTU 47	13307	0.22	*Clostridium papyrosolvens* (NR_026102.1)	86
OTU 48	13152	0.22	*Clostridium papyrosolvens (*NR_026102.1)	87
OTU 49	12978	0.21	*Clostridium papyrosolvens* (NR_026102.1)	87
OTU 50	12418	0.21	*Lactobacillus salivarius* (NZ_AEBA01000145.1)	98

[a] *Greengenes best type strain hit accession numbers are listed in parenthesis.*

TABLE 2 | Relative abundances (%) of the most abundant OTUs in different gut sites of control birds (day 1–28).

OTUs ID	Jejunum mucosa		Jejunum content		Cecum mucosa		Cecum content		JM-JC		CM-CC		JM-CM		JC-CC	
	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]
OTU 49	0	0.02	0.05	0.11	0.01	0.02	0.01	0.02	**0.056**	**0.164**	0.655	0.858	0.366	0.623	0.133	0.326
OTU 25	1.89	4.01	0.06	0.07	1.05	2.95	0.01	0.02	**0.001**	**0.011**	**0.015**	**0.067**	0.365	0.623	**0.001**	**0.011**
OTU 5	12.41	25.84	13.09	21.85	0.28	0.56	0.07	0.09	0.920	0.990	**0.039**	**0.134**	**0.002**	**0.017**	**0.001**	**0.011**
OTU 30	0.45	1.45	0.40	0.72	0.74	1.11	1.25	2.09	0.913	0.990	0.385	0.643	0.808	0.946	**0.059**	**0.170**
OTU 9	0.90	2.72	1.03	3.05	3.56	9.78	5.82	15.04	0.938	0.990	0.514	0.765	0.196	0.401	**0.091**	0.244
OTU 13	1.34	5.22	0.75	1.84	1.36	4.51	1.41	4.43	0.714	0.890	0.831	0.946	0.940	0.990	0.549	0.770
OTU 15	0.07	0.20	0.13	0.32	1.17	1.98	4.21	9.38	0.792	0.942	**0.095**	**0.250**	**0.005**	**0.030**	**0.001**	**0.011**
OTU 18	2.36	8.46	2.34	6.03	0.08	0.23	0.03	0.09	0.961	0.994	0.409	0.665	0.081	0.220	**0.013**	**0.059**
OTU 21	0.65	2.01	0.32	0.87	0.53	1.11	1.08	3.21	0.588	0.787	0.599	0.796	0.812	0.946	0.294	0.526
OTU 33	0.96	1.38	2.57	5.92	0	0	0	0	0.341	0.603			**0.001**	**0.011**		
OTU 12	0.95	2.20	1.25	4.71	2.42	5.21	5.41	11.82	0.776	0.929	0.285	0.519	0.491	0.739	0.131	0.326
OTU 28	3.21	7.19	2.99	7.77	0	0	0	0	0.941	0.990	**0.006**	**0.034**	**0.001**	**0.011**	**0.001**	**0.011**
OTU 37	0.12	0.56	0	0.01	0.39	1.93	0.07	0.28	0.140	0.326	0.939	0.990	0.947	0.991	0.177	0.379
OTU 40	0.05	0.11	1.08	1.53	0	0	0.10	0.44	**0.001**	**0.011**	0.464	0.722	**0.001**	**0.011**	**0.008**	**0.043**
OTU 3	10.36	20.66	18.43	23.28	0.39	0.63	1.52	5.30	0.252	0.475	0.517	0.765	**0.001**	**0.011**	**0.002**	**0.017**
OTU 26	0.03	0.07	0.03	0.06	1.34	2.35	1.75	3.25	0.483	0.737	0.691	0.875	**0.001**	**0.011**	**0.003**	**0.020**
OTU 19	0.80	1.99	0.22	0.57	1.09	2.22	1.01	2.17	0.189	0.395	0.979	0.997	0.961	0.994	0.134	0.326
OTU 44	0.04	0.15	0.02	0.04	0.06	0.12	0.07	0.14	0.748	0.908	0.660	0.858	0.452	0.715	**0.052**	**0.155**
OTU 39	0.02	0.07	0.01	0.02	0.07	0.20	0.08	0.19	0.769	0.927	0.726	0.893	0.253	0.475	**0.046**	**0.147**
OTU 29	0	0	0	0	0	0	0	0			**0.002**	**0.017**				
OTU 2	10.86	14.76	24.38	24.19	0.13	0.26	0.31	1.13	**0.046**	**0.147**	0.817	0.946	**0.001**	**0.011**	**0.001**	**0.011**
OTU 38	1.51	5.31	0.17	0.67	0	0	0	0	0.145	0.326			**0.002**	**0.017**		
OTU 42	0.51	1.20	0.01	0.03	0.67	1.71	0.01	0.03	**0.012**	**0.057**	**0.041**	**0.136**	0.717	0.890	0.669	0.864
OTU 17	0.46	1.30	0.02	0.05	2.79	7.57	0.05	0.15	**0.041**	**0.136**	**0.063**	**0.176**	0.243	0.466	0.571	0.770
OTU 1	14.87	24.08	5.33	7.93	22.17	26.59	13.03	17.69	**0.069**	**0.190**	0.231	0.452	0.295	0.526	**0.062**	**0.176**
OTU 11	0.69	1.32	0.51	0.97	2.63	7.62	3.10	5.11	0.362	0.623	0.692	0.875	0.458	0.719	**0.003**	**0.020**
OTU 4	0.51	2.00	0.17	0.56	1.34	4.60	2.14	7.58	0.571	0.770	0.611	0.806	0.525	0.770	0.147	0.326
OTU 48	0	0	0	0	0	0	0	0			**0.036**	**0.131**				
OTU 14	0.24	0.69	0.22	0.43	3.77	8.30	4.66	10.97	0.542	0.770	0.747	0.908	**0.023**	**0.091**	**0.004**	**0.025**
OTU 46	0	0.02	0.01	0.02	0.26	0.65	0.25	0.50	0.348	0.609	0.991	1.000	**0.028**	**0.106**	**0.039**	**0.134**
OTU 31	0.23	0.55	0.05	0.12	0.87	1.67	0.33	0.51	0.137	0.326	0.170	0.368	0.166	0.364	**0.016**	**0.068**
OTU 45	0.05	0.09	0.01	0.01	0.35	0.56	1.02	1.62	**0.023**	**0.091**	**0.096**	**0.250**	**0.016**	**0.068**	**0.004**	**0.025**
OTU 10	0.37	1.03	0.20	0.46	4.39	11.22	4.97	10.40	0.477	0.736	0.824	0.946	**0.013**	**0.059**	**0.003**	**0.020**
OTU 32	0.12	0.43	0.23	0.79	0	0	0	0	0.540	0.770	0.868	0.975	**0.012**	**0.057**	**0.006**	**0.034**
OTU 8	0.28	1.29	0.03	0.10	1.43	5.01	2.34	7.10	0.537	0.770	0.551	0.770	0.370	0.624	0.138	0.326
OTU 6	1.17	3.54	0.13	0.28	9.20	15.81	1.39	1.56	0.238	0.461	**0.007**	**0.039**	**0.021**	**0.087**	**0.001**	**0.011**
OTU 36	0.03	0.11	0.11	0.22	0.68	1.66	0.25	0.49	0.144	0.326	0.263	0.488	**0.003**	**0.020**	0.220	0.445
OTU 35	0.57	1.28	0.02	0.04	0.90	2.13	0.02	0.08	**0.012**	**0.057**	**0.034**	**0.126**	0.542	0.770	0.867	0.975
OTU 7	1.07	2.73	1.41	2.66	3.52	7.28	6.47	12.19	0.832	0.946	0.282	0.518	0.125	0.320	**0.037**	**0.132**
OTU 16	0.73	3.50	0.27	1.01	1.41	3.77	1.96	4.92	0.905	0.990	0.562	0.770	0.400	0.656	0.146	0.326
OTU 27	0.98	2.31	0.02	0.07	1.56	4.02	0.01	0.05	**0.012**	**0.057**	**0.048**	**0.151**	0.563	0.770	0.719	0.890
OTU 20	1.68	4.52	0.05	0.18	0.86	4.28	0.91	4.07	**0.049**	**0.151**	0.981	0.997	0.448	0.715	0.997	1.000
OTU 50	0.09	0.15	0.84	0.95	0	0.01	0.06	0.23	**0.001**	**0.011**	0.486	0.737	**0.001**	**0.011**	**0.002**	**0.017**
OTU 22	0	0	0	0.02	0	0	0	0.01			0.231	0.452			0.882	0.985
OTU 47	0.12	0.53	0	0.02	0.32	1.55	0.06	0.22	0.191	0.395	0.917	0.990	0.933	0.990	0.180	0.381
OTU 41	0	0.01	0	0	0	0	0	0			0.228	0.452	1.000	1.000		
OTU 23	0.12	0.50	0.01	0.02	0.07	0.20	0.09	0.23	0.136	0.326	0.682	0.874	0.819	0.946	**0.024**	**0.093**
OTU 43	0.01	0.03	0	0	0	0	0	0								
OTU 34	0.03	0.14	0	0	0.06	0.16	0.14	0.38	0.903	0.990	0.417	0.672	0.390	0.645	**0.051**	**0.155**
OTU 24	0.11	0.52	0.18	0.39	1.18	1.89	1.10	1.90	0.567	0.770	0.973	0.997	**0.001**	**0.011**	**0.003**	**0.020**

[a] *q-value: The False Discovery Rate (FDR) adjusted p-value using Benjamini and Hochberg method and the q < 0.25 after FDR correction considered significant. Statistically significant values are formatted in bold.*

TABLE 3 | Relative abundances (%) of the most abundant OTUs in different gut sites of infected birds (days 21 and 28).

