THE ROLE OF ENVIRONMENTAL RESERVOIRS IN CAMPYLOBACTER-MEDIATED INFECTION

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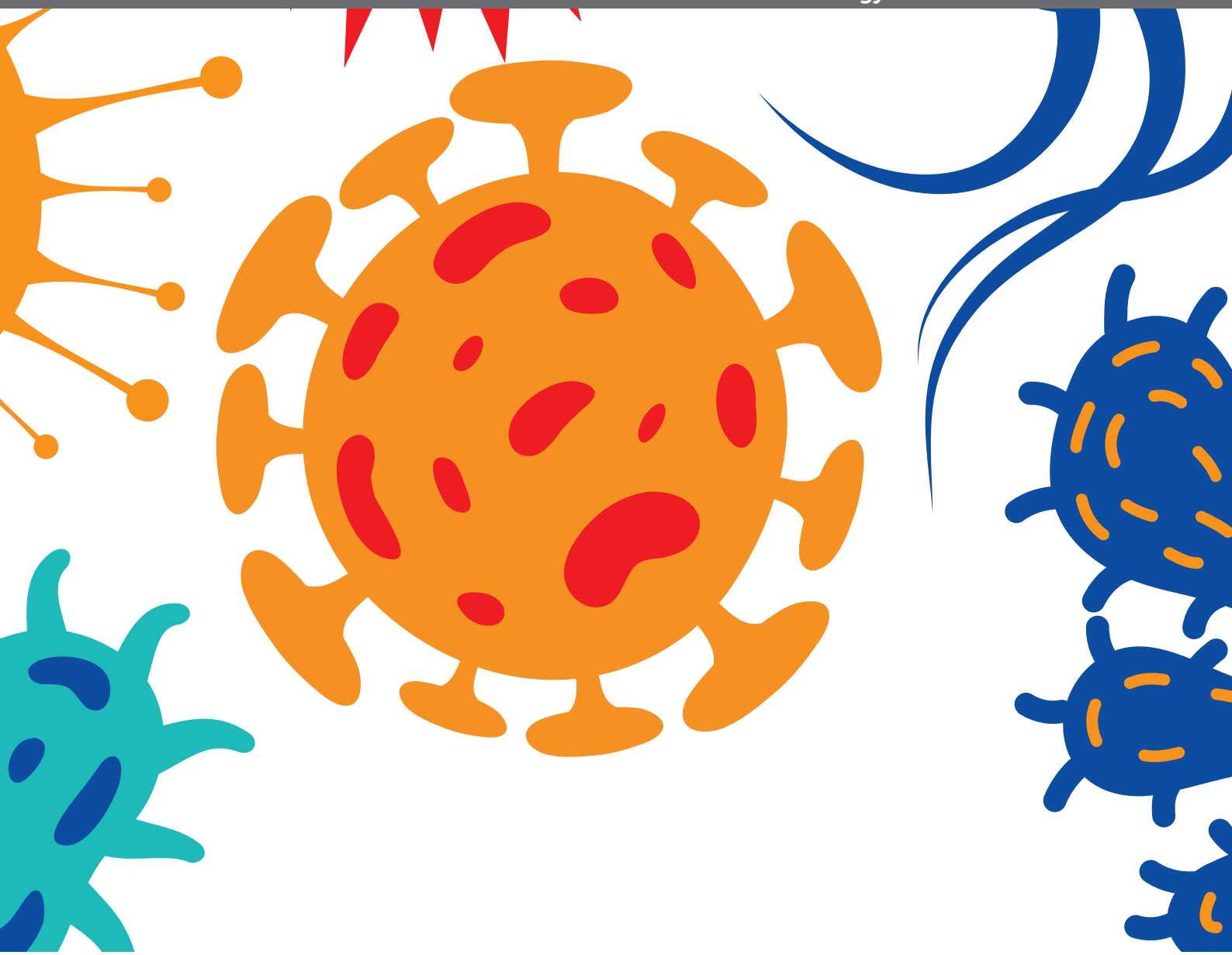

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THE ROLE OF ENVIRONMENTAL RESERVOIRS IN CAMPYLOBACTER-MEDIATED INFECTION

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EDITORIAL
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Editorial: The Role of Environmental Reservoirs in *Campylobacter*-Mediated Infection

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Editorial on the Research Topic

The Role of Environmental Reservoirs in *Campylobacter*-Mediated Infection

Campylobacteriosis is a food-borne infection caused mainly by *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli (C. coli)*. Thermophilic campylobacters are considered the foremost causative agents of bacterial gastroenteritis worldwide and campylobacteriosis represents an important public health problem with numerous socio-economic impacts. Since 2015, approximately 230,000 cases have been reported annually in Europe (Hansson et al., 2018). *C. jejuni* and *C. coli* colonize the digestive tract of multiple animal reservoirs such as birds, sheep, cattle, and pigs, with chicken considered as the main source of human infection in many countries. Yet, epidemiological information regarding the role of other putative disease reservoirs, such as wildlife and environment (*e.g.* soil and water), in disease transmission is limited.

Although *Campylobacter* can grow only inside the animal host, these bacteria can develop survival strategies by adaptation to different environmental stresses such as fluctuations of oxygen, temperature, biotic interactions, and starvation (Mouftah et al., 2021b). Moreover, the biofilm formation and the interaction with other bacteria also affects the bacterial survival outside the host. Studying the biology of *Campylobacter* in different environments may provide a better understanding of the infectivity of surviving bacteria to human.

The review by Kim et al. focused on molecular mechanisms involved in *Campylobacter* resistance to adverse environmental conditions, such as acid (*e.g.* ATR initiation, chaperone proteins DnaK and GroEL, and *rpoS* gene) and thermal stress (*e.g.* cold shock protein CspA), high osmolarity (*e.g.* *RpoN* sigma factor), as well as to antimicrobial agents (*e.g.* tetracyclines, quinolones, and macrolides). In the food production chain, *Campylobacter* is thought to be particularly susceptible to oxidative and desiccation stresses; however, *C. jejuni* has been demonstrated to be able to adapt to aerobic atmosphere by activating a range of enzymes, including KatA catalase. Thermophilic campylobacters are also able to respond to low temperatures encountered in food processing, even though the growth is particularly slow down. One of the food sources allowing a long-term survival of *C. jejuni* is the "chicken juice", in which it is able to form biofilm after expression of the quorum sensing system activated by the *luxS* gene. Under stressful conditions,

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C. jejuni is also able to enter a viable but non-culturable (VBNC) state that, among the others, affects CadF expression and thus its invasion ability.

Molecular strategies used by *Campylobacter* to survive in environmental and/or under stress conditions were also reviewed by Elmi et al. who highlighted the need to employ integrative multi-omics and phenotypic assays in research studies aimed to understand *C. jejuni* pathogenesis in multiple hosts. When exposed to high oxygen tension, limited nutrient availability, heat, acidic pH, temperatures fluctuations and antimicrobials, *Campylobacter* response is mainly due to its (strain-dependent) ability to form biofilms and to switch to the VBNC state. *Campylobacter* does not possess the same genetic repertoire of other bacteria to sense, adapt and survive to stress. *Campylobacter's* ability to form biofilm is thought to be under the control of a complex array of regulatory factors (*e.g.* CsrA, CosR, SpotT and CprRS), whereas heat shock proteins (HSPs) seem to be involved in *C. jejuni* response to temperature stress, and two-component regulatory systems (TCSs), RacRS, and RpoN, to acid stress. Recent studies employing whole genome sequencing (WGS) have suggested that *C. jejuni* host adaptation might be related to *panBCD* genes. Additionally, the *C. jejuni* T6SS seems to play a fundamental role in pathogenesis. *Campylobacter* flagella are well known factors involved in multiple functions including virulence, and recent studies have demonstrated that even minor genetic modifications may influence host colonization and infection. In addition, other factors involved in *Campylobacter* pathogenicity, such as capsule polysaccharide (CPS), adhesins (*e.g.* CadF and FlpA), and major outer membrane proteins (MOMPs) still need to be further explored. *C. jejuni* pathogenicity factors, such as outer membrane vesicles (OMVs), have been recently suggested as important fitness and survival factors as well. Finally, *Campylobacter* has the unique ability to metabolize only a few amino acids that seems to be the main strategy supporting its survival and adaptation in hosts.

Poultry and their products are the main reservoir of *Campylobacter* spp. relevant for public health, and Hakeem and Lu reviewed their transmission, survival, and adaptation in poultry production environments, including farms and processing systems. *Campylobacter* is reported to be widespread in these settings and many sources (soil, water, air, insects, animals, humans, several processing steps, *e.g.* scalding, defeathering, evisceration, neck removal, inside and outside washing) may contribute to its introduction and dissemination along the poultry production chain. Thus, improving on-farm biosecurity and in-plant control strategies are key elements to limit the prevalence of *Campylobacter* in poultry farms and products. One of the most striking aspects of the relationship between poultry and *Campylobacter* is gut colonization occurring at very high levels (100%) and nothing seems to be effective in reducing it, suggesting a commensal relationship between them. Different intervention strategies (*e.g.* vaccination, phage therapy, bacteriocins, probiotics, fatty acids, and essential oils) have been investigated to control *Campylobacter* colonization without any conclusive findings. Currently, only interventions in poultry processing plants seem to decrease the chance of *Campylobacter* contamination of poultry meat and consequently human infection. In their review, Hakeem and Lu presented novel and alternative strategies (*e.g.* plant-based antimicrobials, metal oxide nanoparticles, and antimicrobial synergism) to prevent and control *Campylobacter* in the agro-ecosystem.

However, domesticated birds are not the only source of *Campylobacter*, also wild birds are important source of human enteric pathogens, including bacteria of the genus *Campylobacter*, occurring in their digestive tracts. Interestingly, these species may be vectors of antimicrobial resistance (AMR) in the environment due to contact with antibiotics (Tryjanowski et al., 2020). Therefore, new studies focused on understanding *Campylobacter* sources, visible in this sample of published papers, including development of new molecular diagnostic methods (Carraro et al., 2019; Mouftah et al., 2021a; Saif et al., 2021) and will be useful with prevention (Facciolà et al., 2017).

Morcrette et al. focused on persister cell formation as mechanism used by *C. jejuni* to survive to stress induced by bactericidal concentrations of antibiotics. *C. jejuni* showed the ability to form persister cells at a frequency of 10^{-3} after exposure to 100 × MIC of penicillin G for 24 h. Metabolic activity detected by Redox Sensor Green reagent (RSG) staining suggested that this ability may be the consequence of increased redox protein activity in, or associated with, the electron transport chain. Proteomic analysis showed increased levels of redox proteins, such as reductases, in cells exposed to the antibiotic and indicated a remodelling of the electron transfer chain toward a less electrogenic process in order to moderate membrane hyperpolarization and intracellular alkalization; thus, reducing the antibiotic efficacy and potentially assisting in persister cell formation.

Shagieva et al. investigated the different ability of adhesion and biofilm formation of *C. jejuni* isolates from different sources (surface and wastewater, food, and clinical samples), as well as the potential role of the *luxS* gene (responsible for production of the communication molecule AI-2) in biofilm formation. All *C. jejuni* isolates were able to adhere to a surface, whereas the quantity and the architecture of biofilms were diverse, with wastewater isolates forming more compact biofilms. Not all isolates possessed the *luxS* gene, in particular those originated from surface waters. These isolates formed thinner and sparser biofilms lacking the presence of significant clusters. However, the ability to adhere to the surface was preserved. Overall, this study showed that *C. jejuni* isolated from water can adhere to a surface and subsequently form a spatially structured biofilm. As their adhesion capacity was comparable to the strains of clinical or food origin, they might indeed represent a significant source of contamination in animal husbandry, and as a source of infection in humans.

Although comparative genomic analysis using conventional seven-locus multilocus sequence typing (MLST) has been used as the gold standard over the past decades in attributing different reservoirs of this foodborne pathogen, WGS-based methods enable a better understanding of *Campylobacter* epidemiology, multi-host ecology, host adaptation, and cryptic transmission

networks from animals to humans at the farm-to-fork interface through providing higher-resolution typing, that was not possible to achieve with other previous typing methods. Furthermore, microbial genomics has been recently applied to forecast the genetic determinants involved in *Campylobacter* antimicrobial resistance to detect the complex dynamics of selection and transmission of AMR through a multidisciplinary One Health approach (Mouftah et al., 2021a).

For instance, Nennig at al. implemented WGS gene-by-gene approach to better understand *Campylobacter* epidemiology. In this pilot study, they highlighted the clonal expansion of stable genomes in *Campylobacter* population exhibiting a multi-host profile, suggesting the persistence of this foodborne pathogen in different reservoirs and consequently recommended the need to investigate their survival strategy at a higher resolution.

The role of wild boars as reservoir of multi-drug resistant (MDR) *Campylobacter* species in Italy has been recently elucidated by Marotta et al. through application of whole genome multilocus sequence typing (wgMLST) and genomic AMR determinants characterization. Furthermore, comparison of different genomes from guinea pigs (*Cavia porcellus*) by Parker et al. demonstrated novel genomic alterations, including gene gain and loss, that could be associated with guinea pig host specialization related to guinea pig anatomy, dietary intake, and physiology potentially allowing niche adaptation in this animal species. Furthermore, Davies et al. employed a comparative genomics study using WGS analysis complemented with transcriptional and phenotypic variation within epidemiologically related *C. jejuni* isolates from a waterborne outbreak to highlight the role of water-borne infection. This demonstrated a higher pathogenic potential as revealed by the highest levels of virulence gene expression, adhesion to epithelial cells and interleukin 8 (IL-8) induction. The study provided further evidence of bacterial changes due to niche adaptation in the host and/or the environment.

The advent of next generation sequencing (NGS) technologies enabled Song et al. to conduct metagenomics analysis of wild mice (*Micromys minutus*) gut microbiota. The study provided important insights onto the potential role of wild mice as reservoir for *Campylobacter* transmission with higher sensitivity of detection compared to culture-dependent methods due to ability of WGS to determine VBNC *Campylobacter* species.

Finally, despite intensive research, the mechanisms facilitating *Campylobacter* adaptation and factors influencing the survival in the environment are still unclear. An important factor is that the Czech Republic is among the countries with the highest incidence of the disease. Davies et al. focused on waterborne isolates, as there have been more publications recently on waterborne outbreaks of campylobacteriosis. As far as we know, biofilm formation experiments were mostly conducted on isolates originating from various animal, food, and clinical samples, but excluded environmental isolates. However, the phenotypic features of these isolates might be different due to stress conditions in the environment; in turn, they can help bacteria to survive in some way. Then, findings published in this paper illustrate the necessity for future comprehensive studies of waterborne isolates, as they can transmit infection to the same extent, as isolates from meat or clinical isolates.

CONCLUSIONS AND FUTURE DIRECTIONS

Campylobacter studies presented in this *Frontiers Research Topic* are linked to the following groups: (1) genome sequencing; (2) metagenomics; (3) different mechanisms of *Campylobacter* survival, and (4) biofilm formation and pathogenesis. Biofilm formation and interaction with other bacteria can also have an influence on bacterial survival outside the host. Therefore, comparing the ecology of *Campylobacter* in different environments provides a better understanding of the infectivity of surviving bacteria to humans. Implementing high-throughput technologies, such as genome sequencing in this context, allows a better understanding of the variations in survival strategies among different *Campylobacter* strains. This combined approach underlines the need to clarify the direct and indirect role of *Campylobacter* ecology in the transmission of *Campylobacter* to humans.

AUTHOR CONTRIBUTIONS

All authors contributed to conception and design of the study. All authors wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Carraro, L., Marotta, F., Janowicz, A., Patavino, C., and Piccirillo, A. (2019). Draft Whole-Genome Sequences of 16 *Campylobacter Jejuni* Isolates Obtained From Wild Birds. *Microbiol. Resour Announcements* 8, e00359–e00319. doi: 10.1128/MRA.00359-19

Facciolà, A., Riso, R., Avventuroso, E., Visalli, G., Delia, S. A., and Laganà, P. (2017). *Campylobacter*: From Microbiology to Prevention. *J. Prev. Med. Hyg* 58 (2), E71–E92.

Hansson, I., Sandberg, M., Habib, I., Lowman, R., and Engvall, E. O. (2018). Knowledge Gaps in Control of Campylobacter for Prevention of *Campylobacteriosis*. *Transbound. Emerg. Dis.* 65, 30–48. doi: 10.1111/tbed.12870

Mouftah, S. F., Cobo-Díaz, J. F., Álvarez-Ordóñez, A., Elserafy, M., Saif, N. A., Sadat, A., et al. (2021a). High-Throughput Sequencing Reveals Genetic Determinants Associated With Antibiotic Resistance in *Campylobacter* Spp. From Farm-to-Fork. *PloS One* 16, e0253797. doi: 10.1371/journal.pone.0253797

Mouftah, S. F., Cobo-Díaz, J. F., Álvarez-Ordóñez, A., Mousa, A., Calland, J. K., Pascoe, B., et al. (2021b). Stress Resistance Associated With Multi-Host Transmission and Enhanced Biofilm Formation at 42°C Among Hyper-Aerotolerant Generalist. *Campylobacter jejuni. Food Microbiol.* 95, 103706. doi: 10.1016/j.fm.2020.103706

Saif, N. A., Elserafy, M., El-Shiekh, I., Álvarez-Ordóñez, A., Mouftah, S. F., and Elhadidy, M. (2021). A Pilot Study Revealing Host-Associated Genetic Signatures for Source Attribution of Sporadic *Campylobacter Jejuni* Infection in Egypt. *Transbound. Emerg. Dis.* doi: 10.1111/tbed.14165

Tryjanowski, P., Nowakowski, J. J., Indykiewicz, P., Andrzejewska, M., Śpica, D., Sandecki, R., et al. (2020). *Campylobacter* in Wintering Great Tits Parus Major in Poland. *Environ. Sci. Pollut. Res.* 27, 7570–7577. doi: 10.1007/s11356-019-07502-y

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Genotyping and Antibiotic Resistance Traits in *Campylobacter jejuni* and *coli* From Pigs and Wild Boars in Italy

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The present study investigated the genomic constitution and antimicrobial resistance (AMR) of 238 *Campylobacter* from pigs and wild boars in Italy between 2012 and 2019. *Campylobacter* strains were genotyped using multilocus sequence typing (MLST) and whole genome MLST (wgMLST), screened for antimicrobial resistance genes, and tested for phenotypic susceptibility to six different antibiotics. *C. coli* was detected in 98.31% and 91.66% of pigs and wild boars, while *C. jejuni* was isolated in the remaining cases. MLST assigned 73 STs and 13 STs in pigs and wild boars, respectively, including 44 novel STs. The predominant ST in pigs was ST-854 (12.36%), followed by ST-9264 (6.18%). ST-1055 and ST-1417 were predominant in wild boars (30% and 13.33%, respectively). The minimum spanning tree using 1,121 global MLST profiles showed specific Italian clusters and a clear separation between pig and wild boar profiles. The wgMLST confirmed the MLST clustering and revealed a high genetic diversity within *C. coli* population in Italy. Minimum inhibitory concentrations (MIC) of six antibiotics revealed higher resistance in pigs to ciprofloxacin, nalidixic acid, streptomycin and tetracycline, compared to wild boar. In contrast, most strains were susceptible to gentamicin. Worrying levels of multidrug resistance (MDR) were observed mostly in pig isolates. Molecular screening of AMR mechanisms revealed the predominance of gyrA T86I substitution among fluoroquinolone- and quinolone-resistant isolates, and the 23S rRNA A2075G mutation among macrolide-resistant isolates. Other resistance determinants were observed: (i) *tet (O)* gene was present among tetracycline-resistant isolates; (ii) *rpsL* and *aph*(3')-III genes conferring resistance to aminoglycosides, were identified only in streptomycin or gentamicin-resistant pig isolates; (iii) *cmeA, cmeB, cmeC, cmeR* genes responsible of pump efflux mechanisms, were observed in almost all the strains; (iv) *OXA*-61, encoding β-lactamase, was found in the half of the strains. Genotypic and phenotypic AMR profiling was fairly correlated for quinolones/fluoroquinolones. *Campylobacter* infection is common also in wild boar populations in Italy, suggesting that wild boars could be a reservoir of resistant and multi-resistant *Campylobacter* species, which may be of public health

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concern. The present study adds to our knowledge on the epidemiological and ecological traits of this pathogen in domesticated and wild swine.

Keywords: *Campylobacter*, antimicrobial resistance (AMR), multidrug resistance (MDR), multilocus sequence typing, resistance genes, wgMLST

INTRODUCTION

Campylobacter is known as the most common cause of bacterial gastrointestinal infection in Europe, with the annual number of cases exceeding those of salmonellosis and shigellosis (EFSA & ECDC, 2019). *Campylobacter jejuni* and *Campylobacter coli* are the main causative agents of campylobacteriosis, posing a threat to public health worldwide (EFSA & ECDC, 2019). Fever, bloody diarrhea, headache and abdominal pain, nausea and vomiting are the main symptoms of campylobacteriosis in humans. Generally, the infection is self-limiting after 3-5 days, but in immunocompromised individuals it can spread into the bloodstream and become potentially lethal (Whitehouse et al., 2018). In severe cases, the antibiotic treatment is required, with macrolides and fluoroquinolones being the drugs of first choice (Mourkas et al., 2019). Campylobacteriosis is a mainly food-borne disease in which foods of animal origin, such as poultry meat, beef and pork, play a primary role (Sheppard et al., 2011).

Several studies showed the possibility of wildlife or environmental sources to act as reservoirs of *Campylobacter* infection (Sheppard et al., 2009a; Griekspoor et al., 2013; Cody et al., 2015; Atterby et al., 2018; Marotta et al., 2019; Marotta et al., 2020). In particular, these researchers focused on agricultural settings, especially on wild birds (Sheppard et al., 2009a; Griekspoor et al., 2013; Cody et al., 2015; Atterby et al., 2018; Marotta et al., 2019) small mammals (Sippy et al., 2012) and insects (Hald et al., 2004). However, there are little data on potential spill-over between livestock and wild ungulates (Navarro-Gonzalez et al., 2014). In pig farms, campylobacteriosis often leads to a significant decrease in animal productivity and consequent economic losses (Hansson et al., 2018).

Domestic pigs and wild boars belong to the same species (*Sus scrofa*) making them susceptible to the same pathogens (Ruiz-Fons et al., 2006; Ruiz-Fons et al., 2008). As a result, wild boar populations infected with *Campylobacter* could pose a threat to the pig industry. The Eurasian wild boar is widely distributed throughout most of Europe and in the past 50 years their numbers have increased to an estimatedpopulation of over 2.2 million wild boars (Massei et al., 2015; Meier and Ryser-Degiorgis, 2018). In Italy, it is the most widespread wild ungulate with a consistent presence along the country, due to its high prolificacy, favorable climatic conditions, and to the depopulation of Apennine and Alpine areas (Apollonio et al., 2010; Stella et al., 2018). Wild boars may contract *Campylobacter* from avian species, due to constant contact with soil contaminated with bird droppings (Waldenström et al., 2002; Humphrey et al., 2007; Epps et al., 2013). The increasing communities of wild boars in the anthropized areas as possible reservoirs of different *Campylobacter* species represent a growing challenge for public and veterinary health systems (Jones et al., 2013; Miller and Sweeney, 2013). Numerous studies showed that AMR is still very common in *Campylobacter* strains isolated from farmed animals in many European countries (EFSA & ECDC, 2019). In particular, high level of antibiotic resistance was shown to ciprofloxacin, nalidixic acid and tetracycline (EFSA & ECDC, 2019) followed, especially in *C. coli*, by resistance to macrolides and aminoglycoside antibiotic classes. Moreover, an alarming trend towards multidrug resistance (MDR), particularly among *C. coli*, was also detected (Luangtongkum et al., 2009; Pascoe et al., 2017; Mourkas et al., 2019). In this study, we aimed to evaluate the genotypic diversity of *Campylobacter* in wild boar and domesticated pig populations circulating in Italy and identify AMR genes in the two species investigated in order to understand the extent to which *Campylobacter* species are common, indicating a potential inter-species transmission.

MATERIAL AND METHODS

Bacterial Strains and Species Identification

A total of 238 *Campylobacter* strains isolated using the bacteriological ISO method 10272-1:2017 and stored at the microbial strain collection of the National Reference Laboratory for *Campylobacter* (NRL, http://www.izs.it/IZS/Eccellenza/Centri_nazionali/LNR_-_Campylobacter) were included in the study. The collection comprised 178 *Campylobacter* pig strains isolated from carcasses and from fecal content and 60 *Campylobacter* wild boar strains isolated from liver, muscle and faeces, in Italy between 2012 and 2019. The strains were cultured on Columbia blood agar plates in microaerobic atmosphere at 42°C for 48 h and DNA was extracted using Maxwell instrument (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy). After an initial phenotypic characterization, suspected colonies were confirmed as thermotolerant *Campylobacter* and identified to species level using a multiplex and a simplex PCR, as described previously (Wang et al., 2002; Marotta et al., 2019). Strains used as positive PCR controls were *C. coli* NCTC 11353, *C. fetus* ATCC 19438, *C. jejuni* ATCC 33291, *C. upsaliensis* NCTC 11541 and *C. lari* NCTC 11552.

Sequence Analysis and Identification of Antibiotic Resistance Genes

Total genomic DNA was used to prepare sequencing libraries using Nextera XT Library Preparation Kit (Illumina, Inc., San Diego, CA, USA). The libraries were then sequenced using

Illumina NextSeq 500 sequencer. Sequence reads (150-bp, pair-end) were demultiplexed and the adapters were removed. Subsequently the reads were trimmed with Trimmomatic tool (version 0.36) and *de novo* assembled using SPAdes version 3.11.1 with the "careful" option selected (Bankevich et al., 2012). The sequence reads generated in this study were deposited in NCBI Sequence Read Archive (SRA) in Bioprojects PRJNA638082 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA638082) and PRJNA638084 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA638084).

C. jejuni genome assemblies, were genotyped by MLST. The assemblies were also investigated for the genomic AMR traits.

The MLST profiles were assigned using a *C.jejuni/coli* task template MLST 7 loci, schema available at https://pubmlst.org/Campylobacter/accessible through in Ridom SeqSphere+ v. 6.0.2. Software (RidomGmbH, Münster, Germany). Italian MLST profiles were combined with MLST data of 1,121 pig isolates from Europe, downloaded from PubMLST (http://pubmlst.org/campylobacter/) and analyzed at the time of this analysis. MLST profiles were analyzed using the goeBURST algorithm implemented in PHYLOViZ, version 2.0 (Nascimento et al., 2017). Minimum spanning trees (MST) were created using default software settings.

The wgMLST analysis was performed in Ridom SeqSphere+ v. 6.0.2. The scaffolds were analyzed using two task templates: *C. jejuni/C. coli* cgMLST composed of 637 gene core gene targets and *C. jejuni/C. coli* accessory MLST composed of 958 accessory gene targets. Scaffolds that contained less than 90% good genome targets were excluded from the analysis. UPGMA tree was constructed by pairwise analysis of identified alleles, with missing targets ignored using default settings. The tree and associated metadata were visualized using iTol v5 (Letunic and Bork, 2006).

AMR genes were identified in silico using PointFinder v. 3.1.0 and ABRicate v. 0.8 (https://github.com/tseemann/abricate/) by querying the publicly available Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2016; Zankari et al., 2017). Prokka v1.13 (Seemann, 2014) was used to annotate the assemblies and gyrA sequences were extracted applying the query_pan_genome function in Roary v3.12.0 (Page et al., 2015). gyrA genes were aligned using Uniprot UGENE v1.18.0 (Okonechnikov et al., 2012), from which the gene variants were identified. Only mutations in the quinolone resistance-determining region (QRDR) of gyrA were assessed to be the determinants of resistance, as only these loci have been linked with phenotypic resistance to quinolones. In addition, all the strains studied were deposited in PubMLST database (http://pubmlst.org/campylobacter) and the submissions ids are: BIGSdb_20200511094837_082196_21032, BIGSdb_20200511093337_081290_49754, BIGSdb_20200508081738_149794_16751 and BIGSdb_20200508080706_045922_07760.

Antimicrobial Susceptibility

Antim icrobial susceptibility was tested by the broth microdilution method, using the Sensititre automated system (TREK Diagnostic Systems, Venice, Italy) following the manufacturer's instructions. Briefly, colonies were subcultured on Columbia agar for 24 h and then seeded in Mueller Hinton Broth supplemented with blood (Oxoid, Basingstoke, UK). Then, they were dispensed into Eucamp2 microtiter plates (TREK Diagnostic Systems, Venice, Italy), with known scalar concentrations of the following antibiotics: ciprofloxacin (CIP) (0.12–16 μg/ml), erythromycin (ERY) (1–128 μg/ml), gentamicin (GEN) (0.12–16 μg/ml), nalidixic acid (NAL) (1–64 μg/ml), streptomycin (STR) (0.25–16 μg/ml), and tetracycline (TET) (0.5–64 μg/ml). The distribution % of MIC are reported in brackets. Following bacterial inoculation, the plates were incubated at 42°C in microaerobic atmosphere for 24 h, and then screened. The strains were classified as resistant (R), and susceptible (S) according to MIC breakpoints, by using Swin v3.3 Software (Thermo Fisher Scientific) in accordance with the epidemiological cutoff values (ECOFFs) as defined by EUCAST (European Committee on antimicrobial breakpoints) (www.eucast.org) to interpret their antimicrobial susceptibilities. *C. jejuni* strain NCTC 11351 was used as control. MIC breakpoints of resistance were > 0.5 μg/ml for CIP (*C.jejuni* and *C.coli)*, > 4 μg/ml for STR (*C.jejuni* and *C.coli)*, > 4 μg/ml for ERY (*C.jejuni)* and > 8 μg/ml (*C.coli)*, > 2 μg/ml for GEN (*C.jejuni* and *C.coli)*, > 16 μg/ml for NAL (*C.jejuni* and *C.coli)* and > 1 μg/ml for TET (*C.jejuni*) and > 2 μg/ml (*C.coli*). Details of the pig and wild boar isolates are summarized in **Supplementary Table 1**.