OTUs ID	Jejunum mucosa		Jejunum content		Cecum mucosa		Cecum content		JM-JC		CM-CC		JM-CM		JC-CC	
	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]	*P*-values	*q*-values[a]
OTU 49	0.18	0.28	0.43	0.98	0.61	1.44	0.52	0.91	0.689	0.880	0.936	0.989	0.550	0.791	0.516	0.776
OTU 25	0.02	0.04	0.08	0.09	0.00	0.00	0	0	**0.077**	0.178			**0.063**	0.168		
OTU 5	1.65	2.21	8.89	6.18	0.01	0.01	0	0	**0.009**	**0.043**	0.761	0.927	**0.001**	**0.007**	**0.001**	**0.007**
OTU 30	0.31	0.94	0.06	0.08	0.30	0.38	0.16	0.12	0.957	0.989	0.355	0.596	1.000	1.000	**0.013**	**0.056**
OTU 9	0.17	0.39	0.14	0.14	1.47	4.29	2.06	5.88	0.903	0.987	0.575	0.791	0.530	0.785	0.217	0.408
OTU 13	0.64	1.06	0.72	1.35	2.87	3.77	2.65	2.58	0.955	0.989	0.950	0.989	**0.056**	0.159	**0.020**	**0.071**
OTU 15	0.08	0.13	0.01	0.02	1.24	0.89	2.13	1.23	**0.049**	0.146	**0.040**	0.126	**0.001**	**0.007**	**0.001**	**0.007**
OTU 18	4.67	9.39	6.34	11.77	0.19	0.44	0.14	0.36	0.572	0.791	0.857	0.986	**0.102**	**0.218**	**0.011**	**0.051**
OTU 21	0.28	0.58	0.16	0.19	1.63	2.60	1.93	2.41	0.565	0.791	0.611	0.611	**0.085**	**0.191**	**0.009**	**0.043**
OTU 33	1.11	1.14	0.13	0.13	0.00	0.00	0.00	0.00	**0.008**	**0.041**	1.000	1.000	**0.001**	**0.007**	**0.001**	**0.007**
OTU 12	0.51	0.74	0.06	0.08	0.06	0.09	0.05	0.05	**0.056**	**0.159**	0.929	0.989	**0.062**	**0.168**	0.796	0.949
OTU 28	1.19	1.20	0.20	0.21	0.00	0.00	0.00	0.00	**0.015**	**0.060**	**0.016**	**0.060**	**0.001**	**0.007**	**0.001**	**0.007**
OTU 37	0.14	0.27	0.01	0.02	0.35	0.18	0.51	0.27	**0.074**	**0.178**	**0.077**	**0.178**	0.207	0.394	**0.001**	**0.007**
OTU 40	0.07	0.08	1.35	0.83	0.01	0.01	0.00	0.01	**0.001**	**0.007**	0.881	0.987	**0.008**	**0.041**	**0.001**	**0.007**
OTU 3	11.02	13.33	29.61	19.69	0.52	0.56	0.41	0.45	**0.067**	**0.173**	0.865	0.986	**0.004**	**0.023**	**0.001**	**0.007**
OTU 26	0	0	0.01	0.05	0.01	0.01	0.01	0.01	0.542	0.791	0.801	0.949	**0.070**	**0.176**	0.990	1.000
OTU 19	0.14	0.29	0.31	0.24	3.24	7.48	2.09	5.77	0.372	0.613	0.614	0.823	**0.107**	**0.223**	0.441	0.700
OTU 44	0.43	1.20	0.07	0.12	0.49	0.94	1.06	2.17	0.594	0.809	0.574	0.791	0.552	0.791	**0.014**	**0.059**
OTU 39	0.02	0.05	0.13	0.20	0.59	0.79	1.02	1.34	**0.072**	**0.178**	0.318	0.539	**0.001**	**0.007**	**0.016**	**0.060**
OTU 29	0.30	0.66	0.01	0.03	3.92	6.25	0.38	0.51	**0.052**	**0.152**	**0.049**	**0.146**	**0.036**	**0.115**	**0.005**	**0.028**
OTU 2	19.98	22.32	23.46	10.10	0.85	1.46	0.19	0.30	0.834	0.975	0.285	0.493	**0.002**	**0.012**	**0.001**	**0.007**
OTU 38	0	0.01	0	0	0	0	0	0	0.690	**0.012**						
OTU 42	0	0	0	0	0	0	0	0								
OTU 17	0.62	1.33	0.25	0.31	4.45	6.58	0.12	0.18	0.451	0.703	**0.002**	**0.012**	**0.091**	**0.201**	0.531	0.785
OTU 1	0.11	0.18	0.81	1.36	0.28	0.40	0.19	0.36	0.145	0.292	0.760	0.927	0.280	0.491	0.255	0.455
OTU 11	1.09	1.39	0.10	0.10	2.01	2.44	2.31	3.56	**0.026**	**0.086**	0.761	0.927	0.907	0.987	**0.007**	**0.038**
OTU 4	3.43	6.60	0.13	0.11	12.51	8.03	16.82	10.92	0.154	0.303	0.162	0.315	**0.016**	**0.060**	**0.001**	**0.007**
OTU 48	0.41	1.14	0.01	0.02	2.29	7.23	0.38	1.17	0.225	0.414	0.747	0.927	0.817	0.962	0.222	0.413
OTU 14	0.24	0.65	0.07	0.07	0.45	0.84	1.41	2.08	0.364	0.606	0.122	0.251	0.619	0.824	**0.076**	**0.178**
OTU 46	0.21	0.60	0.01	0.02	0.49	0.81	0.52	0.87	0.306	0.524	0.757	0.927	0.396	0.646	**0.016**	**0.060**
OTU 31	0.03	0.06	0.03	0.04	0.37	0.52	0.08	0.07	0.955	0.989	**0.057**	**0.159**	**0.012**	**0.055**	**0.019**	**0.068**
OTU 45	0	0	0	0	0	0	0	0			1.000	1.000			0.281	0.491
OTU 10	0.26	0.82	0.09	0.12	0.29	0.57	0.22	0.30	0.960	0.989	0.896	0.987	0.891	0.987	0.175	0.336
OTU 32	2.90	3.72	3.80	3.98	0.18	0.46	0.05	0.11	0.872	0.986	0.567	0.791	**0.004**	**0.023**	**0.001**	**0.007**
OTU 8	1.21	2.88	0.05	0.09	7.75	7.99	6.25	8.70	**0.036**	**0.115**	0.917	0.989	**0.021**	**0.073**	**0.001**	**0.007**
OTU 6	0.82	1.99	0.10	0.13	4.11	2.46	1.24	1.97	0.455	0.703	**0.026**	**0.086**	**0.019**	**0.068**	**0.001**	**0.007**
OTU 36	0.21	0.40	0.23	0.21	0.34	0.37	0.19	0.22	0.893	0.987	0.441	0.700	0.473	0.724	0.871	0.986
OTU 35	0	0	0	0	0	0	0	0								
OTU 7	0.24	0.40	0.08	0.17	4.65	13.62	6.23	18.65	**0.095**	**0.205**	0.692	0.880	0.501	0.760	**0.062**	**0.168**
OTU 16	0.19	0.27	0.29	0.47	1.37	1.80	1.55	1.94	0.776	0.932	0.653	0.862	**0.074**	**0.178**	**0.048**	**0.146**
OTU 27	0	0	0	0	0	0	0	0								
OTU 20	0.22	0.67	0.03	0.05	4.32	13.62	0.62	1.88	0.849	0.986	0.770	0.932	0.576	0.791	0.424	0.686
OTU 50	0.10	0.17	1.20	0.73	0.01	0.01	0.01	0.01	**0.001**	**0.007**	0.979	1.000	**0.013**	**0.056**	**0.001**	**0.007**
OTU 22	0.14	0.42	0	0	2.08	5.32	3.60	9.82	**0.107**	**0.223**	0.671	0.877	0.236	0.430	**0.001**	**0.007**
OTU 47	0.03	0.06	0.01	0.01	0.20	0.10	0.31	0.23	0.244	0.440	**0.092**	**0.201**	**0.001**	**0.007**	**0.001**	**0.007**
OTU 41	0.99	2.99	0	0	0.50	0.87	1.28	3.32			0.723	0.913	0.923	0.989		
OTU 23	0.63	1.18	0.02	0.02	2.39	2.31	2.88	2.65	0.124	0.252	0.448	0.703	**0.064**	**0.168**	**0.001**	**0.007**
OTU 43	8.90	28.14	0	0	0	0	0	0								
OTU 34	0.02	0.05	0	0	0.85	0.79	1.54	1.09	0.148	0.294	**0.068**	**0.173**	**0.001**	**0.007**	**0.001**	**0.007**
OTU 24	0	0	0.05	0.07	0.15	0.13	0.12	0.11			0.674	0.877			**0.082**	**0.187**

[a]*q-value: The False Discovery Rate (FDR) adjusted p-value using Benjamini and Hochberg method and the q < 0.25 after FDR correction considered significant. Statistically significant values are formatted in bold.*

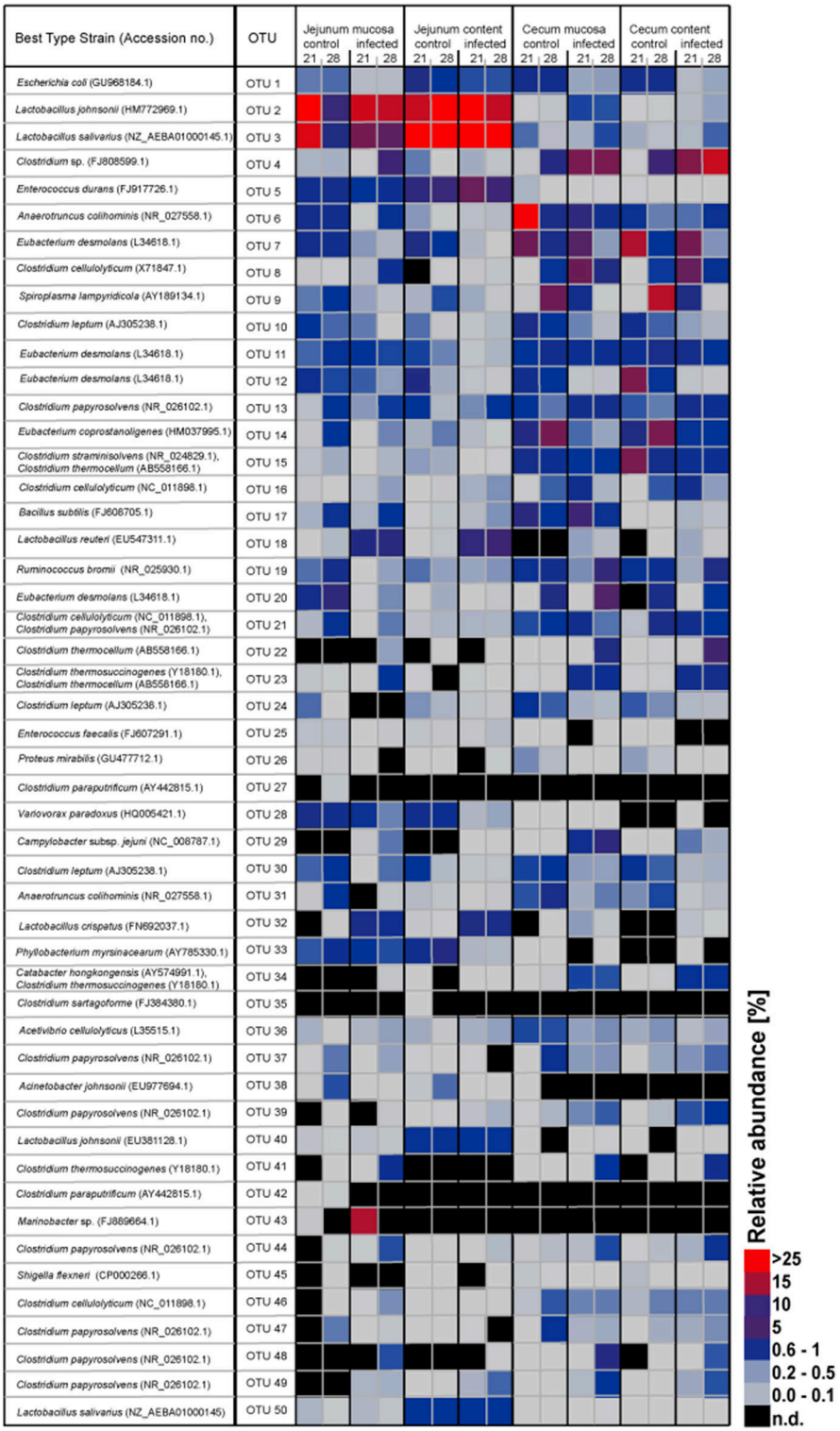

FIGURE 2 | Heatmap showing the relative abundances (%) of the 50 most-abundant OTUs sorted by gut sites of the infected birds compared with the controls at the two sampling points post infection. The heat map integrates relative abundance of a given phylotype. Colour scaling is ranged from 0 to ≥ 25%. n.d, not detected.