Statistical Analysis

The antimicrobial resistance analysis was performed by means of a Chi-square statistic test. All values with $P<0.05$ were considered statistically significant (McHugh, 2013).

RESULTS

Genus and Species Confirmation

We analyzed 178 *Campylobacter* strains isolated from carcasses (53.37%) and fecal content of pigs (46.62%), and 60 *Campylobacter* strains isolated from feces (83.33%), liver (10%), and muscle (6.67%) of wild boars (**Table 1**). *C. coli* was isolated in in 98.31% of pig and 91.66% wild boar strains, while *C. jejuni* was isolated in 1.68% and 8.33% of pig and wild boar strains, respectively (**Table 1**).

TABLE 1 | Percentages of *Campylobacter coli* and *jejuni* isolated from pigs and wild boars.

	Carcass	Feces	Muscle	Liver
Pigs (n=178)	92 (51.68%) *C. coli* 3 (1.68%) *C. jejuni*	83 (46.62%) *C. coli*	–	–
Wild boars (n=60)	–	46 (76.66%) *C. coli* 4 (6.66%) *C. jejuni*	4 (6.7%) *C. coli*	5 (8.33%) *C. coli* 1 (1.66%) *C. jejuni*

MLST Analysis of *C. coli* and *C. jejuni* Isolates

The MLST analysis showed 5 STs among the 8 C. *jejuni* strains studied (**Supplementary Table 1**). One ST (ST-10326) has not been described before in the PubMLST *Campylobacter* database (https://pubmlst.org/campylobacter/). The ST-10326, ST-42, ST-21 were assigned to *C. jejuni* strains isolated from 3 pigs, while ST-267 was assigned to 4 and ST-2863 to one wild boar *C. jejuni* strains (**Supplementary Table 1**). Regarding *C. coli*, 67 and 8 different STs were obtained from pigs and wild boars, respectively (**Supplementary Table 1**). Fifteen STs from pigs (ST- 10304, ST-10305, ST-10307, ST-10319, ST-10323, ST-10324, ST-10325, ST-10326, ST-10327, ST-10328, ST-10329, ST-10330, ST-10331, ST-10332, and ST-10333) and one ST from wild boars (ST-10334) were identified for the first time in this study (**Supplementary Table 1**). In particular, the novel STs contained one or more new allelic genes, and 12 novel alleles were found (aspA547, aspA548, aspA549, glnA754, gltA644, pgm1067, pgm1068, pgm1069, tkt824, tkt825, tkt826, uncA681). Fifty-five STs obtained from both hosts, belonged to the CC-828, only one ST isolated from one pig (ST-5392) belonged to CC-1150, and twenty-eight STs from pigs and wild boars did not belong to any known CC at the time of this analysis (**Supplementary Table 1**). The ST-1055 was the most prevalent ST that grouped 18 strains isolated from wild boars (30%). The second most prevalent ST was ST-1417 assigned to 8 strains isolated from wild boars (13.3%) (**Figure 1**). *C. coli* strains belonging to ST-854 were instead dominant in pigs (12.4%), followed by ST-9264 (6.18%). Out of 70 STs, 42 (60%) were obtained from pigs, and 2 STs out of the 11 STs (18.18%) isolated from wild boars, were represented by only one strain. Only three STs (ST-1016, ST-1055 and ST-1417) were shared between the two animal species (**Figure 1**). In detail, ST-1016 was represented by 14 C. *coli* strains (9 from pigs and 5 from wild boars); ST-1055 was represented by 19 C. *coli* strains (1 from pig and 18 from wild boars) and, finally, ST-1417 was represented by 13 C. *coli* strains (5 from pigs and 8 from wild boars). The MLST analysis with European pig isolates found a substantial number of STs (67) circulating only on Italian territory (**Supplementary Figure 1**). The STs most commonly shared with other European countries were: ST-854 and ST-828 shared with seven European countries (Scotland, Switzerland, Germany, UK, Portugal, Netherlands and Luxemburg), followed by ST-1016 shared with six European countries (Switzerland, Belgium, Scotland, UK, The Netherlands and Portugal). A total of 6 and 7 different STS were common with 3 and 2 other European countries, respectively, and 14 STs were shared with one other European country (**Supplementary Table 2).** The European countries with most STs shared with Italian isolates were Scotland (13 STs), Switzerland (10 STs) and Germany (9 STs).

WgMLST Analysis of *C. coli*

The wgMLST analysis of 213 genomes of *C. coli* revealed wide diversity among the strains circulating in Italy (**Figure 2**). The maximum distance between the pair of wgMLST profiles was 583 genes. The strains isolated from domesticated pigs were scattered along most branches of the phylogenetic tree and few clusters of genetically closely related genotypes could be identified. Interestingly, even within these clusters, we did not observe clear geographic separation as they often contained strains isolated in two or more different locations. Similarly, *C. coli* isolates from wild boar, even though all collected in the Tuscany region, were divided into several separate lineages. The biggest cluster was found in Grosseto province and contained strains assigned to ST-1055. This sequence type was one of the three shared by both, *C. coli* strains from domesticated pigs and from the wild boarHowever, the isolate from the pig was distant from the wild boar ST-1055 complex by more than 400 genes demonstrating that ST determination was not sufficient to find real genetic connections between the strains. Moreover, we did not identify any clusters of closely related wgMLST profiles that contained strains from both the domesticated pig and the wild boar.

Antimicrobial Resistance Phenotypes

The resistance levels of pig isolates to six antibiotics were compared to genomic resistance profiles of isolates of wild boar origin in **Table 2** and **Figures 3** and **4**. Statistically significantly higher levels of AMR in pig isolates in respect to wild boar isolates were observed for TET (89.9% vs 26.7%), CIP (73.1% vs 16%), NAL (68.9% vs 26%) and ERY (36.5% vs 3.3%) (Chi-square test; $p<0.01$). The MIC test revealed that 86.5% of pig and 61.6% of wild boar isolates were resistant to STR. Lower resistance levels were observed for GEN (11.6% for pig isolates; 13.5% for wild boar isolates) (**Figure 3**). MDR, considered as the resistance to at least three different classes of antibiotics (EFSA & ECDC, 2015), was very common (**Figure 4**). Strains isolated from pigs were more often found to display MDR than the strains from the wild boar. The most common MDR profiles were CIP-STR-TET (56% pig isolates; 3% wild boar isolates), followed by NAL-STR-TET (53% pig isolates; 7% wild boar isolates). CIP-ERY-TET was found in the 32% and 3% of pig and wild boar isolates, respectively, while CIP-ERY-STR-TET was present only in 29% of pig isolates (**Figure 4**).

Detection of Resistance Genes, Mutations, and Levels of Concordance

The genome assemblies of all *Campylobacter* were investigated for the genomic AMR genes, 23S rRNA and *gyrA*-associated point mutations and RpsL substitutions. The analysis revealed the presence of 7 AMR genes including: *tet(O)*, *cmeA*, *cmeB, cmeC, cmeR, OXA-61, aph(3')-III*. The resistance genes for the corresponding antibiotic were observed in most but not in all resistant isolates. Regarding resistance to aminoglycosides, resistance traits associated with GEN and STR (*aph(3')-III*) resistance were exclusively found in 9 and 23 pig *C. coli* resistant strains, respectively. RpsL substitution at amino acid 88, involved in STR resistance, was found in only two pig *C. coli* isolates. The concordance rate between the two types of resistances was of 37.5% and 16.3% (**Table 2**). Although we did not test resistance to beta-lactams antibiotics class phenotypically, we detected the *OXA-61* gene in the half of the pig and wild boar isolates. *Tet(O)* gene, conferring resistance to TET, was detected in 88

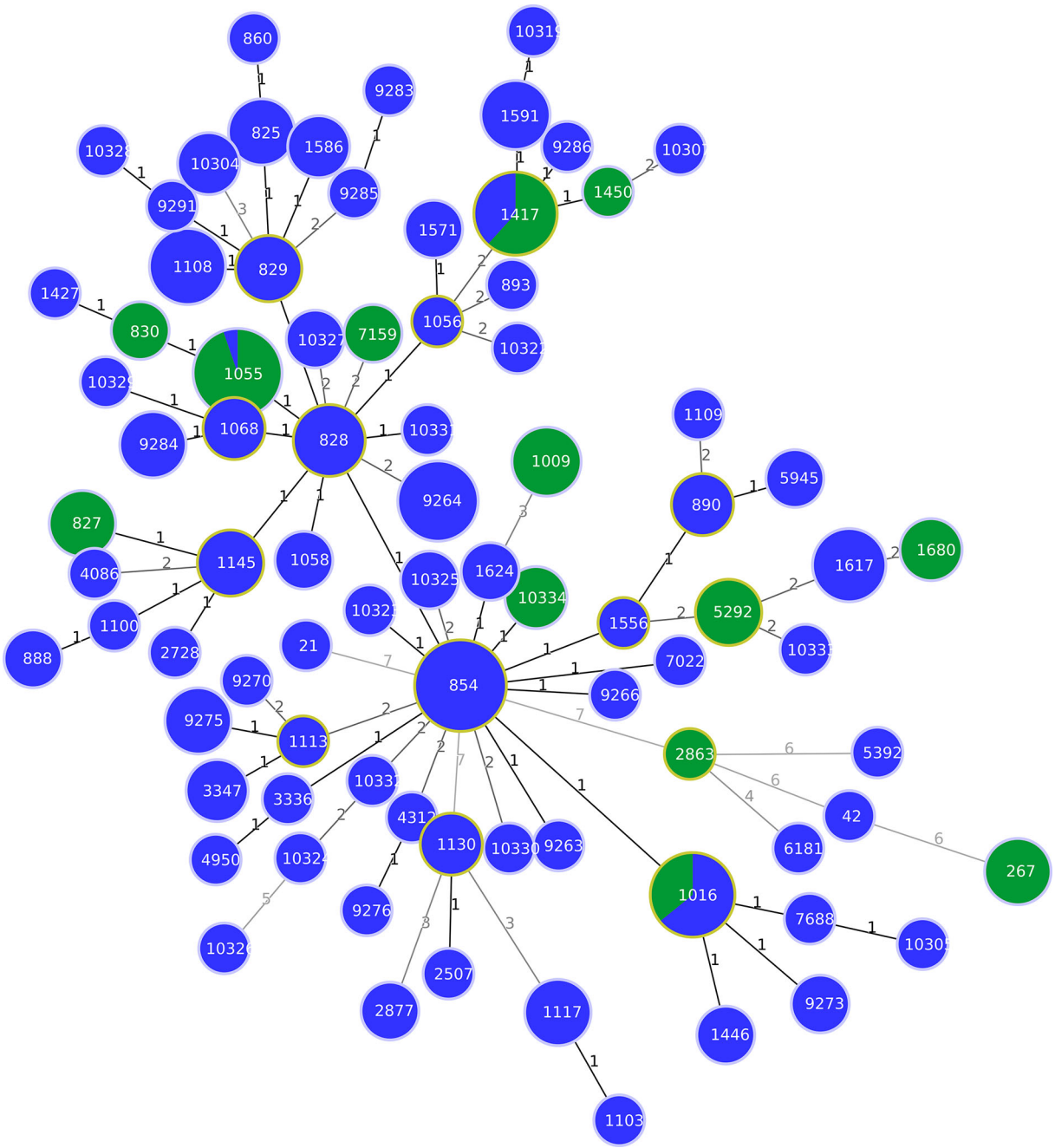

FIGURE 1 | Minimum spanning tree (MST) generated for 238 Italian strains isolated from pigs and wild boars. The tree was generated using the goeBURST algorithm in PHYLOViZ software. The distance labels correspond to the number of discriminating alleles. The blue nodes correspond to pig isolates and the green nodes to wild boar isolates.

pig and 11 wild boar isolates resistant to TET. The concordance rate resulted, respectively, of 55% and 68.7%. The ERY resistant strains were screened for the presence of mutations in 23S rRNA gene. The A2075G mutation was identified in 51 and 2 isolates from pig and wild boar resistant isolates, showing a concordance rate of 78.5% and 100%, respectively. The *cmeA, cmeB, cmeC, and cmeR* genes, associated with efflux pump function, were present in almost all the strains. Finally, isolates resistant to fluoroquinolones and quinolones were screened for mutations in the *gyrA* gene. T86I mutation was detected in 99 and 6 pig and wild boar isolates with CIP resistance phenotype, showing a concordance rate of 76.7% and 60%, respectively, and in 95 and 5 pig and wild boar isolates with NAL resistance phenotype, showing a concordance rate of 78.5% and 31.2% (**Table 2**).

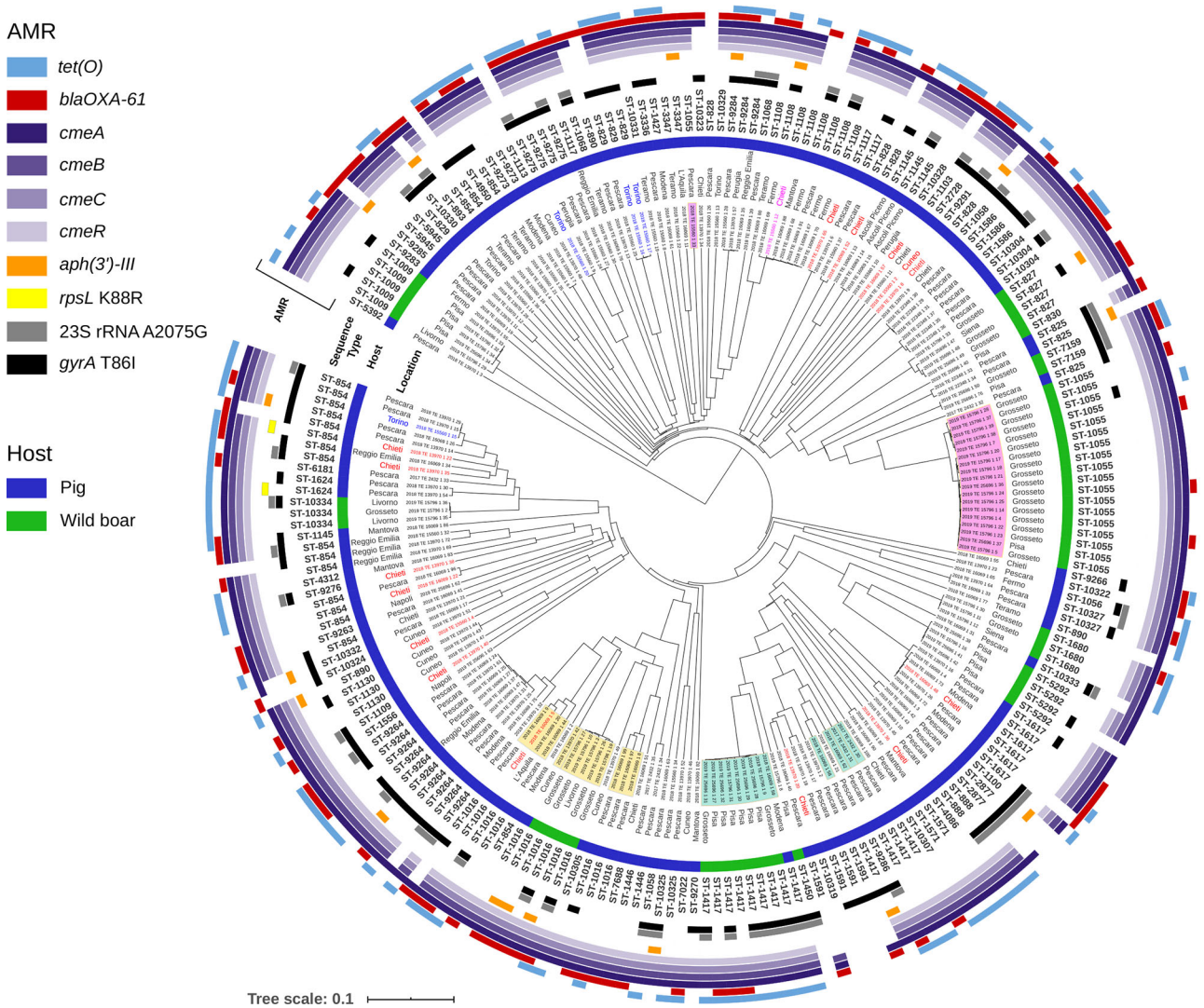

FIGURE 2 | Phylogenetic tree generated for 213 strains of *C. coli* from Italy. The UPGMA tree was constructed based on wgMLST analysis results. The presence and allelic diversity of antimicrobial resistance genes substitutions in *C. coli* genomes are indicated. Strains isolated from domestic pigs are marked with blue color bar and from wild boar with green bar. The isolates highlighted in yellow, green and pink, strains obtained from the two different hosts and belonging to the same MLST sequence types. The isolates in red are of Hungarian origin, those in blue of Danish origin and the only one in fuchsia of French origin. The rest of isolates in black are of Italian origin.

DISCUSSION

Here we presented a cross-sectional study on *Campylobacter* from Italian fattening pigs and wild boars using a multiplex approach that included antimicrobial susceptibility test, MLST, wgMLST, and genetic determination of AMR. The analyzed strains were representative of the Italian pigs and wild boars for the period 2012–2019. A high genomic diversity was observed among *C. coli* isolates in the Italian pig and wild boar populations, with 67 and 11 different STs within 175 and 55 analyzed isolates, respectively. These data are in line with other recent studies (Egger et al., 2012). In this study, MLST revealed the existence of the dominant *C. coli* CC-828 containing 76% of pig and wild boar isolates while the CC-1150 was detected only in one pig isolate. In addition, we observed that *C. coli* strains from pig and wild boar constituted two separate populations. Interestingly, only 3.7% (3/81) of STs were shared between pig and wild boar isolates. However, wgMLST analysis showed that pig isolates belonging to these three STs were genetically distant from the wild boar strains, demonstrating that ST determination was not sufficient to find real genetic connections between the strains of the two animals. In general, we did not identify any clusters of closely related wgMLST profiles that contained strains from both hosts suggesting that no exchange of *Campylobacter* spp. occurred between pigs and the wild boars, possibly due to the segregation of traditional pig farming and wild boar

TABLE 2 | Comparison of genotypic and phenotypic resistance to antibiotics in *C. coli* isolated from Italian pigs and wild boars.

Antibiotic class	Antibiotics	Genes	Animals	No. of isolates with R phenotype[a] (n=178)	No. of isolates with R genotype[b]	Concordance rate[d]
Aminoglycosides	Gentamicin (GEN)	*aph*(3')-III	Pig	n=24	n= 9	37.5
			Wild boar	n=7	n=0	0
	Streptomycin (STR)	*rpsL- aph*(3')-III	Pig	n=154	n=2; n=23	1.3–15
			Wild boar	n=37	n=0	0
Beta-lactams[c]	–	*OXA-61*	Pig	–	n=89	–
			Wild boar	–	n=27	–
Fluoroquinolones/ Quinolones	Ciprofloxacin (CIP)/ Nalidixic acid (NAL)	*gyrA*	Pig	n=129; n=121	n=99; n=95	76.7–78.5
			Wild boar	n=10; n=16	n=6; n=5	60–31.2
Macrolides	Erythromycin (ERY)	*23S rRNA*	Pig	n=65	n=51	78.5
			Wild boar	n=2	n=2	100
Tetracyclines	Tetracycline (TET)	*Tet(O)*	Pig	n=160	n=88	55
			Wild boar	n=16	n=11	68.7
Multidrug CmeABC efflux system and *cmeR*		*cmeA,cmeB,cmeC, cmeR*	Pig	–	n=153; n=132; n=130; n=129	–
			Wild boar	–	n=60; n=58; n=57; n=57	–

[a]*Number of isolates expressing the resistance phenotype for the corresponding antibiotic;*
[b]*Number of isolates expressing the resistance phenotype for the corresponding antibiotic, that have the indicated gene;*
[c]*Antibiotic class does not tested for resistance phenotype;*
[d]*Concordance rate (%).*

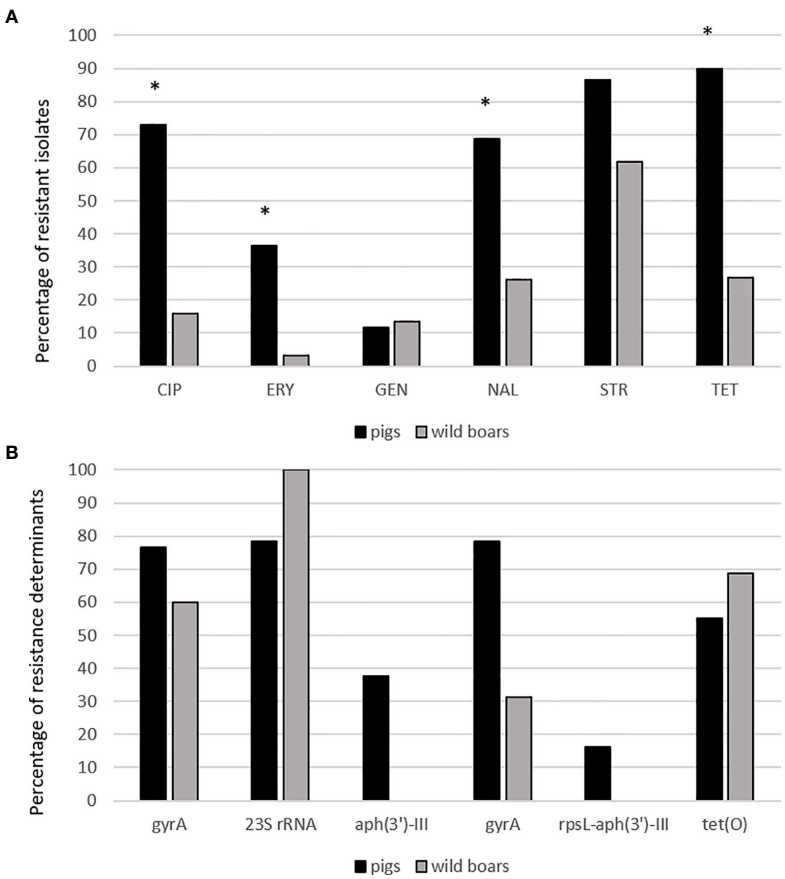

FIGURE 3 | **(A)** Antibiotic resistance pattern between pig and wild boar isolates. CIP, ciprofloxacin, ERY, erythromycin, GEN, gentamicin, NAL, nalidixic acid, STR, streptomycin, TET, tetracycline. *statistically significant vs. wild boar isolates (χ^2-test, p<0.01). **(B)** Percentages of resistance determinants between pig and wild boar antibiotic resistant isolates.

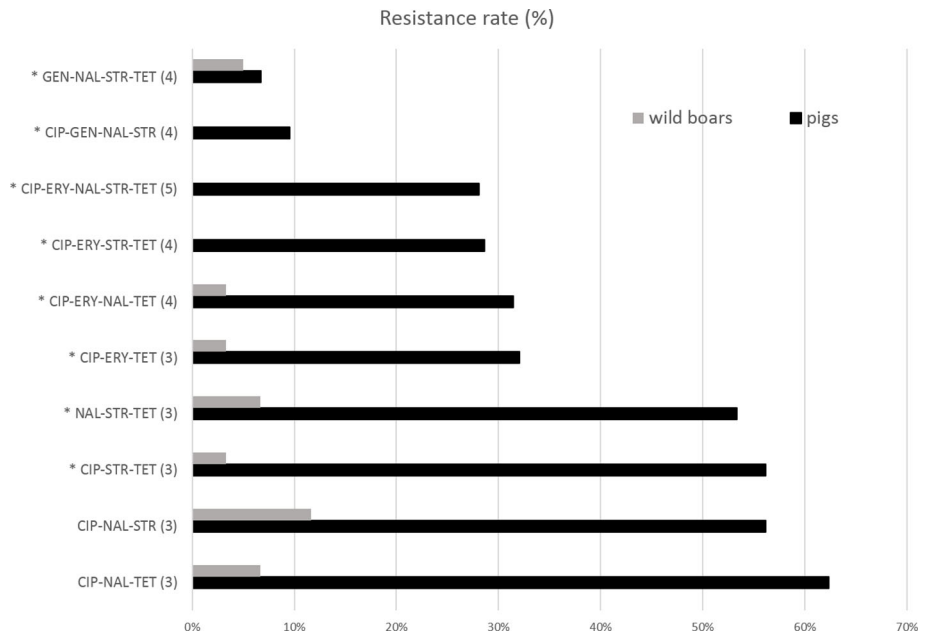

FIGURE 4 | Percentages of resistant isolates to tested antibiotics among *Campylobacter* pig and wild boar isolates. CIP, ciprofloxacin, ERY, erythromycin, GEN, gentamicin, NAL, nalidixic acid, STR, streptomycin, TET, tetracycline. The antibiotic numbers are reported in parenthesis. *= MDR profiles.

population. Interestingly, we noted that three pig strains (ST-829), isolated from pigs born in Denmark, had related wgMLST profiles, although were fattened in 2 different farms located in Pescara and Torino. Similarly, we showed several clusters in pigs with strictly related wgMLST profiles belonging to fattening farms located in different Italian regions. It is likely that fattening farms in Italy and in Europe may share the same feeder pig supplier, which would explainthe genomic relatedness observed in the distant farms. Comparison of our dataset with the strains obtained from *Campylobacter* MLST database revealed that *C. coli* population in Italian pigs and wild boars was different from other European countries. The *C. coli* strains featured with ST circulating only in Italy amounted for 82.7% (67/81) of the entire Italian collection, suggesting a geographical difference between the Italian and European populations. Furthermore, twenty STs were novel, likely representing geographically restricted clones, as reported also by other authors (Stone et al., 2013). Although the lack of WGS data hampered the verification of the genomic relatedness, it was surprising to observe a numerous STs shared between Italy and Scotland, indicating a possible internationally spread driven by the pig industry. However, a limitation of the study was the underrepresentation of *Campylobacter* isolates from wild boars in the PubMLST. As suggested in many studies we likely found several host-associated alleles that are present in *Campylobacter* (French et al., 2005; Miller et al., 2006; Litrup et al., 2007).

In this study, we revealed a clear separation between pig and wild boar *Campylobacter*, as shown by the presence of only three shared STs out of 83. It was also previously suggested that host preference or niche adaptation for certain STs play a role in acquisition and maintenance of specific clones in different host species (Schouls et al., 2003). Although our study did not allow us to draw conclusions on host association, it is likely that wild boars harbour *Campylobacter* STs that are rarely, if ever, transmitted to domestic pigs, possibly due to rare contact between the two hosts. Although wild boars are an environmentally destructive invasive species acting as a reservoir for zoonotic pathogens, our findings suggest that they might not be the primary source of infection of *Campylobacter* for traditional bio-secured domestic pig farms in Italy.

Despite the ban on the application of antibiotics as growth promoters in animal farms in the EU, *C. jejuni* and *coli* isolated from humans and animal sources show high levels of resistance to the most important antimicrobials used to treat campylobacteriosis (Castanon, 2007; EFSA & ECDC, 2019). As well as fluoroquinolones and tetracyclines, *C. coli* strains show a higher resistance to macrolide erythromycin and to aminoglycoside streptomycin, compared to *C. jejuni* (EFSA & ECDC, 2019). This is worrying because the use of fluoroquinolones, known to be the first-choice treatment for campylobacteriosis, has been recently shifted to erythromycin, against which *Campylobacter* resistance seemed to develop more slowly, in respect to fluoroquinolones-resistance (Lapierre et al., 2016). *Campylobacter* resistance mechanisms against the principal antibiotic classes are well known. Fluoroquinolone resistance is rapidly developed in *Campylobacter* strains because it requires only a single point mutation in *gyrA* gene (Luangtongkum et al., 2009). On the contrary, erythromycin resistance is due to specifics mutation in 23S rRNA and also depends on an rRNA methylation enzyme (*erm B*) (Wang et al.,

2014). Tetracycline resistance is associated with the presence of *tet(O)* gene, encoding for a ribosomal protection protein (Sougakoff et al., 1987), while aminoglycosides resistance is due to several genes including *rpsL* and *aph(3')-III* (Iovine, 2013; Zhao et al., 2016). *Campylobacter* is also known as a bacterium naturally resistant against Beta-lactams, (owning the ubiquitous gene *OXA-61*)used in combination with beta-lactamase inhibitors, when fluoroquinolones and macrolides are inefficacious (Griggs et al., 2009). Furthermore, among *C. coli*, which usually harbor AMR genes, a worrying trend towards MDR have been displayed. For all these reasons, *Campylobacter* has been categorized as a high priority pathogen on the list of bacteria for which new antimicrobials are urgently needed (WHO, 2017). In the present study, high levels of resistance to streptomycin, ciprofloxacin and tetracycline were detected in *C. coli* isolated from pigs, with resistance to streptomycin frequently found also among *C. coli* isolated from wild boars. Although the erythromycin resistance levels were lower, the existence of 36% of pig strains resistant to this antibiotic, which is the first-choice drug in the treatment of campylobacteriosis, is alarming. These resistance rates are in line with those reported by other European studies (García-Fernández et al., 2018; Di Donato et al., 2020).