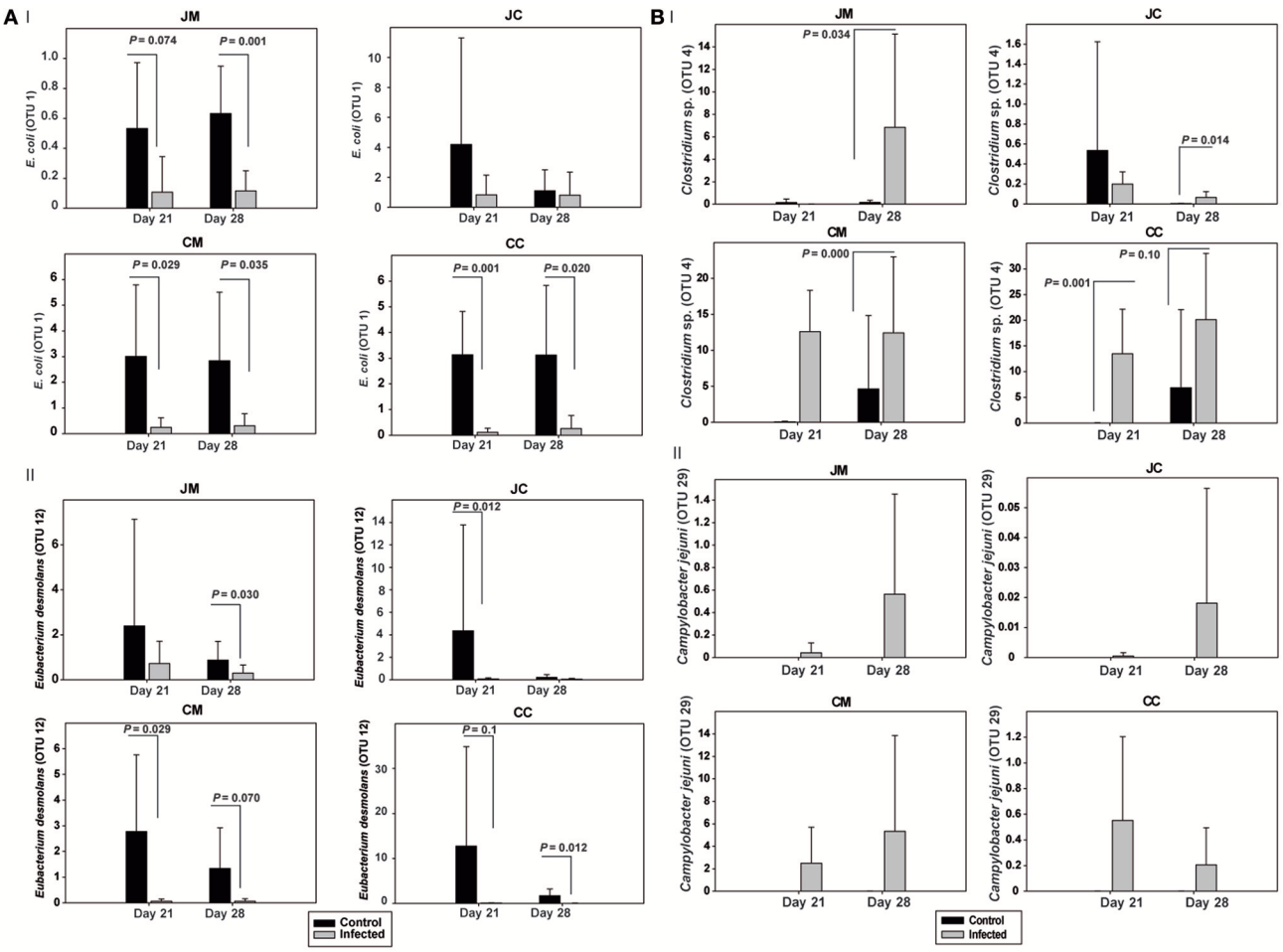

FIGURE 3 | Relative abundances (%) of the most relevant OTUs in the infected birds compared with the controls at the two sampling points post infection (A) OTUs 1 and 12, **(B)** OTUs 4 and 29. JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content.

microbial diversity in older chickens is more consistent, as there was no difference in diversity indices comparing samples from days 14 to 28. The results also revealed significant differences in the microbial diversity among jejunum and cecum as the chicken aged, supported by Sobs ($P < 0.001$), Chao1 ($P < 0.001$), ACE ($P < 0.001$), Shannon's index ($P < 0.001$), and Simpson index ($P = 0.060$) with a more complex diversity in the cecum compared with the jejunum. Furthermore, a difference in species richness among the luminal and mucosa-associated gut microbiota, independent of the age, was detected in all birds as supported by Sobs ($P = 0.017$), Chao1 ($P = 0.015$), ACE ($P = 0.022$), respectively.

In the infected birds, significant differences in the microbial diversity among jejunum and cecum supported by Sobs ($P < 0.001$), Chao1 ($P < 0.001$), ACE ($P < 0.001$), Shannon's index ($P < 0.001$), and Simpson index ($P = 0.011$) were found. Additionally, an increase in the species richness among luminal and mucosa-associated gut microbiota of the infected birds at 14 dpi compared with those from 7 dpi was obtained. Diversity indices were not significantly different among the gut sites of infected and control birds. Exceptional to this, a higher species richness was noticed in the cecum content of infected birds at 14 dpi, supported by Sobs, Chao1, and ACE ($P = 0.047$, **Figure 4C**), indicating that the *Campylobacter* infection increased the microbiota complexity.

Similarity and Stability of the Gut Microbiota Composition Over Time

The microbial community similarity among all samples over time was assessed by calculating a Bray-Curtis similarity matrix. Community similarity analysis based on the Bray-Curtis index showed clear differences between gut sites and age, indicating strong shifts in microbial community structures (**Figure 5**). In addition, the Bray-Curtis index suggested that the birds at the first day of age displayed a high degree of dissimilarity compared with the other ages. It was also apparent that microbiota compositions of older birds were more similar compared with young birds.

The Bray-Curtis index revealed clear differences between jejunum and cecum from infected birds at the two sampling

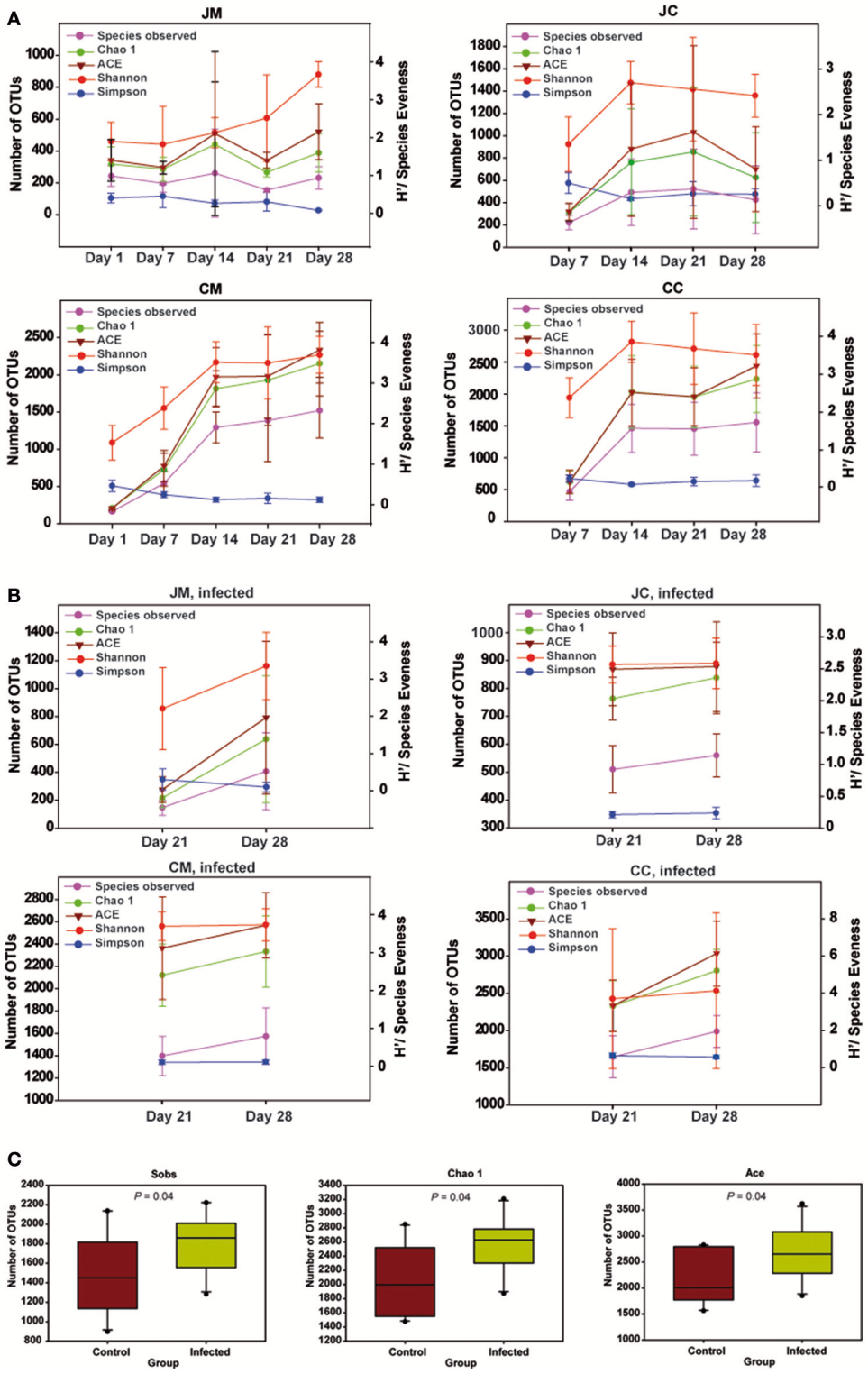

FIGURE 4 | Species richness and diversity measures of the microbial community at all gut sites in the control (A), infected birds **(B)**, and **(C)** species richness and diversity estimates for bacteria from cecum content of the infected birds compared with the controls. Left Y-axis for number of observed OTUs (Sobs), Chao 1 and ACE, and Right Y-axis for Shannon and Simpson. Significant differences were calculated with Kruskal-Wallis-tests and Mann-Whitney-tests, and significance was declared at $P < 0.05$. Data are presented as the mean values and *SD*. JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content.

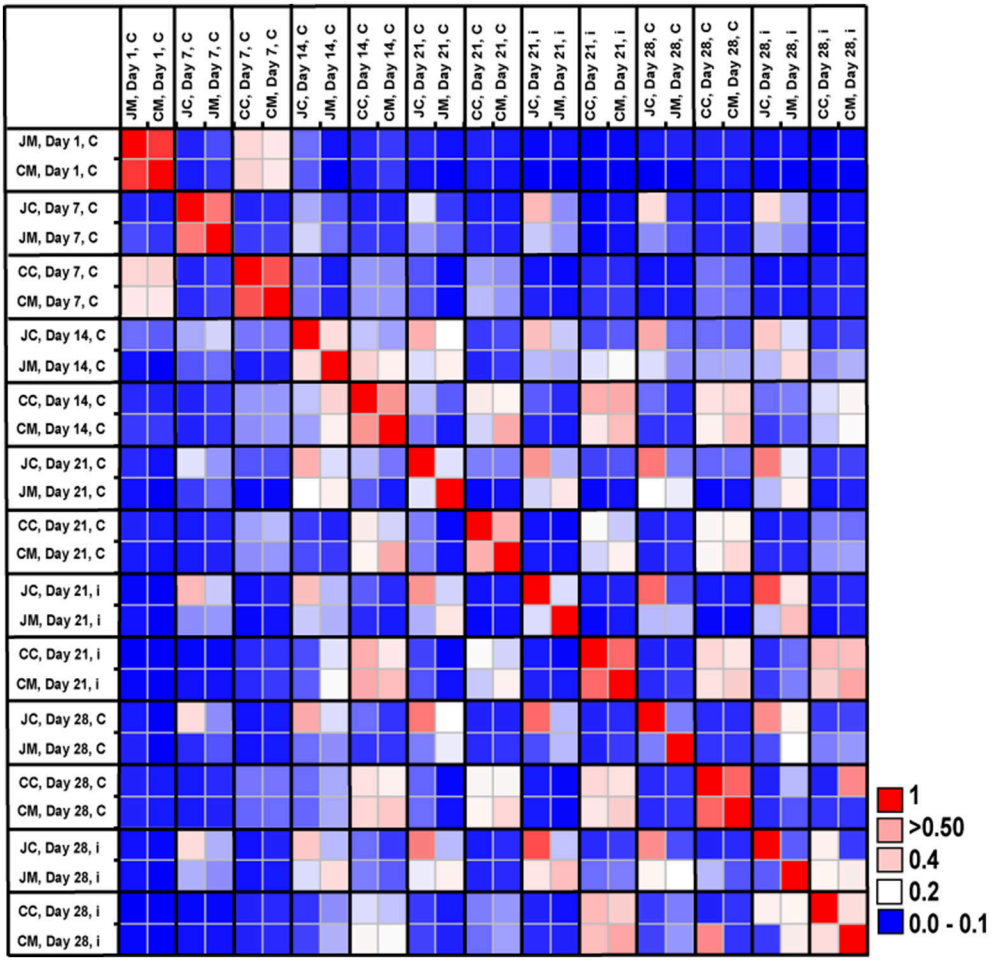

FIGURE 5 | Microbial community similarity between all samples calculated with Bray-Curtis similarities, which displays the similarity results between the control and infected groups according to age and gut sites. JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content.

time points post infection. Furthermore, the comparison of the microbiota between control and infected birds showed that community structures were more dissimilar at the OTUs level, demonstrating that the gut microbial communities changed as a result of infection.