In our study, we found a good correlation between phenotypic resistance to erythromycin, tetracycline, fluoroquinolones and quinolones and the presence of one or more resistance genes or nucleotide polymorphisms expected to confer resistance to the respective antimicrobials. For erythromycin, we found a correlation of 100% and 78.5% between the two types of resistances in pigs and wild boars, respectively. It is possible, that determinants of erythromycin resistance that were not analyzed in our study, such as mutations in L4 and L22 or in the regulatory region of CmeABC efflux pump, could be responsible for enhanced resistance in absence of mutations in 23S rRNA genes (Bolinger and Kathariou, 2017). For tetracyclin, the correlation varied between 68.7% and 55% of the presence of putative resistance genes and observed resistance phenotype respectively in pigs and wild boars. For fluoroquinolones and quinolones, the concordance rate varied between 77% and 45%, in pigs and wild boars. Discrepancies were found for rpsL mutation and the observed phenotype and for aminoglycosides, which could be explained with the existence of the efflux pump mechanisms or other unknown resistance mechanisms. These results suggest that, on one hand, the incidence of AMR in *C. coli* isolated from wild boars could be still considered low, showing that pigs, animals reared for food production, are much more exposed to antimicrobials. On the other hand, the results obtained show us the hazardous spread of AMR genes through the environment. A reassuring finding from our study was that *C. coli* isolated from wild boars have MDR profiles lower than 10%, in respect to MDR profiles of pigs, which were 5 times higher.

In conclusion, a rational and moderate use of antimicrobials, combined with a continuous monitoring of AMR bacteria spread in the environment, should be guaranteed to fight the increase in antibiotic resistance rates, extremely dangerous for human and animal health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LDM and AA carried out the experiment. FM and AJ wrote the manuscript with support from GG. EDG, RN, and GG helped supervise the project. FM and EDG conceived the original idea. GG supervised the project. GDD, AJ, and FP analyzed the data and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.592512/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Minimum spanning tree (MST) generated for 1121 European and Italian strains isolated from pigs and wild boars. The tree was generated using the goeBURST algorithm in PHYLOViZ software. The distance labels correspond to the number of discriminating alleles. The blue nodes correspond to Italian isolates and the red nodes to European isolates.

SUPPLEMENTARY TABLE 1 | List of pig and wild boar isolates with MLST and AMR genes profiles, phenotypic resistance and mutations.

REFERENCES

(OIE) *W.O.f.A.H. Classical Swine Fever*. Available at: https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/CLASSICAL_SWINE_FEVER.pdf (Accessed on 08/05/2020).

Apollonio, M., Andersen, R., and Putman, R. (2010). *European ungulates and their management in the 21st century* (UK: Cambridge University Press), 201–222.

Atterby, C., Mourkas, E., Méric, G., Pascoe, B., Wang, H., Waldenström, J., et al. (2018). The potential of isolation source to predict colonization in avian hosts: a case study in Campylobacter jejuni strains from three bird species. *Front. Microbiol.* 9, 591. doi: 10.3389/fmicb.2018.00591

Bankevich, A., Nurk, S., and Antipov, D. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19 (5), 455–477. doi: 10.1089/cmb.2012.0021

Bolinger, H., and Kathariou, S. (2017). The Current State of Macrolide Resistance in Campylobacter spp.: Trends and Impacts of Resistance Mechanisms. *Appl. Environ. Microbiol.* 83 (12), e00416–e00417. doi: 10.1128/AEM.00416-17

Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult. Sci.* 86, 2466–2471.

Centers for Disease Control and Prevention *Campylobacter (Campylobacteriosis)*. Available at: https://www.cdc.gov/campylobacter/index.html (Accessed on 08/05/2020).

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

Di Donato, G., Marotta, F., Nuvoloni, R., Zilli, K., Neri, D., Di Sabatino, D., et al. (2020). Prevalence, Population Diversity and Antimicrobial Resistance of Campylobactercoli Isolated in Italian Swine at Slaughterhouse. *Microorganisms* 8 (2), 222. doi: 10.3390/microorganisms8020222

EFSA & ECDC (2019). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. *EFSA J.* 17, 1–278.

Egger, R., Korczak, B. M., Niederer, L., Overesch, G., and Kuhnert, P. (2012). Genotypes and antibiotic resistance of Campylobacter coli in fattening pigs. *Vet. Microbiol.* 155 (2-4), 272–278. doi: 10.1016/j.vetmic.2011.08.012

Epps, S. V., Harvey, R. B., Hume, M. E., Phillips, T. D., Anderson, R. C., and Nisbet, D. J. (2013). Foodborne Campylobacter: infections, metabolism, pathogenesis and reservoirs. *Int. J. Environ. Res. Public Health* 10, 6292–6304. doi: 10.3390/ijerph10126292

European Food Safety Authority (EFSA), Aerts, M., Battisti, A., Hendriksen, R., Kempf, I., Teale, C., et al. (2019). Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. *EFSA J.* 17 (6), 5709. doi: 10.2903/j.efsa.2019.5709

French, N., Barrigas, M., Brown, P., Ribiero, P., Williams, N., Leatherbarrow, H., et al. (2005). Spatial epidemiology and natural population structure of Campylobacter jejuni colonizing a farmland ecosystem. *Environ. Microbiol.* 7, 1116–1126. doi: 10.1111/j.1462-2920.2005.00782.x

García-Fernández, A., Dionisi, A. M., Arena, S., Iglesias-Torrens, Y., Carattoli, A., and Luzzi, I. (2018). Human Campylobacteriosis in Italy: Emergence of Multi-Drug Resistance to Ciprofloxacin, Tetracycline, and Erythromycin. *Front. Microbiol.* 9, 1906. doi: 10.3389/fmicb.2018.01906

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

Griggs, D. J., Peake, L., Johnson, M. M., Ghori, S., Mott, A., and Piddock, L. J. V. (2009). Lactamase-mediated ß-lactam resistance in Campylobacter species: prevalence of Cj0299 (bla. OXA-61) and evidence for a novel β-lactamase in C. jejuni. *Antimicrob. Agents Chemother.* 53, 3357–3364.

Hald, B., Skovgard, H., Bang, D., Pedersen, K., Dybdahl, J., Jespersen, J., et al. (2004). Flies and Campylobacter infection of broiler flocks. *Emerg. Infect. Dis.* 10, 1490–1492. doi: 10.3201/eid1008.040129

Hansson, I., Sandberg, M., Habib, I., Lowman, R., and Olsson Engvall, E. (2018). Knowledge gaps in control of Campylobacter for prevention of campylobacteriosis. *Transbound Emerg. Dis.* 65 (1), 30–48. doi: 10.1111/tbed.12870

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

Iovine, N. M. (2013). Resistance mechanisms in Campylobacter jejuni. *Virulence* 4, 230–240.

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., et al. (2016). CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. doi: 10.1093/nar/gkw1004

Jones, B. A., Grace, D., Kock, R., Alonso, S., Rushton, J., Said, M. Y., et al. (2013). Zoonosis emergence linked to agricultural intensification and environmental change. *Proc. Natl. Acad. Sci. U. S. A.* 110, 8399–8404. doi: 10.1073/pnas.1208059110

Lapierre, L., Arias, M. L., and Fernández, H. (2016). Antimicrobial Resistance in Campylobacter spp. In: *Campylobacter spp. and Related Organisms in Poultry*. Eds. B. Fonseca, H. Fernandez and D. Rossi (Cham: Springer). doi: 10.1007/978-3-319-29907-5_10

Letunic, I., and Bork, P. (2006). Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23 (1), 127–128.

Litrup, E., Torpdahl, M., and Nielsen, E. M. (2007). Multilocus sequence typing performed on Campylobacter coli isolates from humans, broilers, pigs and cattle originating in Denmark. *J. Appl. Microbiol.* 103 (1), 210–218. doi: 10.1111/j.1365-2672.2006.03214.x

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

Marotta, F., Garofolo, G., di Marcantonio, L., Di Serafino, G., Neri, D., Romantini, R., et al. (2019). Antimicrobial resistance genotypes and phenotypes of Campylobacter jejuni isolated in Italy from humans, birds from wild and urban habitats, and poultry [published correction appears in PLoS One. 2019 Nov 7;14(11):e0225231]. *PLoS One* 14 (10), e0223804. doi: 10.1371/journal.pone.0223804

Marotta, F., Janowicz, A., Di Marcantonio, L., Ercole, C., Di Donato, G., Garofolo, G., et al. (2020). Molecular Characterization and Antimicrobial Susceptibility of C. jejuni Isolates from Italian Wild Bird Populations. *Pathogens* 9 (4):304. doi: 10.3390/pathogens9040304

Massei, G., Kindberg, J., Licoppe, A., Gačić, D., Šprem, N., Kamler, J., et al. (2015). Wild boar populations up, numbers of hunters down? A review of trends and implications for Europe. *Pest Manage. Sci.* 71, 492–500. doi: 10.1002/ps.3965

McHugh, M. L. (2013). The Chi-square test of independence: Lessonsin biostatistics. *Biochem. Med.* 23, 143–149. doi: 10.11613/BM.2013.018

Meier, R. K. (2015). *Investigations of Health and Abundance of Free-ranging Wild Boar (Sus Scrofa) in Switzerland in a European Context* (Vetsuisse-Fakultät Bern).

Meier, R., and Ryser-Degiorgis, M. (2018). Wild boar and infectious diseases: evaluation of the current risk to human and domestic animal health in Switzerland: A review. *Schweiz Arch. Tierheilkd.* 160 (7–8), 443–460. doi: 10.17236/sat00168

Miller, R. S., and Sweeney, S. J. (2013). Mycobacterium bovis (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. *Epidemiol. Infect.* 141, 1357–1370. doi: 10.1017/s0950268813000976

Miller, W. G., Englen, M. D., Kathariou, S., Wesley, I. V., Wang, G., Pittenger-Alley, L., et al. (2006). Identification of host-associated alleles bymultilocus sequence typing of Campylobacter coli strainsfrom food animals. *Microbiology* 152, 245–255. doi: 10.1099/mic.0.28348-0

Mourkas, E., Florez-Cuadrado, D., Pascoe, B., Calland, J. K., Bayliss, S. C., Mageiros, L., et al. (2019). Gene pool transmission of multidrug resistance among Campylobacter from livestock, sewage and human disease. *Environ. Microbiol.* 21 (12), 4597–4613. doi: 10.1111/1462-2920.14760

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A. P., Carriço, J. A., and Vaz, C. (2017). PHYLOViZ 2.0: Providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* 33, 128–129. doi: 10.1093/bioinformatics/btw582

Navarro-Gonzalez, N., Ugarte-Ruiz, M., Porrero, M. C., Zamora, L., Mentaberre, G., Serrano, E., et al. (2014). Campylobacter shared between free-ranging cattle and sympatric wild ungulates in a natural environment (NE Spain). *Ecohealth* 11 (3), 333–342. doi: 10.1007/s10393-014-0921-3

Okonechnikov, K., Golosova, O., and Fursov, M. (2012). The UGENE team. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 28, 1166–1167. doi: 10.1093/bioinformatics/bts091

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31 (22), 3691–3693. doi: 10.1093/bioinformatics/btv421

Pascoe, B., Méric, G., Yahara, K., Wimalarathna, H., Murray, S., Hitchings, M. D., et al. (2017). Local genes for local bacteria: evidence of allopatry in the genomes of transatlantic Campylobacter populations. *Mol. Ecol.* 26, 4497–4508. doi: 10.1111/mec.14176

Ruiz-Fons, F., Vicente, J., Vidal, D., Höfle, U., Villanúa, D., Gauss, C., et al. (2006). Seroprevalence of six reproductive pathogens in European wild boar (Sus scrofa) from Spain: the effect on wild boar female reproductive performance. *Theriogenology* 65, 731–743. doi: 10.1016/j.theriogenology.2005.07.001

Ruiz-Fons, F., Segalés, J., and Gortázar, C. (2008). A review of viral diseases of the European wild boar: effects of population dynamics and reservoir rôle. *Vet. J. (Lond. Engl. 1997)* 176, 158–169. doi: 10.1016/j.tvjl.2007.02.017

Schouls, L. M., Reulen, S., Duim, B., Wagenaar, J. A., Willems, R. J., Dingle, K. E., et al. (2003). Comparative genotyping of Campylobacter jejuni byamplified fragment length polymorphism, multilocussequence typing, and short repeat sequencing: strain diver-sity, host range, and recombination. *J. Clin. Microbiol.* 41, 15–26. doi: 10.1128/JCM.41.1.15-26.2003

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinform* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Sheppard, S. K., Dallas, J. F., MacRae, M., McCarthy, N. D., Sproston, E. L., Gormley, F. J., et al. (2009a). Campylobacter genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6. *Int. J. Food Microbiol.* 134, 96–103. doi: 10.1016/j.ijfoodmicro.2009.02.010

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

Sippy, R., Sandoval-Green, C. M. J., Sahin, O., Plummer, P., Fairbanks, W. S., Zhang, Q., et al. (2012). Occurrence and molecular analysis of Campylobacter in wildlife on livestock farms. *Vet. Microbiol.* 157, 369–375. doi: 10.1016/j.vetmic.2011.12.026

Sougakoff, W., Papadopoulou, B., Nordmann, P., and Courvalin, P. (1987). Nucleotide sequence and distribution of gene tetO encoding tetracycline resistance in Campylobacter coli. *FEMS Microbiol. Lett.* 44, 153–159.

Stella, S., Tirloni, E., Castelli, E., Colombo, F., and Bernardi, C. (2018). Microbiological Evaluation of Carcasses of Wild Boar Hunted in a Hill Area of Northern Italy. *J. Food Prot.* 81 (9), 1519–1525. doi: 10.4315/0362-028X.JFP-18-077

Stone, D., Davis, M., Baker, K., Besser, T., and Roopnarine, R. (2013). Sharma R. MLST genotypes and antibiotic resistance of Campylobacter spp isolated from poultry in Grenada. *BioMed. Res. Int.* 794643. doi: 10.1155/2013/794643

Waldenström, J., Broman, T., Carlsson, I., Hasselquist, D., Achterberg, R. P., Wagenaar, J. A., et al. (2002). Prevalence of Campylobacter jejuni, Campylobacter lari, and Campylobacter coli in different ecological guilds and taxa of migrating birds. *Appl. Environ. Microbiol.* 68, 5911–5917. doi: 10.1128/aem.68.12.5911-5917.2002

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Woodward, D. L., et al. (2002). Colony multiplex PCR assay for identification and differentiation of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and C. fetus subsp. fetus. *J. Clin. Microbiol.* 40 (12), 4744–4747. doi: 10.1128/jcm.40.12.4744-4747.2002

Wang, Y., Zhang, M., Deng, F., Shen, Z., Wu, C., and Zhang, J. (2014). Emergence of multidrug-resistant Campylobacterspecies isolates with a horizontally acquired rRNA methylase. *Antimicrob. Agents Chemother.* 58, 5405–5412.

Whitehouse, C. A., Zhao, S., and Tate, H. (2018). Antimicrobial Resistance in Campylobacter Species: Mechanisms and Genomic Epidemiology. *Adv. Appl. Microbiol.* 103, 1–47. doi: 10.1016/bs.aambs.2018.01.001

WHO media centre. (2017). WHO publishes list of bacteria for which new antibiotics are urgently needed. Available at: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/ [cited 2017 May 6].

Zankari, E., Allesøe, R., Joensen, K. G., Cavaco, L., Lund, O., and Aarestrup, F. M. (2017). PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J. Antimicrob. Chemother.* 72, 2764–2768. doi: 10.1093/jac/dkx217

Zhao, S., Tyson, G. H., Chen, Y., Li, C., Mukherjee, S., Young, S., et al. (2016). Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in Campylobacter spp. *Appl. Environ. Microbiol.* 82, 459–466.

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Campylobacter jejuni 11168H Exposed to Penicillin Forms Persister Cells and Cells With Altered Redox Protein Activity

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The formation of persister cells is one mechanism by which bacteria can survive exposure to environmental stresses. We show that *Campylobacter jejuni* 11168H forms persister cells at a frequency of 10^{-3} after exposure to 100 × MIC of penicillin G for 24 h. Staining the cell population with a redox sensitive fluorescent dye revealed that penicillin G treatment resulted in the appearance of a population of cells with increased fluorescence. We present evidence, to show this could be a consequence of increased redox protein activity in, or associated with, the electron transport chain. These data suggest that a population of penicillin G treated *C. jejuni* cells could undergo a remodeling of the electron transport chain in order to moderate membrane hyperpolarization and intracellular alkalization; thus reducing the antibiotic efficacy and potentially assisting in persister cell formation.

Keywords: *Campylobacter jejuni*, proteomics, persister cell, antibiotic, electron transport

INTRODUCTION

Campylobacter jejuni is the leading bacterial cause of gastroenteritis in the world estimated be causing almost 100 million cases worldwide (Asuming-Bediako et al., 2019) with 1 million cases a year in the US, and over 250,000 cases in the European Union (Chlebicz and Slizewska, 2018). In many areas of the world the reported incidence of disease appears to be increasing (Chlebicz and Slizewska, 2018). The avian gut is the normal site of carriage of the bacterium and most cases in humans arise from the ingestion of contaminated poultry (Chlebicz and Slizewska, 2018; Igwaran and Okoh, 2019; Rossler et al., 2019). In developing countries, *Campylobacter* is hyper-endemic, a leading bacterial cause of diarrhoeal disease and a major cause of infant mortality (Asuming-Bediako et al., 2019; Igwaran and Okoh, 2019). The bacterium is notoriously fastidious and survives poorly under laboratory conditions, yet it appears to be ubiquitous in the environment, forming reservoirs of infection (Murphy et al., 2006; Bronowski et al., 2014). Environmental survival of the bacterium is essential for transmission to new hosts. Survival outside of the host is also evidenced in the ability of the bacterium to tolerate abiotic stresses encountered during food processing (Ligowska et al., 2011; Garcia-Sanchez et al., 2017).

One mechanism employed by bacteria to survive otherwise lethal stresses is the formation of persister cells (Lewis, 2005, 2007). Persistence has been defined as the ability of a subset of the

population (perisister cells) to survive exposure to a bactericidal drug concentration (Balaban et al., 2019). It is an example of phenotypic switching; the ability of a genetically identical population of organisms to display diverse phenotypes under a given environment (Balaban et al., 2004; Lewis, 2007). There is good evidence that persister cells are present in the bacterial population before exposure to the stress. They can be revealed by exposing the population to a supra-lethal dose of a bactericidal antibiotic (Lewis, 2007; Balaban et al., 2019), which kills most of the bacterial population, except for the persister cells. The ability of persister cells to survive exposure to abiotic stresses, including multiples of the minimum inhibitory concentration (MIC) of antibiotics, biocides and killing by toxic metals, is ascribed to their low growth rates or dormancy (Harrison et al., 2005; Lewis, 2008; Harms et al., 2016). There is also increasing evidence that persister cells make up a subpopulation of antibiotic-resistant cells in biofilms (Lewis, 2007, 2008; Jayaraman, 2008; Harrison et al., 2009; Kim et al., 2009).

The mechanisms by which persister cells form and their molecular makeup have been studied intensively over the past few years and reveal some common mechanisms but have also revealed that a diverse range of molecular events are associated with persister cell formation (Wilmaerts et al., 2019). Toxin-antitoxin systems may play a role by regulating metabolism and targeting functions such as transcription, translation and DNA replication (Harms et al., 2016). Another mechanism involves energy metabolism: a number of reports linking the electron transport chain to persister cell formation (Harms et al., 2016). Drug efflux pumps may contribute to their formation too (Harms et al., 2016). We have previously shown that oxygen availability can influence persister cell formation (Hemsley et al., 2014). At the time of submitting this manuscript there were no reports of the formation of persister cells by *Campylobacter*.

Here, we set out to investigate whether *C. jejuni* 11168H is able to form persister cells and to investigate the molecular makeup of these cells. *C. jejuni* strain 11168H is a strain, selected as representative, for the first Campylobacter genome sequencing project (Parkhill et al., 2000). This should provide new insight into the mechanisms by which *C. jejuni* 11168H survives exposure to antibiotic stresses and could reveal mechanisms that it uses to survive in the environment.

MATERIALS AND METHODS

Growth of *C. jejuni*

C. jejuni 11168H was cultured on Columbia agar plates (CBA) supplemented with either 5–9% (v/v) horse blood or with Skirrow selective supplement (Oxoid Ltd., Basingstoke, UK) and 5–9% (v/v) horse blood in a variable atmosphere incubator (VAIN) (Don Whitley Scientific, Bingley, UK) under microaerobic conditions (5% O_2, 85% N_2, 10% CO_2) at 37°C for 24 or 48 h. The bacteria were sub-cultured into 25 ml of Mueller-Hinton broth (Oxoid) and grown under microaerobic conditions as before (Champion et al., 2010).

Staining With BacLight™

Bacteria were enumerated using *Bac*Light™ Live/Dead Bacterial Viability Kit (Life Technologies, Paisley, UK) according to the manufacturers' instructions. Previous studies have demonstrated the utility of *Bac*Light™ for the differentiation of live and dead *C. jejuni* (Alonso et al., 2002; He and Chen, 2010; Kim et al., 2014). Briefly, aliquots of bacterial cells were treated with premixed stain, mixed and incubated for 15 min in the dark. Two μl of the resulting bacterial suspension was then placed on poly-L-lysine coated glass slides (Sigma, Dorset, UK) along with 2 μl of 1 μm polystyrene latex beads (Sigma), and fluorescence viewed using a Zeiss Fluorescence Microscope with an FITC filter. The total number of cells and the number of specifically stained cells was calculated using Image J software (Schneider et al., 2012).

Staining With Redox Sensor Green

Aliquots of the bacterial cell suspension in 1 ml PBS were stained with 4 μl of *Bac*Light™ Redox Sensor Green reagent (RSG) (Life Technologies) for 10 min in the dark at room temperature and then visualized after excitation at 490 nm by microscopy as described above or flow cytometry as detailed below.

Exposure to Penicillin G

The MIC of penicillin G toward *C. jejuni* 11168H was measured using ETEST® antimicrobial susceptibility test strips (Biomerieux, Basingstoke, UK). Overnight broth cultures were diluted to the equivalent of a McFarland turbidity standard of 0.5 and used to create a lawn on Mueller-Hinton agar plates. The ETEST® strips were added and the plates were incubated at 37°C for 24 h under microaerobic conditions and zones of growth inhibition recorded. We found that the MIC of penicillin G was 11.43 μg/ml. For studies where *C. jejuni* 11168H was exposed to penicillin G, bacteria were first grown in MHB broth for 14 h and the cell harvested by centrifugation and re-suspended in fresh MHB or in MHB containing 100 × the MIC of penicillin G (1,143 μg/ml).

Bacterial Killing by Penicillin G

Broth cultured bacteria were incubated for 24 h with either 100 × the MIC of penicillin G (Sigma) in Mueller Hinton broth or in Mueller Hinton broth alone. At intervals, samples of the cultures were taken, centrifuged and the cell pellet re-suspended in phosphate buffered saline (PBS; pH 7.2). Bacteria were then enumerated as described above. To calculate the number of colony forming units (CFU), bacteria were serially diluted in PBS, plated onto Mueller Hinton agar plates (Oxoid) and incubated at 37°C under microaerobic conditions. The frequency of persister cell formation was calculated as the number of *C. jejuni* cells cultured after 24 h exposure to 100 × MIC of penicillin/number of *C. jejuni* cells cultured before exposure to antibiotic. Assays were carried out in triplicate.

Flow Cytometry

Aliquots of untreated and penicillin G-treated *C. jejuni* 11168H were stained with Redox Sensor Green as described above, and analyzed in a BD FACS Aria III (Becton Dickenson fluorescence-activated cell sorting (FACS) cytometer using a 488 nm laser.

Emission of fluorescence was detected at 530 ± 30 nm. Three distinct RSG stained populations (bright, dim and unstained, 3 × 10^6 cells) were sorted into 10 ml falcon tubes and kept on ice.

The populations of bacterial cells were collected as described above, concentrated by centrifugation at 11,337 × g for 30 min at 4°C and then pooled and lysed in 70 µl of Bugbuster reagent (Merck Millipore, Dorset, UK). After shaking at room temperature for 20 min the bacterial lysates were stored at −80°C.

Mass Spectrometry

Proteomics was performed as described previously (Goggs et al., 2013). Briefly, an UltiMate™3000 nano HPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) was used. The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against UniProt *C. jejuni* strain 11168H database using the SEQUEST algorithm. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5%. Abundance of each protein in each sample was calculated using the average area measurements of the three most abundant peptides matching to each protein (Top3 method) (Ahrne et al., 2013). Normalization of the mass spectrometric data (protein abundances) was performed globally at protein level. Abundance of each protein was expressed as the fraction of the signal derived from the total abundance detected in each sample (Ting et al., 2009). This value was then compared for each protein in the penicillin G treated and control samples.

Proteins with significantly differential abundance were identified using the R packages limma and *q*-value (Smyth, 2004), when proteins with constant abundance across replicates were removed from the analysis. The former package was used for significance analysis, calculating *p*-values. The latter package was used for false discovery rate control, calculating *q*-values. Proteins with a $q < 0.05$ and more than 2-fold change difference in abundance were considered significant.

Online Tools

Cellular localization of the proteins encoded in the *C. jejuni* strain 11168H genome was predicted using PSORTb v.3.0.2 (https://www.psort.org/psortb/) (Yu et al., 2010). *C. jejuni* strain 11168H proteins were classified into functional categories based on clusters of orthologous gene (COG) designations; COG categories were assigned to each protein using eggNOG-mapper (http://eggnog-mapper.embl.de/) (Huerta-Cepas et al., 2017, 2019).

RESULTS AND DISCUSSION

C. jejuni 11168H Forms Persister Cells

We exposed *C. jejuni* 11168H to penicillin G, because beta-lactam antibiotics were previously used successfully to reveal persister cells in other species (Balaban et al., 2019) and globally the penicillins are widely used in poultry farming, as growth promoters and to treat disease (Allen and Stanton, 2014; Manyi-Loh et al., 2018). Therefore, *C. jejuni* in the avian gut could be exposed to high doses of penicillin.

We grew *C. jejuni* 11168H in broth for 14 h (mid-log phase), and then exposed cultures to 100 × the MIC (11.43 µg/ml). At intervals the numbers of total cells, culturable cells, cells strained by *Bac*Light™ and cells stained using RSG were determined for up to 48 h post dosing (**Figure 1**). Staining with RSG reveals metabolic activity because in metabolically active cells redox sensor green is reduced as a consequence of electron transport chain function, forming a green fluorescent dye (Jaen et al., 2019).

The numbers of culturable cells (CFU) and of cells that fluoresced after staining with RSG showed bi-phasic reductions, which is characteristic of the killing of drug-sensitive cells followed by the much slower decline in the number of drug tolerant persister cells (Lewis, 2005). The numbers of cells that stained green with *Bac*Light™ declined over the course of the experiment but did not show a biphasic reduction. When we used older cultures (24 or 48 h instead of than 14 h) for this experiment, we saw similar patterns of changes in total cells numbers, culturable cells, or *Bac*Light™ stained cells (data not shown). The total number of cells did not show the same pattern of decline. When the population that survived exposure to antibiotic was re-cultured in fresh broth, we found that the MIC of penicillin G (11 µg/ml) was similar to that of the population at the start of the experiment. This is consistent with the non-inherited and antibiotic-tolerant phenotype of persister cells. Based on the number of cells that could be cultured after exposure of the population to 100 × MIC of penicillin G for 24 h, we calculate the persister cell frequency to be 5.25×10^{-4}. It is reported (Lewis, 2007) that the frequency of persister cell formation in other bacterial species is typically 10^{-3} to 10^{-6} (Keren et al., 2004; Lewis, 2007). The high frequency with which *C. jejuni* 11168H forms persister cells might explain the ability of this bacterium to survive a wide range of environmental insults when exposed to antibiotics. Since our manuscript was submitted Ovsepian et al. (2020) reported that *C. jejuni* strains 81–176 and RM1221 form persister cells which are revealed by exposure to ciprofloxacin at frequencies of 10^{-5} to 10^{-7} after exposure to this drug for 22 h. This finding confirms that a range of strains of *C. jejuni* can form persister cells. However, these authors were unable to demonstrate persister cells resistant to 100 × MIC of ampicillin. This could indicate strain-specific differences in persister cell formation or differences in the responses to ampicillin or penicillin G exposure. Strain-specific differences in persister cell formation and differences in survival after exposure to different drugs is well-established (Fisher et al., 2017). Further work is required to investigate these possibilities in *C. jejuni*.