To measure the similarity between microbial communities in all birds at different ages, principal component analysis (PCA) was performed (**Figure 6**). PCA analysis showed that there was a clear clustering of the birds at days 1 and 7 of age in the jejunum and cecum compared with the other days. In addition, the microbial community of the older chickens clustered with less variation compared to young birds. PCA plots also demonstrate that, the microbial community was more separated in the ceca than in the jejunum.

To delineate the shared species among the groups, a Venn diagram displaying the overlaps between gut sites at different ages and groups was performed (**Figure S3**). The proportions of shared OTUs appear to be low at each gut site from day 1 to day 28 of age. These shared species, however, varied from one site to another.

Furthermore, the analysis showed that only 399 OTUs (n = 1847 OTUs) were shared among the jejunal mucosa in the control and infected birds, while 745 OTUs (n = 2401 OTUs) were shared between the jejunal content in the control and infected birds at the two time points post infection (**Figure 7A**). In the cecal mucosa and the cecal content, the comparison revealed that only 2218 OTUs (n = 6736 OTUs) and 2617 OTUs (n = 6860 OTUs) were shared, among control and infected birds combining the two time points post infection (**Figure 7B**). These data demonstrated that 25–36% of the observed OTUs in the jejunum and cecum were shared between the control and infected birds, respectively.

DISCUSSION

The intestinal microbiota acts as a physical barrier for incoming pathogens and plays an important role in the host resistance against infections by both direct interactions with pathogenic bacteria via competitive exclusion, such as occupation of attachment sites or consumption of nutrient sources, and

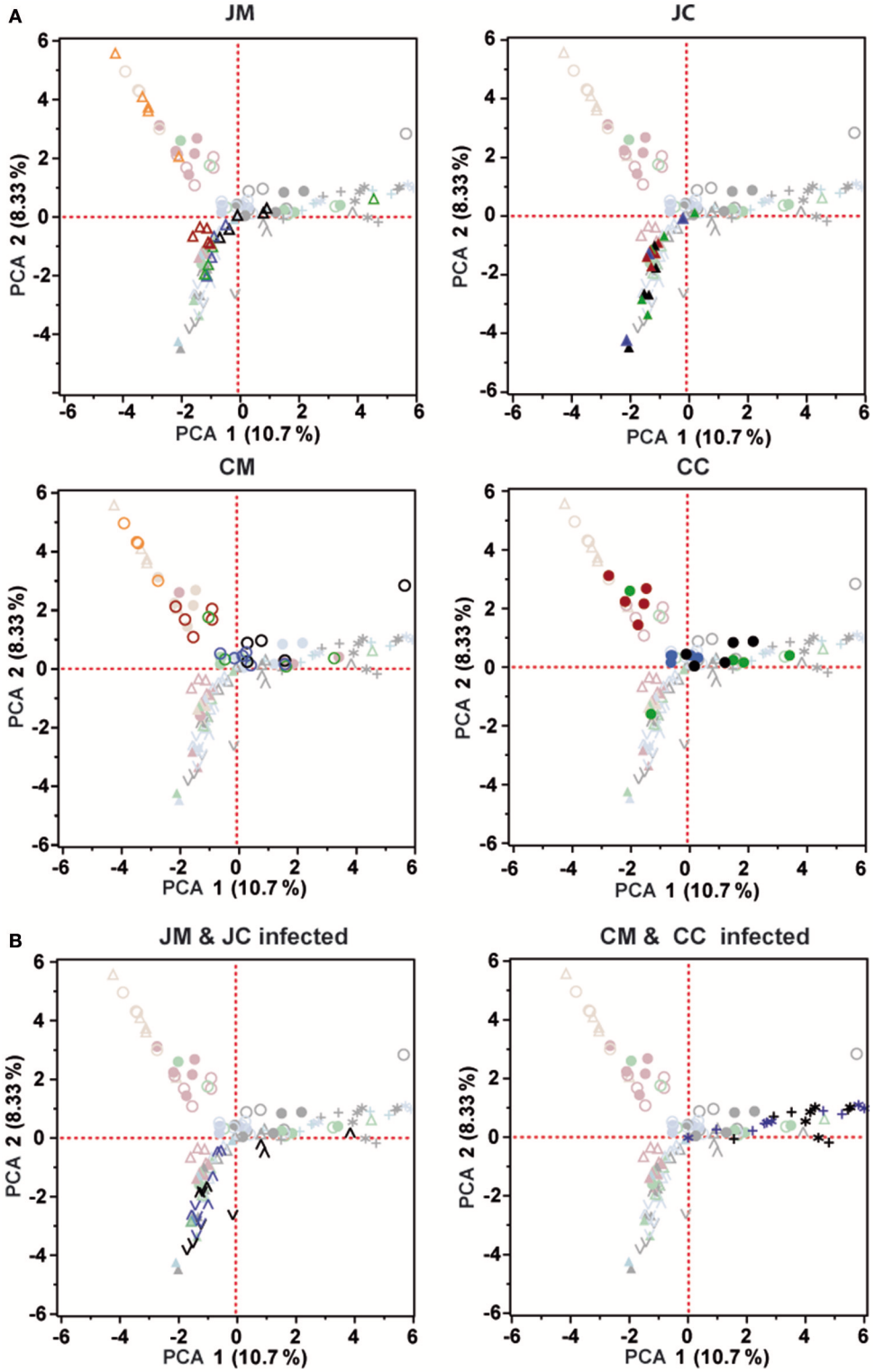

FIGURE 6 | Principal component analysis (PCA) was analyzed for the control (A) and infected birds **(B)**. Orange (day 1), Red (day 7), Green (day 14), Blue (day 21), and Black (day 28). Each symbol indicates an individual bird. All PCA plots include the data of all samples; symbols not belonging to the sample group indicated in the header are displayed faded. JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content.

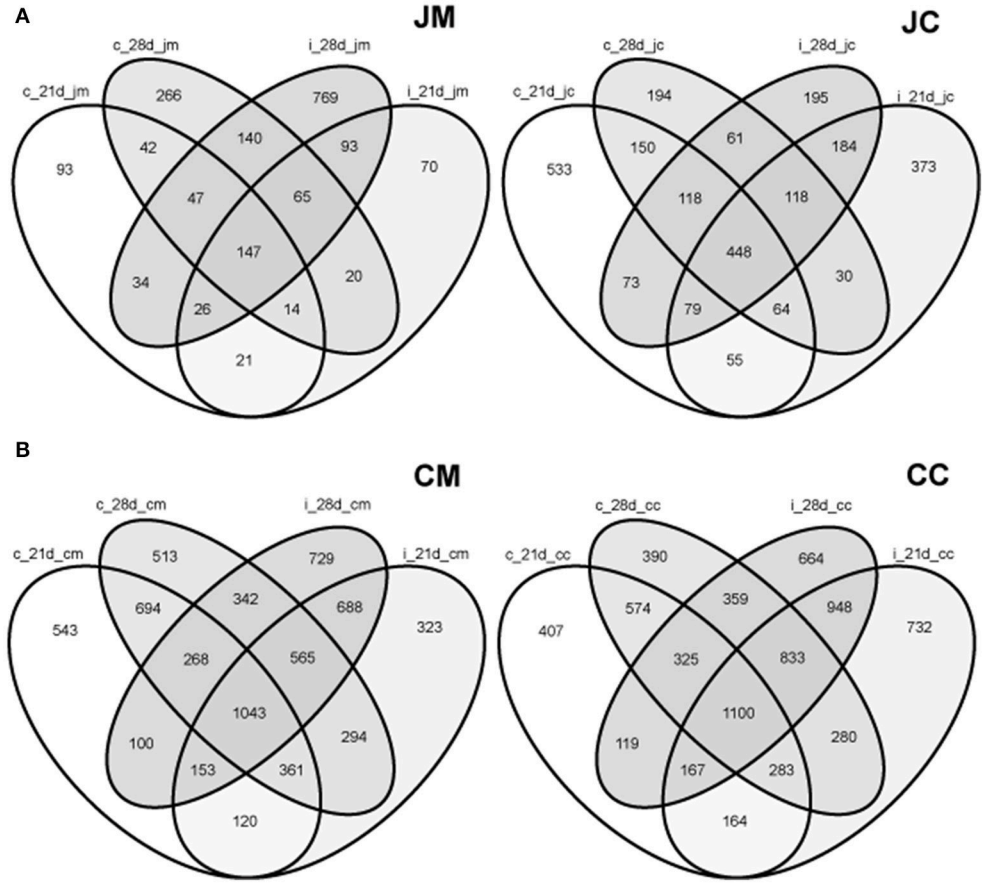

FIGURE 7 | Venn diagrams showing the shared OTUs between the control and infected birds at different gut sites at the two sampling points post infection (A) jejunum; **(B)** cecum. JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content; control (c); infected (i); d, day.

indirectly by influencing the immune system via production of antimicrobial substances (Sekirov et al., 2010). Development of the gut microbiota in chickens occurs immediately after hatching and by getting older, this microbiome becomes very diverse until it reaches a relatively stable dynamic state (Pan and Yu, 2014).

Interactions of the intestinal microbiome with the host and certain microorganisms have profound effects on bird health, and are therefore of great importance for poultry production. Consequently, in the present study, the composition of the gut microbiota of chickens in a longitudinal study from day 1 to day 28 of age was analyzed and the differences between content and mucosa-associated gut microbiota were investigated. In order to extend the range of analyses comparisons were performed between control chickens and chickens infected at 14 days of age with *C. jejuni*.

In this study, a high diversity of phyla (15 in the jejunal and 4 in the cecal mucosal samples) was found at day 1 of life, indicating a rapid intake of environmental organisms after birth. In addition, the composition of the gut microbiota differed substantially between young and older birds, with *Proteobacteria* being significantly more present at the first day of life and decreasing thereafter, whereas the *Firmicutes* were the predominant phylum in older birds. This is in agreement with Lu et al. (2003) who found that the gut is firstly colonized by the phylum *Proteobacteria*, particularly by the family *Enterobacteriaceae*. In older birds, the phylum *Firmicutes* mainly represented by *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, and *Lactobacillaceae* dominated. As a consequence, the chicken gut is firstly colonized by facultative aerobes which are substituted later on by anaerobes. Obviously, oxygen consumption by the aerobic bacteria alters the gut ecosystem toward more reducing conditions, which facilitates subsequent growth and colonization of the obligate anaerobes (Wise and Siragusa, 2007).

Besides *Proteobacteria* and *Firmicutes,* also lower abundant phyla (e.g., *Actinobacteria and Tenericutes*) changed significantly with time, indicating high dynamics in the re-organization of the whole microbiome through time. Taken together, the present study revealed that the chicken gut is largely dominated by the phyla *Proteobacteria* and *Firmicutes*, with lower proportions of *Actinobacteria*, *Bacteroidetes*, and *Tenericutes.* Similarly, previous studies have also shown that *Firmicutes*, *Bacterioidetes*, and *Proteobacteria* are the most common phyla in the chicken ceca (Wei et al., 2013; Oakley et al., 2014; Sergeant et al., 2014).

Interestingly, jejunal, and cecal microbiota were found to be distinct and certain acid-tolerant bacteria, mostly *Acidobacteria,* were present in the jejunum only. Altogether, the results demonstrated that the abundance of bacteria varied between the jejunum and the cecum, with some species more present in the jejunum (e.g., *Acinetobacter* and *Acidobacteria*) and others (e.g., *Bacteroides* and *Clostridium)* being predominant in the cecum of chickens. This and other variations can be explained by the fact that feed passes quickly through the foregut and is retained for hours in the hindgut. In addition, the small intestine is mainly responsible for food digestion and absorption, while the large intestine, especially the cecum, is responsible for microbial fermentation, further nutrient absorption and detoxification of substances that are harmful to the host (Gong et al., 2002).