Flow Cytometry Reveals Three Populations After Exposure to Penicillin G

We next compared the flow cytometry histograms of cultures of *C. jejuni* 11168H that had been cultured for 14 h in MHB (T0 culture) with the histograms of cells that were subsequently incubated at 37°C under micro-aerobic conditions in either MHB or in MHB containing 100 × MIC of penicillin G. The histograms showed cells grown in MHB, had a normally distributed range of fluorescence signal intensities, ranging from

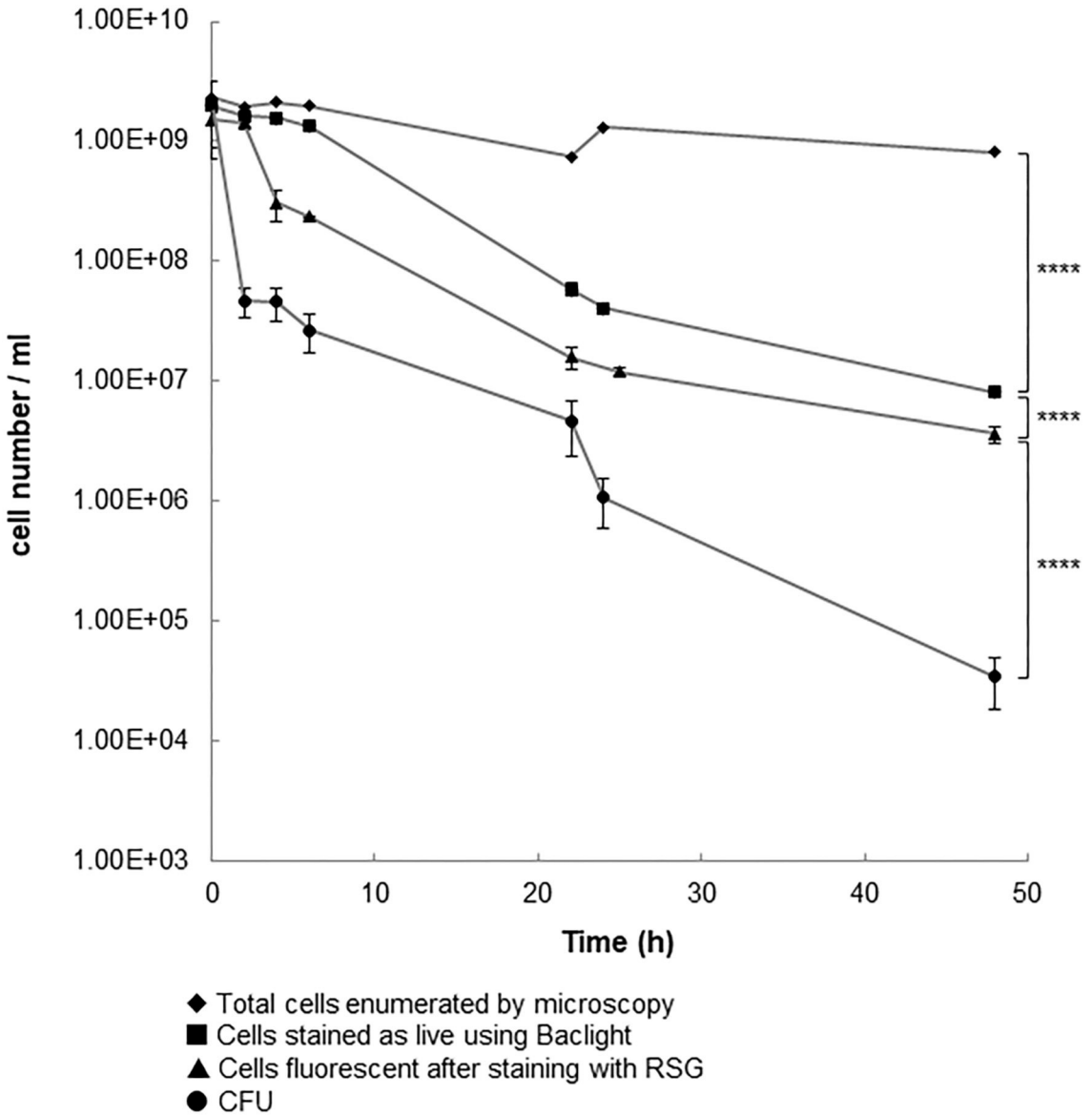

FIGURE 1 | Cell populations following the addition of penicillin G to 14 h old cultures of *C. jejuni* 11168H. A log phase culture (14 h) of *C. jejuni* was incubated for 24 h with 100 × the MIC of penicillin G. Results shown are the mean of three replicates with bars corresponding to the standard error of the mean shown at each data point. ****$p < 0.0001$ (unpaired *t*-test).

dim to bright (**Figure 2**; T0). A small sub-population of cells were unstained.

A parallel culture was not stained or analyzed by flow cytometry, and instead these cells were collected by centrifugation and re-suspended in fresh MHB and incubated for 24 h before staining with RSG and then analyzed using flow cytometry (**Figure 2**; +24 h MHB). In this antibiotic-free control we found a population of cells with a normally distributed range of fluorescence signal intensities from dim to bright. We found that the median signal intensity was reduced compared to the T0 culture.

In the test culture, we processed cells as detailed above but after centrifugation we re-suspended them in MHB containing 100 × MIC of penicillin G and then incubated for 24 h before staining with RSG. After flow cytometry analysis the penicillin G treated culture separated into three populations, labeled P1-P3 (**Figure 2**; +24 h penicillin G).

Proteomic Analysis of *C. jejuni* With Different RSG Fluorescence Signals

We next analyzed the proteomic makeup of three biological replicates of cells incubated for 24 h in MHB (control) or

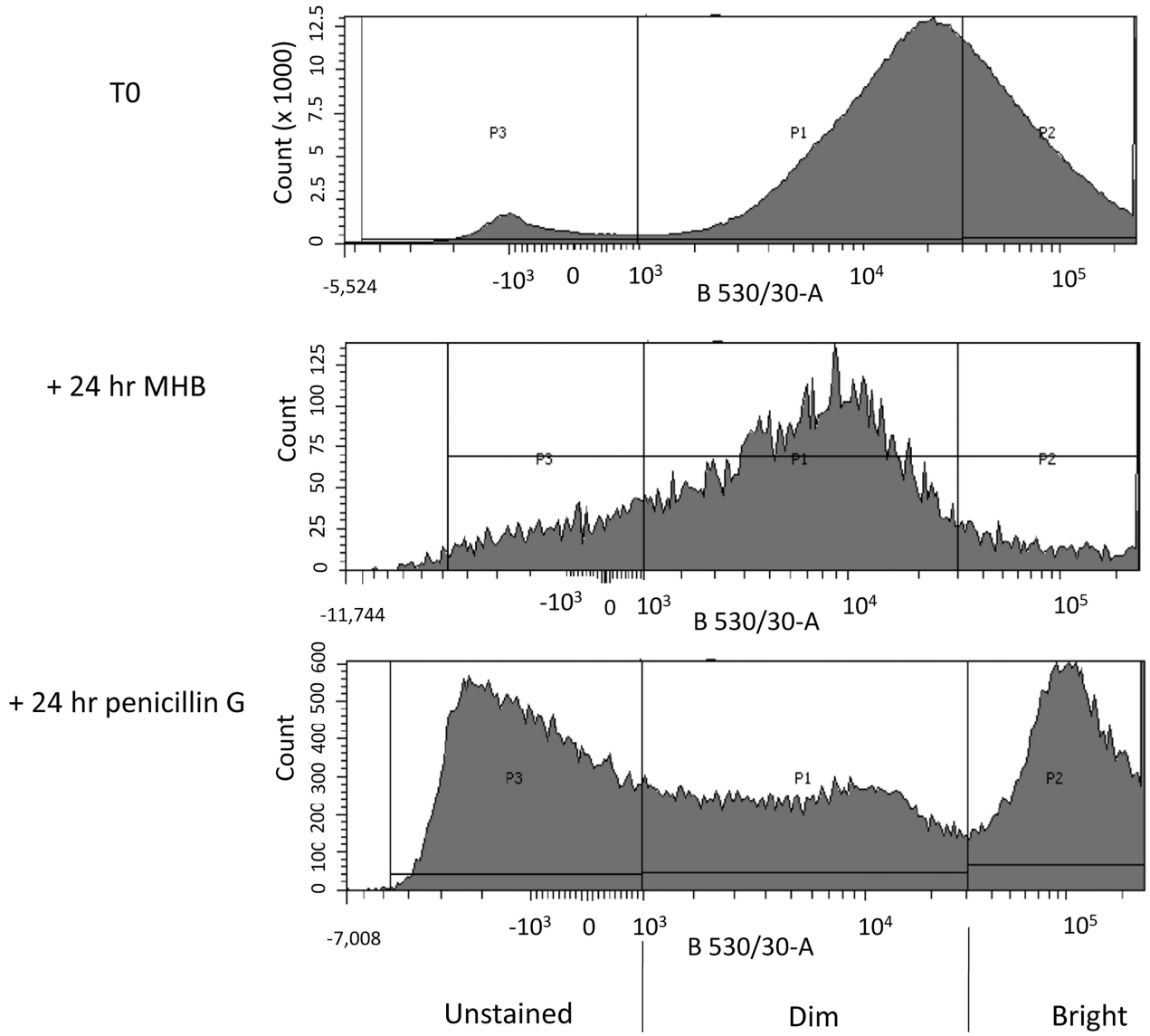

FIGURE 2 | FACS profiles of *C. jejuni* 11168H after staining with RSG. Three biological replicates of each of the following: A log phase (14 h growth) *C. jejuni* culture (T0), a log phase culture incubated for a further 24 h in MHB (control + 24 h MHB) and a log phase culture incubated for a further 24 h in MHB containing 100 × MIC of penicillin G (+ 24 h Penicillin G), were stained with Redox Sensor Green (RSG). The stained cultures were then analyzed by FACS. Fluorescence intensity of RSG stained *C. jejuni* cells at 530 ± 30 nm (x-axis) after excitation with a 488 nm laser is plotted against bacterial cell count (y-axis). Different populations of cells were gated according to the intensity of the RSG fluorescence of each population (P1–stained, P2–brightly stained, and P3–unstained).

three biological replicates of cells incubated for 24 h in MHB containing 100 × MIC of penicillin G. The control or penicillin-treated cultures were stained with RSG and sorted using FACS into cells that did not fluoresce, cells that fluoresced weakly or cells that fluoresced brightly (**Figure 2**). We collected broadly similar numbers of cells (1–3 × 10^6) events in each of these groups and the collected cells were lysed and subjected to tryptic digests to release proteins and analyzed by mass spectrometry. Our results were also normalized according to the abundance of each protein relative to the total protein detected by mass spectrometry in each sample. The *C. jejuni* 11168H genome encodes 1,572 proteins and 95% (1,493) of these were detected in the combined untreated and penicillin G treated samples.

In the control cultures, we first compared the proteomes of the dimly stained cells and brightly stained cells. We did not identify differentially produced proteins, confirming that these were essentially the same population of cells. Considering that the dimly stained cells were the predominant population in the control culture, we next carried out a detailed comparison of the proteome of these cells with the proteome of the brightly stained cells in the penicillin G treated culture (**Supplementary Table 1**).

We identified 1,331 proteins in the dimly stained cells from the control culture and 1,217 proteins in the brightly stained cells from the penicillin G treated culture. A comparison of these two datasets (**Table 1**) revealed 44 significantly more abundant and 87 significantly less abundant proteins in the brightly stained cells from the penicillin G treated culture. These proteins were assigned to COG functional categories (**Figure 3**). The cell membrane/envelope biogenesis and the energy production and conversion groups had the greatest number of proteins with increased abundance. These proteins included the CmeA and CmeC components of the CmeABC efflux pump, which has previously been associated with antibiotic, bile, heavy metals and other antimicrobials in *C. jejuni* (Iovine, 2013). The upregulation of this efflux pump has also been reported after phage infection (Sacher et al., 2018). Our proteome analysis also highlighted MurG (Cj1039) to be over-produced, which catalysis the final intracellular step of peptidoglycan synthesis (Shaku et al., 2020), indicating a possible role of this enzyme in restoring cell wall integrity after exposure to penicillin G. The majority of the proteins with increased abundance were associated with translation and amino acid metabolism and transport.

The increase in RSG fluorescence signal from bright cells after treatment with penicillin G indicates reduction of the dye within the cell. RSG stain will fluoresce when modified by bacterial reductases (Jaen et al., 2019) which are predominantly located in, or associated with, the electron transport chain of the inner membrane.

Analysis of the proteome of the penicillin G treated cells reveals that a component (NuoG, Cj1573c) of an unusual flavodoxin driven membrane-bound quinone-reductase Complex I (an NADH-quinone oxidoreductase but lacking the NADH dehydrogenase module subunits *nuoE* and *nuoF*) (Weerakoon and Olson, 2008) and a component (SdhA Cj0437) of a fumarate reductase (Cj0437-0439) (Taylor and Kelly,

TABLE 1 | Top 20 proteins significantly over-produced (left hand side) or under-produced (right hand side) in *C. jejuni* cells that strained brightly with RSG after exposure to 100 × MIC of penicillin G for 24 h.

Significantly over-produced proteins				Significantly under-produced proteins			
Locus	**Gene name**	**Protein name**	**Fold change**	**Locus**	**Gene name**	**Protein name**	**Fold change**
Cj0755	*cfrA*	Ferric enterobactin uptake receptor	12.4733	Cj1430c	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	∞
Cj1039	*murG*	UDP-N-acetylglucosamine–N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	7.8637	Cj1567c	*nuoM*	NADH-quinone oxidoreductase I subunit M	0.0225
Cj0367c	*cmeA*	Multidrug efflux pump protein CmeA	4.7408	Cj1685c	*bioB*	Biotin synthase	0.0282
Cj0926		Membrane protein	4.1717	Cj1378	*selA*	L-seryl-tRNA(Sec) selenium transferase	0.0415
Cj0365c	*cmeC*	Multidrug efflux pump protein CmeC	3.9992	Cj0117	*pfs*	Aminodeoxyfutalosine nucleosidase	0.056
Cj1357c	*nrfA*	Cytochrome c nitrite reductase cytochrome c552 subunit	3.8977	Cj0314	*lysA*	Diaminopimelate decarboxylase	0.0587
Cj0318	*fliF*	Flagellar MS-ring protein	3.7812	Cj0002	*dnaN*	DNA polymerase III subunit beta	0.0627
Cj0946		Lipoprotein	3.6892	Cj1725		Periplasmic protein	0.0859
Cj0842		Lipoprotein	3.6419	Cj1171c	*ppi*	Peptidyl-prolyl cis-trans isomerase	0.0899
Cj0329c	*plsX*	Phosphate acyltransferase	3.4946	Cj1315c	*hisH*	Imidazole glycerol phosphate synthase subunit HisH	0.0975
Cj1215		Peptidase M23 family protein	3.4565	Cj1720		Hypothetical protein Cj1720	0.1062
Cj0277	*mreC*	Rod shape-determining protein MreC	3.3915	Cj0405	*aroE*	Shikimate 5-dehydrogenase	0.1123
Cj1573c	*nuoG*	NADH-quinone oxidoreductase subunit G	3.3741	Cj1041c		ATP/GTP-binding protein	0.1129
Cj0508	*pbpA*	Penicillin G-binding protein	3.2216	Cj1250	*purD*	Phosphoribosylamine–glycine ligase	0.1131
Cj0151c		Periplasmic protein	3.1399	Cj1670c	*cgpA*	Glycoprotein CpgA	0.1286
Cj1207c		Lipoprotein thiredoxin	3.1258	Cj1601	*hisA*	1-(5-phosphoribosyl)-5	0.1415
Cj0268c		Transmembrane protein	3.1233	Cj0440c		Transcriptional regulator	0.1449
Cj0734c	*hisJ*	Histidine-binding protein	3.0803	Cj0234c	*frr*	Ribosome recycling factor	0.1472
Cj0090		Lipoprotein	3.0552	Cj1516		Oxidoreductase	0.158
Cj0853c	*hemL*	Glutamate-1-semialdehyde aminotransferase	2.8635	Cj0014c		Integral membrane protein	0.159

The proteome of these cells was compared to the proteome of a control culture not exposed to antibiotic. The proteins listed showed an abundance change of 2-fold or more and the q value was < 0.05.