Chickens investigated in the current study had a high abundance of *E. coli* and *E. faecalis* (best type strain hits) in the first week of life which might potentially increase their resistance to other bacterial infections. *E. coli*, a facultative anaerobe bacterium, was the dominant species in the early life of chickens. Thus, a depletion of *E. coli* during the second week of life could potentially affect the host susceptibility to enteric pathogen infections, representing a key role for these gut microbiota in host resistance. This decrease in *E. coli* abundance has been attributed with a beginning dominance of anaerobes (Zhu and Joerger, 2003). It may be possible that such disturbances in the community structure allow a pathogen to colonize and proliferate. Anyhow, it remains hypothetical whether these diversity changes influence the susceptibility to pathogens and the outcome of infection.

The current results revealed that *E. coli, E. faecalis, C. paraputrificum*, and *C. sartagoforme* (best type strain hits) were more predominant in the mucosa than in the lumen, suggesting significant implications for birds' health, considering that the mucosa-associated bacteria are of great importance in the host mucosal responses with consequences for the mucosal barrier (Ott et al., 2004).

Despite the high prevalence of *Campylobacter* in chickens the mechanism of colonization in the gut is still poorly understood. The high bacterial load in the gut and the establishment of a latent infection characterized by continuous shedding indicates that *Campylobacter* in chickens can modify the microbiota composition. In the current study it could be shown that *Campylobacter* colonization shifted the two major phyla towards an enrichment of *Firmicutes* with concomitant reduction of *Proteobacteria*. Interestingly, a reverse correlation between *Firmicutes* and *Proteobacteria* was observed, suggesting a possible antagonistic interaction between these two phyla. According to Pan and Yu (2014) alterations in one phyla or species may not only affect the host directly, but can also disrupt the entire microbial community. Notably, bacterial taxa belonging to the phyla *Firmicutes* are known to be involved in the degradation of complex carbohydrates (not absorbed by the host) and in the production of SCFAs (Thibodeau et al., 2015). Thus, the SCFAs production by *Firmicutes* might, at least partially, explain their dominance in the infected birds, which have a high SCFAs requirement as a source of energy for *C. jejuni* to colonize the chicken gut. Furthermore, Brown et al. (2012) reported that members of the phylum *Firmicutes* can inhibit the growth of opportunistic pathogens, such as *E. coli,* which has also been shown in the present study.

Besides these major shifts, also low abundant phyla (e.g., *Actinobacteria* and *Tenericutes*) were affected by the *Campylobacter* infection, which could also disequilibrate the microbiome composition. Similarly, Johansen et al. (2006) found in a denaturing gradient gel electrophoresis (DGGE) based experiment that *C. jejuni* colonization affected the development and complexity of the microbial communities of the ceca over 17 days of age. Furthermore, Qu et al. (2008) noted that the community structure of the cecal microbiome from the *C. jejuni* challenged chicken has greater diversity and evenness with a higher abundance of *Firmicutes* at the expense of the *Bacteroidetes* and other taxa. Sofka et al. (2015) also reported that *Campylobacter* carriage, assessed in samples from slaughter houses, was associated with moderate modulations of the cecal microbiome as revealed by an increase in *Streptococcus* and *Blautia* relative abundance in birds of 56 days of age, originating from different farms and production types. Recently, Thibodeau et al. (2015) found also that *C. jejuni* colonization induced a moderate alteration of the chicken cecal microbiome beta-diversity at 35 days of age.

This study's results strongly suggest that the *Campylobacter* associated alterations of the gut microbiota were a direct effect due to the interaction of *C. jejuni* with the microbiota or a consequence of the host responses or even a combination of both (Barman et al., 2008; Mon et al., 2015). The obtained results indicate that the influence of a *Campylobacter* infection on microbial communities was more pronounced at 14 dpi than at 7 dpi. This could be explained by an increased load of *Camplyobacter* at the later time point as demonstrated in recent studies using the same *C. jejuni* strain (Awad et al., 2014, 2015a,b, 2016).

We also found significant differences in the abundance of certain bacterial species in the infected birds compared with the controls. *C. jejuni* caused a significant decrease in *E. coli* (best type strain hit) in the microbiota of infected birds in both jejunum and cecum. This is in agreement with our previous study which showed that *Campylobacter* colonization decreased *E. coli* loads in the jejunum and cecum at 7 dpi and at 14 dpi, but increased *E. coli* translocation to the liver and spleen of the infected birds as determined by conventional bacteriology (Awad et al., 2016). Thus, the current results pointed out that the relative abundance of *E. coli* could be an important determinant of susceptibility for a *Campylobacter* infection in particular and Gram-negative pathogens in general.

In contrast to the *Campylobacter -E. coli interaction*, it was found that the relative abundance of *Clostridium* spp. was higher in the infected birds compared with the negative controls, indicating a link between *C. jejuni* and *Clostridium*. This confirms data from an earlier study in which a positive correlation between high levels of *Clostridium*

perfringens (>6 log) and the colonization of *C. jejuni* were found by real-time quantitative PCR (Skånseng et al., 2006; Thibodeau et al., 2015). This might be due to the fact that *C. jejuni* acts as a hydrogen sink leading to improved growth conditions for some *Clostridia* through increased fermentation (Kaakoush et al., 2014). This link can also be explained by the fact that the *Clostridium* organic acid production could be used by *C. jejuni* as an energy source. Furthermore, it was found that a *Campylobacter* infection induces excess mucous production in the intestine (Molnár et al., 2015) which consequently may enhance *Clostridium* proliferation due to the fact that an increase in mucin secretion in the gut provides an opportunity for *Clostridium* spp. to proliferate (M'Sadeq et al., 2015). Overall, the higher abundance of *Campylobacter* and *Clostridium* spp. might result in a higher endotoxin production with subsequent increase in intestinal permeability that facilitates the colonization and enhances bacterial translocation from the intestine to the internal organs, which is well in agreement with our pervious results (Awad et al., 2015a, 2016).

Finally, the strong shifts in the bacterial microbiome in the current study might help to explain why a *Campylobacter* infection is age dependent and chickens in the field become mainly colonized at an age of two to 4 weeks (Newell and Fearnley, 2003; Conlan et al., 2007). In agreement with this, Bereswill et al. (2011) demonstrated that a shift of intestinal microbiota in humans was linked with an increased susceptibility for *C. jejuni*. Finally, Haag et al. (2012) demonstrated that *C. jejuni* colonization in mice depends on the microbiota of the host and *vice versa* and *Campylobacter* colonization induces a shift of the intestinal microbiota. This was also observed in the present study as community structures were more dissimilar at the OTUs level in the infected birds compared with the controls. Moreover, in the infected birds, the population of beneficial microbes, such as *E. coli* and *E. desmolans* were comparatively lower than the potentially pathogenic bacteria, such as *Clostridium* spp., rendering the need for modulation of the gut microbiota to improve the gut health of the infected birds.

CONCLUSION

In the current study a substantial change in the composition of luminal and mucosa-associated gut microbiota in broiler chickens from day 1–28 was noticed. It could also be demonstrated that a *C. jejuni* infection in chickens was associated with significant changes in the composition of the intestinal ecosystem. Furthermore, these changes of the gut microbiota could lead to intestinal dysfunction, which has been evidenced in our previous studies. In this context, the results provide new insights into the microecological divergence of the intestinal microbiota with and without a *Campylobacter* infection and illustrate the *C. jejuni*–host crosstalk within the gut of broiler chickens. Understanding the relationship between disruption of the normal gut microbiota and *Campylobacter* infection may lead to improve in control strategies in order to minimize the consequences for the chicken host and the risk of bacterial spread to humans.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2016.00154/full#supplementary-material

Figure S1 | Relative abundances (%) of the most abundant bacterial phyla of (A) control and **(B)** infected birds. Data are presented as the mean values and SD. Left Y-axis for *Firmicutes, Proteobacteria* and others (unclassified); Right Y-axis for *Tenericutes, Acidobacteria, Actinobacteria, Bacteroides,* and *Cyanobacteria.* JM, jejunal mucosa; JC, jejunal content; CM, cecum mucosa; CC, cecum content.

Figure S2 | Heatmap showing the relative abundances of the 50 most-abundant OTUs sorted by gut sites and age in the control birds. The heat map shows relative abundance of a given phylotype. Colour scaling is ranged from 0 to higher than 25%. n.d, not detected; n.a, not analyzed.

Figure S3 | Venn diagrams are showing the shared OTUs for the control at different gut sites from day 1–28 (A) jejunum; **(B)** cecum. jm, jejunal mucosa; jc, jejunal content; cm, cecum mucosa; cc, cecum content; (c), control; (i), infected; d, day.

Table S1 | Relative abundances (%) of bacterial phyla in the control birds from day 1–28 (A) jejunal mucosa, **(B)** jejunal content, **(C)** cecal mucosa, **(D)** cecal content.

Table S2 | Relative abundances (%) of bacterial phyla in different gut sites of control birds (day 1–28).

Table S3 | Relative abundances (%) of bacterial phyla in different gut sites of infected birds (days 21 and 28).

Table S4 | Relative abundances (%) of bacterial phyla in the infected birds at the two sampling points post infection (A) jejunal mucosa, **(B)** jejunal content, **(C)** cecal mucosa, **(D)** cecal content.

Table S5 | The 50 most abundant OTUs in the control birds from day 1–28 (A) jejunal mucosa, **(B)** jejunal content, **(C)** cecal mucosa, **(D)** cecal content.

Table S6 | The 50 most abundant OTUs in the infected birds at the two sampling points post infection (A) jejunal mucosa, **(B)** jejunal content, **(C)** cecal mucosa, and **(D)** cecal content.

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Lack of Evidence That Selenium-Yeast Improves Chicken Health and Modulates the Caecal Microbiota in the Context of Colonization by *Campylobacter jejuni*

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Faced with ever-increasing demand, the industrial production of food animals is under pressure to increase its production. In order to keep productivity, quality, and safety standards up while reducing the use of antibiotics, farmers are seeking new feed additives. In chicken production, one of these additives is selenium. This element is expected to confer some advantages in terms of animal health and productivity, but its impact on chicken intestinal microbiota as well as on the carriage of foodborne pathogens is unknown. In this study, chickens raised in a level 2 animal facility were fed or not 0.3 ppm of in-feed selenium-yeast until 35 days of age and were inoculated or not with the foodborne pathogen *Campylobacter jejuni* at the age of 14 days. At the end of the study, body weight, seric IgY, intestinal IgA, seric gluthatione peroxydase activity, the caecal microbiota (analyzed by MiSeq 16S rRNA gene sequencing), and *C. jejuni* caecal levels were analyzed. The experiment was completely replicated twice, with two independent batches of chickens. This study revealed that, for healthy chickens raised in very good hygienic conditions, selenium-yeast does not influence the bird's body weight and lowers their seric gluthatione peroxidase activity as well as their intestinal IgA concentrations. Furthermore, selenium-yeast did not modify the caecal microbiota or the colonization of *C. jejuni*. The results also showed that *C. jejuni* colonization does not impact any of the measured chicken health parameters and only slightly impacts the caecal microbiota. This study also clearly illustrated the need for true biological replication (independent animal trials) when assessing the microbiota shifts associated with treatments as the chickens microbiotas clearly clustered according to study replicate.

Keywords: chicken, selenium, *Campylobacter jejuni*, microbiota, health

INTRODUCTION

Worldwide, the demand for food is increasing at an exponential rate (National Academy of Science, 2015), putting an ever-increasing pressure on meat production. In Canada, compared to 1991, beef and pork consumption *per capita* is declining while chicken consumption is increasing (Agriculture and Agrifood Canada [AAFC], 2016). Chicken meat production is following the same trend; in Canada, 610 million live birds were produced in 2005 compared to 661 million in 2015 (Agriculture and Agrifood Canada [AAFC], 2016). This increased production is challenging the food production system. In response to these challenges, the "One Health" approach – which recognizes and puts emphasis on the direct link between production animal health and public health – is gaining momentum in animal husbandry. For example, in Canada, the on-farm use of antibiotics is becoming more controlled in an effort to promote the judicious use of antibiotics (Gazette du Canada, 2016) and surveillance of foodborne pathogens is being tightened (Canadian Food Inspection Agency [CFIA], 2016a). Therefore, to continue the production of high-quality chicken proteins while ensuring the lowest possible contamination of products by foodborne pathogens, producers must adapt their rearing practices. One avenue is to modify animal feed composition by the inclusion of selected feed additives aimed at increasing the health of chicken flocks.

One of these feed additives is selenium. Selenium can be added to chicken feed in the organic or inorganic form (Zoidis et al., 2014). It has been observed that selenium in-feed supplementation increases carcass weight, oxidative stress response, immune response and it appears to be most effective in limiting morbidity when the broilers are exposed to disease or environmental stress (Zhou and Wang, 2011; Chen et al., 2014; Markovic et al., 2014; Boostani et al., 2015; Xu et al., 2015; Sun et al., 2016). One interesting form of selenium that is commercially available is yeast grown in a media enriched with selenium. This selenium-enriched yeast provides a source of organic selenium to chickens. Furthermore, yeast has been observed to be a good feed additive for chickens as they may act as a probiotic (Caly et al., 2015; Mountzouris et al., 2015; Nawaz et al., 2016), making selenium-yeast an interesting product. The impact of selenium on healthy chickens is not fully characterized while its potential impact on chicken intestinal health, in particularly the intestinal microbiota, remains poorly characterized.

The chicken microbiota is defined as the microorganism community that inhabits the chicken (Oakley et al., 2014). The caecal microbiota is becoming increasingly characterized and was observed to be modulated by a plethora of factors, ranging from feed composition to disease (Thibodeau et al., 2015b; Ajuwon, 2016; Antonissen et al., 2016). An optimal microbiota, conferring optimal health and growth, has yet to be defined in chickens, illustrating the need for deeper research in this field. One particularly interesting member of the chicken microbiota is one that often colonizes the chicken caecum and is a zoonotic foodborne pathogen for humans: *Campylobacter jejuni* (Chen and Jiang, 2014).

Campylobacter jejuni is the most common bacterial foodborne pathogen worldwide (Kirk et al., 2015). It colonizes the chicken caecum at very high loads and therefore easily contaminates chicken food products during processing (Meunier et al., 2016). Reducing chicken *C. jejuni* caecal carriage would lessen the health burden associated with this particular pathogen (Meunier et al., 2016). *C. jejuni* colonization of chickens was observed to be strain dependent and to be mediated by colonization genes that have yet to be completely identified (Thibodeau et al., 2015a). *C. jejuni* mainly uses amino acids and organic acids for its energy production (Hofreuter, 2014). It has been shown that *C. jejuni* colonization can be reduced by competitive exclusion caused by modifications to the chicken microbiota (Laisney et al., 2004). Food additives that could modify the chicken microbiota, such as selenium-yeast, are of particular interest in that context.

Consequently, the aim of this study was to assess the effect of selenium-yeast in-feed supplementation on broiler chicken health parameters and on the caecal microbiota, with a specific focus on *C. jejuni* colonization.

MATERIALS AND METHODS

In vivo Chicken Experimentations

All animal experimentations were approved by the ethics committee of the Faculty of Veterinary Medicine of the University of Montreal, certificate number 14-Rech-1730. Newly hatched broiler chickens (Ross 308) were purchased at a local hatchery and transported to the avian research center (level 2 confinement facility) of the veterinary medicine faculty. All chickens were vaccinated at the hatchery against Marek's disease and infectious bronchiolitis. The exact number of analyzed chickens per group per experiment is presented in each table (**Tables 1–7**). Chickens were placed in two different rooms: the chickens housed in the first room were inoculated with *C. jejuni* while the chickens housed in the second room were not to be inoculated with *C. jejuni*. The chickens housed in each room were further separated into two groups: one group received an in-feed supplementation of a selenium-yeast commercial preparation at 0.3 ppm and the other did not. This selenium-yeast concentration is the maximum supplementation allowed by the Canadian Food Inspection Agency (Canadian Food Inspection Agency [CFIA], 2016b). All chickens were fed a standard mash diet and had *ad libitum* access to water and feed.

At day 12, fresh caecal droppings were collected from each group to confirm the absence of *C. jejuni* colonization. To differentiate the effect of selenium-yeast from the eventual effect of *C. jejuni* carriage on the chicken health parameters evaluated, at 14 days of age, one room was inoculated with an oral suspension of two deeply characterized *C. jejuni* strains (A2008a and G2008b) (Thibodeau et al., 2013, 2015a,b) while the other was not. The oral suspension was obtained from an overnight blood agar culture of each strain that was suspended in PBS phosphate buffered saline (PBS) to an optic density of 1.0 (at 630 nm) and further diluted to obtain a final concentration of 10^4 CFU per strain per inoculation.

TABLE 1 | Observed mean body weight (g) for chickens according to the use of selenium-yeast or the inoculation of *C. jejuni*.

Condition	In-feed selenium-yeast		*C. jejuni* inoculation	
	No additive	Selenium-yeast	*C. jejuni* neg	*C. jejuni* pos
Replicate 1	2190 (160.7)	2249 (111.9)	2202 (128.1)	2235 (151.1)
Replicate 2	2190 (221.7)	2248 (214)	2254 (202.5)	2186 (230)
Replicate 1+2	2190 (190.3)	2248 (168.6)	2248 (169.2)	2211 (192.8)

Standard deviation reported in parenthesis. Replicate 1+2: combined analysis of biological replicates. Chickens analyzed per group for Replicate 1: No additive/C. jejuni neg = 9, No additive/C. jejuni pos = 11, Selenium-yeast/C. jejuni neg = 10, Selenium-yeast/C. jejuni pos = 10. Chickens per group for Replicate 2: No additive/C. jejuni neg = 10, No additive/C. jejuni pos = 9, Selenium-yeast/C. jejuni neg = 9, Selenium-yeast/C. jejuni pos = 11.

TABLE 2 | Observed seric GPX activity (U/ml) for chickens according to the use of selenium-yeast or the inoculation of *C. jejuni*.

Condition	In-feed selenium-yeast		*C. jejuni* inoculation	
	No additive	Selenium-yeast	*C. jejuni* neg	*C. jejuni* pos
Replicate 1	1532 (996) ***a**	680 (523) ***b**	1275 (1194)	1073 (354)
Replicate 2	1264 (673) ***a**	825 (845) ***b**	1131 (750)	958 (830)
Replicate 1+2	1394 (843) ***a**	755 (701) ***b**	1202 (993)	949 (662)

*Standard deviation reported in parenthesis; On the same row, *a different from *b. Replicate 1+2: combined analysis of biological replicates. Chickens analyzed per group for Replicate 1: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8. Chickens per group for Replicate 2: No Additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8.*

At 35 days of age, chickens were weighed prior to being stunned by electronarcosis and euthanized by bleeding. On each animal, a 10 ml blood sample, a 10 cm segment of the ileum measured from the ileum-caecal junction, as well as the whole caecum were collected. All samples were sent on ice to the laboratory for immediate processing. The *in vivo* experiment was replicated once more with a distinct lot of birds.

Sample Treatment

Caecal matter was collected from the caecum. A 1 g portion was used for the enumeration of *C. jejuni* while another 1 g was flash-frozen in liquid nitrogen and kept at −80°C for DNA extraction (Thibodeau et al., 2015b).

Blood samples were kept 1 h at room temperature and then centrifuged at 100 × *g* for 15 min. The supernatant was collected and divided into two distinct samples that were kept at −20°C. One sample was used to determine the seric IgY concentrations and the other one was sent to the diagnostic laboratory of the Veterinary medicine faculty for the determination of the total glutathione peroxidase activity (GPX).

The 10 cm ileal segment was opened longitudinally and emptied of its contents with a gloved finger. A sterile microscopic glass slide was then used to scrape off the mucus which was resuspended in 10 ml of cold PBS. The mucus suspension was then kept at −20°C until used for the determination of the intestinal mucus IgA concentration.

Seric IgY and Intestinal IgA Levels

The concentration of the total seric IgY and intestinal IgA was assessed by ELISA using commercial kits from Bethyl laboratories (Bethyl Laboratories, Montgomery, AL, USA) for eight chickens per experimental group. Protocols were performed according to the manufacturer recommendations. Serum samples were used at a dilution of 1:50,000 while the intestinal samples were used at a dilution of 1:20. For IgY, the secondary antibody was used at a dilution of 1:20,000 while a dilution of 1:40,000 was used for the IgA.

DNA Extraction, Amplicon MiSeq Sequencing, and Bioinformatics

Total DNA was extracted from all the caecal samples kept at −80°C using a combination of a beads-beating lysis and phenol–chloroform purification as previously described (Thibodeau et al., 2015b). A sample without caecal matter was extracted at the same time as a negative control for use in the downstream molecular biology analysis. DNA concentration was assessed using the Qubit BR assay (Fisher Scientific, Ottawa, ON, Canada). The DNA samples were diluted to a concentration of 10 ng/μl, separated in aliquots, and kept at −20°C until use.

A survey of the chicken caecal microbiota was performed by amplifying and sequencing the V4 region of the 16S

TABLE 3 | Seric IgY concentrations (μg/ml) for chickens according to the use of selenium-yeast or the inoculation of *C. jejuni*.

Condition	In-feed selenium-yeast		*C. jejuni* inoculation	
	No additive	Selenium-yeast	*C. jejuni* neg	*C. jejuni* pos
Replicate 1	1.68 (0.80)	2.24 (0.92)	2.01 (1.01)	1.91 (0.79)
Replicate 2	1.59 (0.66)	1.59 (1.12)	1.28 (0.74)	1.90 (0.96)
Replicate 1+2	1.64 (0.72)	1.92 (1.06)	1.64 (0.95)	1.91 (0.87)

Standard deviation reported in parenthesis. Replicate 1+2: combined analysis of biological replicates. Chickens analyzed per group for Replicate 1: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8. Chickens per group for Replicate 2: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8.

TABLE 4 | Observed mean intestinal IgA concentrations (μg/ml) for chickens according to the use of selenium-yeast or the inoculation of *C. jejuni*.

Condition	In-feed selenium-yeast		*C. jejuni* inoculation	
	No additive	Selenium-yeast	*C. jejuni* neg	*C. jejuni* pos
Replicate 1	3.83 (2.39)	3.19 (1.36)	2.96 (1.39)	4.06 (2.28)
Replicate 2	7.10 (5.83) ***a**	3.49 (2.27) ***b**	3.55 (3.0) ***c**	7.03 (5.54) ***d**
Replicate 1+2	5.47 (4.69) ***a**	3.34 (1.85) ***b**	3.26 (2.32) ***c**	5.54 (4.43) ***d**

*Standard deviation reported in parenthesis; On the same row, *a different from *b, *c different from *d. Replicate 1+2: combined analysis of biological replicates. Chickens analyzed per group for Replicate 1: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8. Chickens per group for Replicate 2: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8.*

TABLE 5 | Comparison of identified differences in diversity metrics regarding the use of selenium-yeast, *C. jejuni* inoculation or the study's replicate.

Replicate	Replicate 1				Replicate 2			
Condition	Selenium-yeast		*C. jejuni*		Selenium-yeast		*C. jejuni*	
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Coverage	0.98 (0.01)	0.98 (0.01)	0.98 (0.01)	0.98 (0.01)	0.986 (0.005) **a**	0.982 (0.006) **b**	0.97 (0.01)	0.97 (0.01)
Sobs	394 (100)	498 (149)	454 (153)	415 (106)	604 (201) **a**	755 (231) **b**	691 (257)	684 (208)
Simpson inverse	16.4 (2.6)	15.4 (3.5)	15.6 (3.3)	16.3 (2.8)	14.4 (4.5)	16.2 (6.4)	15.2 (4.8)	15.6 (6.4)
Shannon	3.5 (0.1)	3.5 (0.2)	3.5 (0.2)	3.5 (0.1)	3.5 (0.2)	3.7 (0.3)	3.6 (0.3)	3.6 (0.2)
Shannon even	0.59 (0.03)	0.58 (0.03)	0.58 (0.06)	0.58 (0.06)	0.60 (0.04)	0.60 (0.04)	0.56 (0.04)	0.56 (0.03)

*Condition's effect on the metric for selenium and C. jejuni inoculation; On the same row, *a different from *b; Neg: negative control, Pos: positive; standard deviation in parenthesis. Chickens analyzed per group for Replicate 1: No additive/C. jejuni neg = 8, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 7. Chickens per group for Replicate 2: No additive/C. jejuni neg = 5, No additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8.*

rRNA gene from the DNA extracted from the chicken caecal samples, according to Illumina's "16S Metagenomic Sequencing Library Preparation" guide (Part # 1504423 Rev. B). In each experimental group, the DNA extracted from the ceacum of eight chickens was used. The 16S rRNA gene PCR mastermix (25 μl final volume per reaction) consisted of 1x KAPA HiFi HotStart ReadyMix (Kappa Biosystems, Willington, MA, USA), 600 nM of each primer (Caporaso et al., 2012), 0.4 mg/ml BSA, and 12.5 ng of DNA. The following PCR cycling conditions were used: initial denaturation at 95°C for 5 min followed by 25 cycles consisting of a 30 s denaturation at 95°C, a 30 s annealing at 55°C, and an elongation of 180 s at 72°C that ended with a final elongation of 10 min at 72°C. The amplicons were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer protocol.

Purified amplicons were barcoded using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) using an eight cycles PCR: initial denaturation at 95°C for 3 min, followed by cycles consisting of a 30 s denaturation at 95°C, a 30 s annealing at 55°C, and an elongation of 30 s at 72°C, and then by a final elongation of 5 min at 72°C. Reactions consisted of 1x KAPA HiFi HotStart ReadyMix (Kappa Biosystems), 2.5 μl of each index, and 5 μl of the purified 16S rRNA gene amplicons. The index PCR was also purified using AMPure XP beads (Beckman Coulter) according to the manufacturer protocol. The purified indexed amplicons were quantified with Qubit HS kit (Fisher Scientific) and diluted to 2 ng/μl. Five microliters of each index PCR was pooled in a

TABLE 6 | Common OTUs associated with *C. jejuni* inoculation in both replicates.

C. jejuni	OTU classification	LDA score Replicate 1	LDA score Replicate 2
Neg	*B(100);F*(100);*C*(100);*Cles*(100);*Lachno*(100);*Eisenbergiella*(92)	4.2	4.2
	B(100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100);*Tyzzerella_3*(100)	2.9	2.6
	B(100);*F*(100);*C*(100);*Cles*(100);*Lachno*(99);*[Eubacterium]_hallii_group*(84)	3.7	3.8
	B(100);*F*(100);*C*(100);*Cles*(100);*Ruminoc*(100)	3.3	3.0
	B(100);*F*(100);*C*(100);*Cles*(100);*Ruminoc*(100);*Ruminoc_UCG-014*(100)	3.8	3.7
	B(100);*F*(100);*C*(100);*Cles*(100);*Ruminoc*(99)	4.1	3.8
	B(100);*F*(100);*C*(100);*Cles*(100);*Ruminoc(99)Anaerotruncus*(98)	2.7	2.6
Pos	*B*(100);*F*(100);*C*(100);*Cles*(100);*Cles_vadinBB60_group*(100)	2.9	3.0
	B(100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100)	4.1	4.4
	B(100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100)	3.0	3.4
	B(100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100)	3.1	2.8
	B (100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100)	3.1	2.5
	B (100);*F*(100);*C*(100);*Cles*(100);*Lachno*(100)	2.3	2.8
	B (100);*F*(100);*C*(100);*Cles*(100);*Ruminoc*(100);*Anaerotruncus*(98)	4.0	3.5
	B (100);*F*(100);*C*(100);*Cles*(100);*Ruminoc*(100);*Oscillibacter*(99)	3.4	4.0
	B(100);*F* (100);*C*(100);*Cles*(100);*Ruminoc*(100);*Ruminoc_UCG-14*(100)	2.0	2.1
	B(100);*ProteoB*(100);*EpsilonproteoB*(100);*Campyr*(100);*Campylo*(100);*Campylobacter*(100)	4.5	3.5

The last returned identification for common OTUs is displayed; (): percentage of classification; OTU classification by increased alphabetical order; B = Bacteria; F = Firmicutes; C = Clostridia; Cles = Clostridiales; Lachno = Lachnospiraceae; Ruminoc = Ruminococcaceae, Campyr = Campylobacterales; Campylo = Campylobacteraceae; LDA: Linear Discriminant Analysis to estimate the effect size of each differentially abundant feature. Chickens analyzed per group for Replicate 1: No Additive/C. jejuni neg = 8, No Additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 7. Chickens analyzed per group for Replicate 2: No Additive/C. jejuni neg = 5, No Additive/C. jejuni pos = 8, Selenium-yeast/C. jejuni neg = 8, Selenium-yeast/C. jejuni pos = 8.

single tube and sent to NRC Montréal for MiSeq Sequencing, using the Miseq reagent 500 V2 kit (Illumina) for a 2x 250 bp length, as specified by Illumina.

Raw demultiplexed reads were received from the sequencing center and processed using Mothur version 1.38 (Schloss et al., 2009), following the online MiSeq SOP[1]. Prior to OTU clustering, the negative control sample and some samples containing too few or suspicious reads were removed.

To determine OTUs, reads were clustered using Vsearch with the AGC method at the 0.03 level. From this point on, results were analyzed separately for birds raised during the first and second replicate experiment. Reads were aligned and classified using the Silva database (version 123).

For diversity analysis, reads were subsampled or rarefied to the lowest number of reads found in a single sample. The following alpha-diversity indices were computed and compared across conditions: coverage, Sobs, Inverse Simpson, Shannon, and Shannon's evenness. Beta-diversity analysis was performed by comparing the bird's microbiota structure using Yue and Clayton diversity index and analyzed by AMOVA and HOMOVA. The composition was analyzed by LDA effect size (LEFSE) (Segata et al., 2011) according to the experimental conditions. The bird's microbiota compositions were also compared by LEFSE using the phylotype approach. For LEFSE, only significant OTU with a LDA score over 2 were reported. Raw reads for each chicken caecal microbiota analyzed in this study are available through the NCBI SRA database under accession SRP094491.

C. jejuni Enumeration and PCR

For all chickens, a 1 g of fresh caecal matter was homogenized in 9 ml of a tryptone-salt solution composed of 0.1% (w/v) tryptone (LabM, Heywood, UK) and 0.85% (w/v) NaCl (Fisher Scientific). For the birds that were not inoculated with *C. jejuni*, 100 µl of this suspension was plated on mCCDA (LabM) and immediately incubated at 42°C in a microaerobic atmosphere using chemical gas pack generators (Oxoïd, Ottawa, ON, Canada) (Macé et al., 2015). This protocol was also used for confirming the absence of *C. jejuni* in all birds at day 12.

For the *C. jejuni* inoculated birds, the caecal suspensions were diluted up to 10^6 and the last four dilutions were plated on mCCDA (LabM) and immediately incubated at 42°C in a microaerobic atmosphere (Oxoïd). The positive control used for monitoring the adequate *C. jejuni* growth was *C. jejuni* strain ATCC 33291. After 48 h of incubation, typical colonies were enumerated and the results were log 10 transformed to assess the effect of selenium-yeast supplementation on the chicken *C. jejuni* carriage.

To corroborate the colonization status determined by culture, all culture negative and culture positive *C. jejuni* samples were confirmed by PCR (Yamazaki-Matsune et al., 2007). The PCR mix (25 µl) was composed of primers (C412F at 200 nM, C1228R at 200 nM, C-1 at 800 nM, and C-3 at 800 nM), 1 unit of Taq DNA polymerase (Bio Basic, Markham, ON, Canada), $MgSO_4$ at 2 mM, and dNTPs at 200 mM. PCR amplicons were visualized on a 1% agarose (Fisher Scientific) gel stained with Sybrsafe (Fisher Scientific). Positive control consisted of DNA extracted from *C. jejuni* strain ATCC 33291 while the negative control contained no DNA.

[1]https://www.mothur.org/wiki/MiSeq_SOP

Statistical Analysis

Comparison of the chicken's body weight, *C. jejuni* colonization levels, seric total GPX activity, seric IgY concentrations, intestinal IgA concentrations, and alpha-diversity indices were analyzed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Prior to selecting the correct statistical analysis, the distribution of the data was inspected. When the normality was confirmed, data were analyzed using parametric tests. Otherwise, non-parametric analyses were conducted. An alpha of 0.05 was set to assess significance. Results, with the exception of the microbiota, were analyzed separately for the first or second replicate according to the selenium-yeast or *C. jejuni* status before being pooled for the analysis of the global effect observed in this study.

RESULTS

Chicken Body Weight

After 35 days of growth, no significant difference ($p > 0.05$) was observed in regards to the chicken final body weight, though the use of selenium-yeast consistently yielded chickens with higher mean body weight values in both replicates (**Table 1**).

Seric GPX Levels

Seric GPX levels were observed to be significantly lower ($p < 0.05$) for the chickens fed selenium-yeast (**Table 2**). No significant difference ($p > 0.05$) was observed for chickens inoculated with *C. jejuni*, although consistent lower mean values for the inoculated chickens were observed. A large inter-individual variation was also observed, as exemplified by the high standard deviation values.

Immunoglobuline Concentrations

No significant difference ($p > 0.05$) was measured regarding total seric IgY concentrations (**Table 3**). For the intestinal IgA recovered from the ileal mucus layer (**Table 4**), significantly lower concentrations were observed in selenium-yeast supplemented chickens, but only for the second experimental replicate ($p < 0.05$). Similarly, IgA concentrations were significantly lower in chickens not inoculated with *C. jejuni* ($p < 0.05$), but only for the second experimental replicate.

Microbiota

A total of 8,438,914 sequences were obtained after assembly. Prior to OTU clustering, a total of 4,978,059 sequences remained, representing 308,896 unique sequences. The two negative controls included in this study contained 260 and 427 sequences. Two chicken caecal samples returned numbers of sequences similar to the negative controls (328 and 348) and were discarded from the analysis. Two more samples, originating from chickens not inoculated with *C. jejuni,* returned unexpectedly high numbers of reads classified as *Campylobacter*, in similar

proportions to all other samples originating from inoculated birds. Further PCR analyses confirmed the samples negative for *C. jejuni* while all *C. jejuni* inoculated birds returned a strong PCR signal (data not shown); these samples were removed from the analysis. After this, the lowest number of sequences in a chicken sample was 14,482 while the highest was 161,901.

Alpha-diversity indices (**Table 5**) were first compared based on the use of selenium-yeast in the feed or the inoculation with *C. jejuni*. Coverage over 97% was observed in all treatments. In experimental replicate 2, the use of yeast-selenium significantly increased ($P < 0.05$) the coverage and richness (Sobs) of the bird's microbiota but this was not observed in experimental replicate 1. Richness (Sobs) was significantly different between the two experimental replicates regardless of the experimental condition ($p < 0.001$), with mean values of 435 ($\pm$130) and 687 ($\pm$225) for experimental replicate 1 and 2, respectively. The observed mean Shannon indices (3.50 ± 0.15 and 3.62 ± 0.25 for experimental replicates 1 and 2, respectively) and Shannon Evenness indices (0.58 ± 0.03 and 0.56 ± 0.03 for experimental replicates 1 and 2, respectively) were also significantly different ($p = 0.001$) between replicates.

The difference in the microbiota structure according to the experimental conditions was investigated. In both replicates, selenium-yeast supplementation (**Figure 1**) did not influence the microbiota structure, while the inoculation with *C. jejuni* (**Figure 2**) did influence the caecum bacterial community. When analyzing all chickens together, the bacterial community structure was significantly different between the experimental replicates (AMOVA $p < 0.001$, HOMOVA $p = 0.2$).

The LEFSE analysis identified OTUs that were consistently associated with the inoculation of *C. jejuni* or the use of selenium-yeast. When using *C. jejuni* inoculation as a class and selenium-yeast as a subclass, 75 OTUs were identified in replicate 1 and 71 OTUs in replicate 2. Of these, only 17 OTUs were consistently associated in both replicates (**Table 6**). On the contrary, when using selenium-yeast as the class and the inoculation with *C. jejuni* as a subclass, for replicate 1, five OTUs were identified compared to two OTUs in replicate 2. For selenium-yeast, no OTU were common to both replicates. All LEFSE results, broken down per replicate, are available in the Supplementary Material.

Using phylotype analysis with genera as the cutoff (mothur taxlevel = 1), 23 OTUs were found to be associated with *C. jejuni* inoculation for experimental replicates 1 and 2, and the six following OTUs were found for both replicates: unclassified_ *Ruminococcaceae*, *Eisenbergiella*, *Tyzzerella_3*, and *[Eubacterium]_hallii_group* were associated with the non-inoculated birds while *Lachnoclostridium* and *Campylobacter* were associated with the inoculated birds. For supplementation with selenium-yeast, two OTUs were identified in each of the experimental replicates, but none were shared. Apart from the obvious association of sequences from the *C. jejuni* lineage (*Proteobacteria*; *Epsilonproteobacteria; Campylobacterales*; *Campylobacteraceae*) with *C. jejuni* inoculated chickens, no other association with treatments were found when using different taxonomical levels (mothur taxlevel 2, 3, 4 or 5) in LEFSE analysis. All LEFSE phylotype results, broken down per replicate, are available in the Supplementary Material.

C. jejuni Colonization

In experimental replicate 1, the *C. jejuni* caecal concentrations were found to be slightly but significantly higher for the chicken supplemented with selenium-yeast (**Table 7**) ($p < 0.05$). The opposite effect was observed for experimental replicate 2. When all the birds from the two replicates were used together in the same analysis, no significant difference remained ($p > 0.05$). Confirmation of the presence of *C. jejuni* in the inoculated chickens was carried out by PCR and culture, which confirmed that all inoculated chickens were infected by *C. jejuni* while the all the non-inoculated chickens were not.

DISCUSSION

The aim of this study was to assess the impact of a selenium-yeast feed additive on some chicken health related parameters as well as on the caecal colonization of *C. jejuni*. Based on the observations made during this study, it cannot be concluded that the use of yeast-selenium positively modulated the parameters measured in healthy chickens.

Nevertheless, a non-significant trend of increased performances in terms of weight gain was observed in the present study, an observation also reported by some other studies (Jensen and Mc, 1960; Choct et al., 2004; Markovic et al., 2014; Suchy et al., 2014). The present study was conducted in a level 2 facility that allows precise control of the chickens' rearing environment, therefore reducing confounding factors and allowing the study of individual conditions. Raising chickens with high biosecurity clearly maximizes chicken growth, limiting the chances that a supplement will further increase the performance of chickens.

In this study, it was also observed that the use of selenium-yeast could lower slightly, but significantly seric GPX levels in both biological replicates as well as the intestinal IgAs in replicate 2 and when combining both replicate results. We also observed a high and unexpected variation between individual samples. This observation is in opposition with the current literature where the use of selenium is usually associated with increased levels of immunoglobulins and GPX, factors associated

TABLE 7 | Observed *C. jejuni* caecal colonization levels (log 10) for chickens according to the use of selenium-yeast.

Condition	In-feed selenium-yeast	
	No additive	Selenium-yeast
Replicate 1	7.1 (0.63) ***a**	7.8 (0.51) ***b**
Replicate 2	6.0 (0.71) ***a**	5.2 (0.60) ***b**
Replicate 1+2	6.57 (0.84)	6.43 (1.41)

*Standard deviation reported in parenthesis. On the same row, *a different from *b. Replicate 1+2: combined analysis of biological replicates. Chickens analyzed per group for Replicate 1: No additive/C. jejuni pos = 11, Selenium-yeast/C. jejuni pos = 10. Chickens analyzed per group for Replicate 1: No additive/C. jejuni pos = 9, Selenium-yeast/C. jejuni pos = 11.*

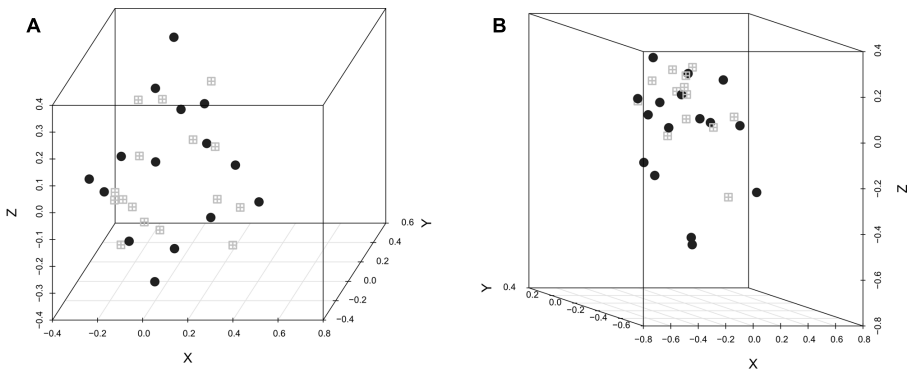

FIGURE 1 | NMDS graphic representation of caecal microbiota diversity according to the use of selenium-yeast or not. The gray windows represent the chickens that did not receive selenium-yeast while the black circles represent the chickens that did. **(A)** Replicate 1: AMOVA: $p > 0.05$; HOMOVA: $p > 0.05$; lowest stress: 0.091; R^2: 0.939. **(B)** Replicate 2, AMOVA: $p > 0.05$; HOMOVA: $p > 0.05$; lowest stress: 0.098; R^2: 0.958.

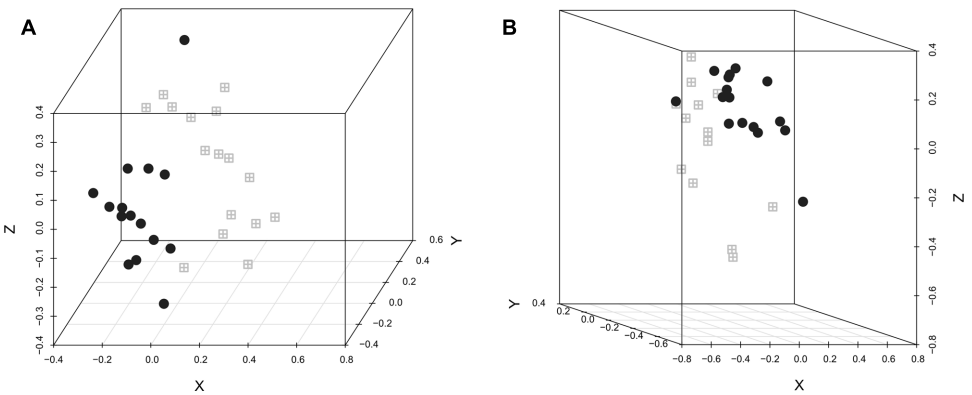

FIGURE 2 | NMDS graphic representation of caecal microbiota diversity according to *C. jejuni* inoculation or not. The gray windows represent the chickens that were not inoculated with *C. jejuni* while the black circles represent the chickens that were. **(A)** Replicate 1, AMOVA: p < 0.001; HOMOVA: p > 0.05; lowest stress: 0.091; R2: 0.939. **(B)** Replicate 2, AMOVA: p < 0.001; HOMOVA: p > 0.05; lowest stress: 0.098; R2: 0.958.

with healthy chickens (Chen et al., 2014; Placha et al., 2014; Boostani et al., 2015). Under our experimental settings, the exact mechanisms driving these unexpected results remained unidentified.

This study was also the first to look at the potential impact of selenium-yeast on the chicken microbiota. Despite the observation of some biological modifications due to selenium-yeast supplementation, no impact on both alpha and beta diversity was observed. This study was conducted under controlled conditions where the available bacteria that might contribute to the development of the chicken microbiota are expected to be limited and quite different from the ones field chickens could encounter. Our experiment should therefore be replicated in commercial settings to fully understand the effects of selenium-yeast supplementation of the chicken microbiota.

When using plate counts, no significant differences in *C. jejuni* colonization were observed when the selenium-yeast was used as a feed-additive, which is in agreement with the lack of modulation of the caecal microbiota observed in selenium-yeast supplemented chickens. Under experimental conditions less favorable to chicken health, selenium supplementation was reported to be beneficial for chicken (Xu et al., 2015). It has been observed that the competition for zinc within the chicken microbiota plays a role in the effective colonization of *C. jejuni* (Gielda and DiRita, 2012). It could therefore be interesting to monitor *C. jejuni* colonization under various levels of competition for selenium acquisition. Using different *C. jejuni* strains might also lead to different results since the impact of selenium on the overall *C. jejuni* population has yet to be fully characterized.

Campylobacter jejuni colonization of the caecal microbiota was confirmed here to slightly reorganize the caecal microbiota, in keeping with past results obtained using the same *C. jejuni* strains and with birds raised within similar parameters (Thibodeau et al., 2015b). However, when analyzing the shift in microbiota composition associated with *C. jejuni* colonization, different results were obtained. In our previous study, *C. jejuni* colonization was associated with changes in the relative abundance of the genera *Streptococcus*, *Blautia*, *Anaerofilum*, *Faecalibacterium*, *Clostridium*, *Coprobacillus,* and *Anaeroplasma,* which was not the case in the current study.

The few studies that evaluated the impact of *C. jejuni* colonization on the chicken caecal microbiota all showed that the microbiota structure is somewhat affected, but different conclusions were reached when comparing microbiota compositional changes (Johansen et al., 2006; Sofka et al., 2015; Saint-Cyr et al., 2016). This might be due to the use of different DNA extraction methods, different 16S rRNA gene regions being sequenced, and different bioinformatics pipelines used to process raw sequences (de la Cuesta-Zuluaga and Escobar, 2016). Even when taking in account technical discrepancies and the differences observed between replicates in the present study, these results, when taken together, clearly indicate that the existing chicken caecal microbiota reacts somewhat to the presence of *C. jejuni,* regardless of the initial microbiota of the chicken. This indicates a potential commensal lifestyle for *C. jejuni* that would act as a super colonizer. This strongly suggests that the on-farm control of *C. jejuni* via modification of the caecal microbiota only is a titan's task, which is reflected in the lack of recent significant advances in the control of the colonization of chicken by *C. jejuni* using feed additives.

CONCLUSION

We observed here that selenium-yeast supplementation modified, but did not improve the general health of broiler chickens at slaughter age and that selenium-yeast supplementation could even be somewhat detrimental when chickens are raised in controlled conditions maximizing their health. These changes were not associated with any modification of the caecal microbiota. This suggests that the microbiota is not always linked to the animal's health parameters and that healthy animal do not exhibit a common and defined microbiota, highlighting the need for further studies to define a truly healthy microbiota. Moreover, the use of selenium-yeast supplementation did not modify the chicken colonization by *C. jejuni*. This study also confirmed that *C. jejuni* colonization can slightly modify the caecal microbiota, an observation in line with the potential commensal lifestyle of *C. jejuni*. This study also illustrated the importance of true biological replicates when studying the chicken intestinal microbiota, especially when the observed changes are subtle.

AUTHOR CONTRIBUTIONS

AT designed the experiments, did all experimentations, analyzed all results, discussed the results and wrote the manuscript. AL, EY, and PF designed the experiments, discussed the results and revised the manuscript. GL-G did some experimentations, discussed the results and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00451/full#supplementary-material

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