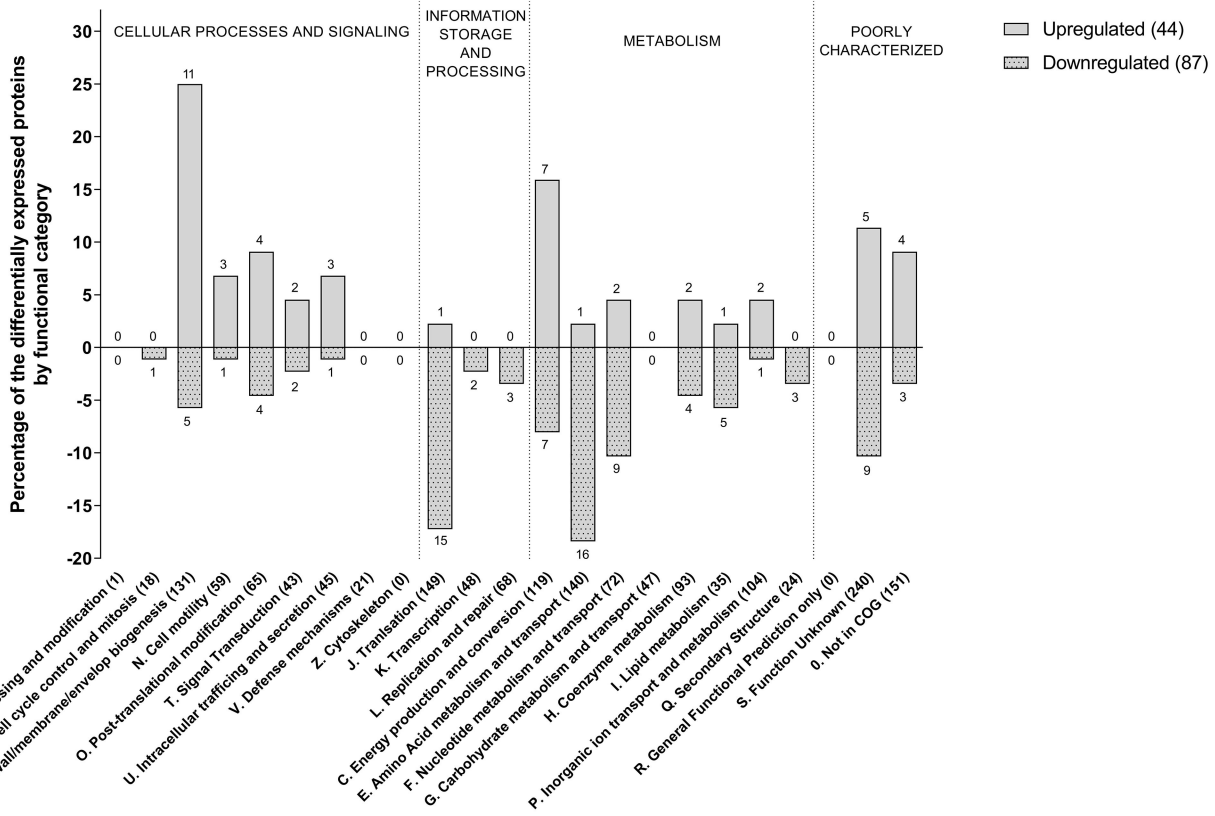

FIGURE 3 | Functional COG categories of differentially produced proteins in penicillin G treated *C. jejuni* cells compared to untreated cells. Only significant produced proteins ($q < 0.05$) with abundance changes of 2-fold or more are shown.

2019) (previously mis-annotated as a succinate dehydrogenase) are significantly over-produced. These two key respiratory complexes are responsible for electron transfer to/from the menaquinone pool during respiration. The β (AtpD Cj1355) and δ (AtpH Cj0104) subunits of the F1 ATP synthase are also significantly over-produced.

In addition, the periplasmic facing cytochrome *c* nitrite reductase (NrfA, Cj 1357c) (Baymukhametov et al., 2018) was over-produced in penicillin treated cells. It is a periplasmic respiratory enzyme that couples to the formate dehydrogenase (Cj1508-1511), via menaquinol oxidation, in order to generate a membrane potential (Δp). NrfA is a pentaheme containing *c*-type cytochrome that catalysis the six-electron reduction of nitrite to ammonium by receiving electrons from menaquinol (Sellars et al., 2002). In *E. coli*, the NrfA nitrite reductase is normally produced during both anoxic and micro-oxic conditions, and in addition to reducing nitrite has also been shown to play a defensive role in NO detoxification (Poock et al., 2002). Our results show that NrfA in *C. jejuni* 11168H is significantly over-produced in penicillin G treated cells with a fold change of 3.9, and could provide a route to an alternative electron acceptor and protect against nitrosative stress (Pittman et al., 2007). The combined effects of these over-produced redox proteins undoubtedly contributes to the enhanced RSG intensity seen in the antibiotic treated cells and indicate a remodeling of the electron transfer chain toward a less electrogenic process. A potential shift from hydrogen or formate oxidation (which provides the highest measurable values of membrane potential of the available electron donors) to utilizing the flavodoxin driven membrane-bound quinone-reductase (Complex I) could help to dissipate the high membrane potential. Similarly, using periplasmic facing electroneutral terminal reductases such as NrfA or fumarate reductase, that accept electrons directly from the menaquinol pool rather than a QCR (Cj1184c−1186c), will also function to reduce the Δp as their $H^+/e^- = 0$ (Taylor and Kelly, 2019). The net effect of these changes would modify the bioenergetics of the system and reduce the number of protons translocated per electron transfer, and thus aid to moderate Δp, ATP production and intracellular pH homeostasis. The reduction in the hyperpolarization of the cytoplasmic membrane could lead indirectly to restrain the ROS formation and reduce intracellular alkalization (Voskuil et al., 2018).

The efficacy of antibiotics being linked to changes in cellular respiration has been reported previously (Lobritz et al., 2015). In *E. coli* and *S. aureus* exposure to that treatment with bactericidal antibiotics gave rise to acceleration in respiration rate, and that inhibition of cellular respiration by creating a knockout mutant deficient in cytochrome *c* oxidase was sufficient to attenuate drug

bactericidal activity. Furthermore, it was demonstrated that when the basal rate of electron transfer was accelerated, by uncoupling the electron transfer chain from ATP synthesis, the effectiveness of the antibiotic was increased. There is a general consensus that, for aerobes, the generation of reactive oxygen species (ROS) and the subsequent oxidative damage of many macromolecules is a known secondary effect of many bactericidal antibiotics including fluoroquinolones, beta-lactams and aminoglycosides (Dwyer et al., 2014).

The reason that potentiation of beta-lactam activity is less pronounced in bacteria grown at low oxygen levels may be because ROS levels are much lower under low oxygen aerobic conditions (Oh et al., 2015). Consistent with this suggestion, we (Hemsley et al., 2014) and others (Hamad et al., 2011) have shown that beta-lactam antibiotics are less effective in killing the Gram-negative bacterium *Burkolderia pseudomallei* under anaerobic compared to aerobic conditions. We would expect low levels of ROS in *C. jejuni* grown under microaerobic conditions. Also, the bacterium has a number of ROS detoxification enzymes (Taylor and Kelly, 2019) including; superoxide dismutase SodB (Cj0169), alkyl hydroxide reductase AhpC (Cj0334), catalase KatA (Cj1385), thiol peroxidases Tpx (Cj0779), bacterioferritin comigratory protein Bcp (Cj0271), two cytochrome *c* peroxidases (Cj0020c and Cj0358) and methionine sulfoxide reductases MsrA and MsrB (Cj0637 and Cj1112), none of which are significantly regulated upon treatment with penicillin G. Therefore, in summary we believe that unlike most bacteria studies to date, ROS do not play a major role in antibiotic killing in *C. jejuni* 11168H.

Persister cell formation in bacteria has previously been associated with reduced levels of metabolic activity and reduced membrane potential (Δp) and can be induced by perturbing the intracellular ATP levels. Although, recently it has been shown that the addition of salicylate can induce persister cell formation in *E. coli* via a mechanism that generates ROS (Wang et al., 2017). It has been suggested that the salicylate induces ROS generation and causes a decrease in membrane potential, which in turn leads to reduced metabolism and an increase in persistence. Further studies with *E. coli* using moderate levels of hydrogen peroxide (300–600 μM H_2O_2) as a direct source of ROS showed a protection against a lethal dose of ofloxacin by increasing persister cell formation by an order of magnitude (Vega et al., 2012). The metabolic burst we saw in penicillin G treated *C. jejuni* cells under microaerobic conditions could result from a remodeling of the electron transport chain to prevent hyperpolarization of the inner membrane and potentially curb intracellular alkalization and limit ROS formation. Therefore, the combination of these changes might play a broader role in supporting persister cell formation.

CONCLUSION

In this study we set out to investigate how antibiotics affect *C. jejuni* 11168H and how this in turn might influence environmental survival. We report that *C. jejuni* 11168H forms persister cells after exposure to penicillin G. Another important finding from our study is the appearance of a population of cells with increased levels of redox proteins in cells exposed to penicillin G, resulting in a greater signal from cells stained with RSG. It is not clear if cells with increased levels of redox proteins are a feature of persister cells and further work would be required to explore this possibility.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021418.

AUTHOR CONTRIBUTIONS

The project was conceived by DS, OC, OS, and RWT. Experimental work was carried out by HM, OC, and RKT. Flow cytometry was enabled and supported by JL and RKT. Data analysis was carried out by AK-S, CB, HM, RKT, RWT, and ZY. Manuscript writing was carried out AK-S, CB, DS, HM, OS, RKT, RWT, SW, and ZY. All authors have read and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.565975/full#supplementary-material

REFERENCES

Ahrne, E., Molzahn, L., Glatter, T., and Schmidt, A. (2013). Critical assessment of proteome-wide label-free absolute abundance estimation strategies. *Proteomics* 13, 2567–2578. doi: 10.1002/pmic.201300135

Allen, H. K., and Stanton, T. B. (2014). Altered egos: antibiotic effects on food animal microbiomes. *Annu. Rev. Microbiol.* 68, 297–315. doi: 10.1146/annurev-micro-091213-113052

Alonso, J. L., Mascellaro, S., Moreno, Y., Ferrús, M. A., and Hernández, J. (2002). Double-staining method for differentiation of morphological changes and membrane integrity of *Campylobacter coli* cells. *Appl. Environ. Microbiol.* 68 5151–5154. doi: 10.1128/AEM.68.10.5151-5154.2002

Asuming-Bediako, N., Parry-Hanson Kunadu, A., Abraham, S., and Habib, I. (2019). *Campylobacter* at the human-food interface: the african perspective. *Pathogens* 8:87. doi: 10.3390/pathogens8020087

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17, 441–448. doi: 10.1038/s41579-019-0196-3

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science (New York, N.Y.)* 305, 1622–1625. doi: 10.1126/science.1099390

Baymukhametov, T. N., Chesnokov, Y. M., Pichkur, E. B., Boyko, K. M., Tikhonova, T. V., Myasnikov, A. G., et al. (2018). Three-dimensional structure of cytochrome c nitrite reductase as determined by cryo-electron microscopy. *Acta Naturae* 10, 48–56. doi: 10.32607/20758251-2018-10-3-48-56

Bronowski, C., James, C. E., and Winstanley, C. (2014). Role of environmental survival in transmission of *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 356, 8–19. doi: 10.1111/1574-6968.12488

Champion, O. L., Karlyshev, A. V., Senior, N. J., Woodward, M., La Ragione, R., Howard, S. L., et al. (2010). Insect infection model for *Campylobacter jejuni* reveals that O-methyl phosphoramidate has insecticidal activity. *J. Infect. Dis.* 201, 776–782. doi: 10.1086/650494

Chlebicz, A., and Slizewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int. J. Environ. Res. Public Health* 15:863. doi: 10.3390/ijerph15050863

Dwyer, D. J., Belenky, P. A., Yang, J. H., MacDonald, I. C., Martell, J. D., Takahashi, N., et al. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U.S.A* 111, E2100–E2109. doi: 10.1073/pnas.1401876111

Fisher, R. A., Gollan, B., and Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* 15, 453–464. doi: 10.1038/nrmicro.2017.42

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

Goggs, R., Harper, M. T., Pope, R. J., Savage, J. S., Williams, C. M., Mundell, S. J., et al. (2013). RhoG protein regulates platelet granule secretion and thrombus formation in mice. *J. Biol. Chem.* 288, 34217–34229. doi: 10.1074/jbc.M113.504100

Hamad, M. A., Austin, C. R., Stewart, A. L., Higgins, M., Vazquez-Torres, A., and Voskuil, M. I. (2011). Adaptation and antibiotic tolerance of anaerobic *Burkholderia pseudomallei*. *Antimicrob. Agents Chemother.* 55, 3313–3323. doi: 10.1128/AAC.00953-10

Harms, A., Maisonneuve, E., and Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science (New York, N.Y.)* 354:aaf4268. doi: 10.1126/science.aaf4268

Harrison, J. J., Ceri, H., Roper, N. J., Badry, E. A., Sproule, K. M., and Turner, R. J. (2005). Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology (Reading, England)* 151, 3181–3195. doi: 10.1099/mic.0.27794-0

Harrison, J. J., Wade, W. D., Akierman, S., Vacchi-Suzzi, C., Stremick, C. A., Turner, R. J., et al. (2009). The chromosomal toxin gene yafQ is a determinant of multidrug tolerance for *Escherichia coli* growing in a biofilm. *Antimicrob. Agents Chemother.* 53, 2253–2258. doi: 10.1128/AAC.00043-09

He, Y., and Chen, C. Y. (2010). Quantitative analysis of viable, stressed and dead cells of *Campylobacter jejuni* strain 81-176. *Food Microbiol.* 27, 439–446. doi: 10.1016/j.fm.2009.11.017

Hemsley, C. M., Luo, J. X., Andreae, C. A., Butler, C. S., Soyer, O. S., and Titball, R. W. (2014). Bacterial drug tolerance under clinical conditions is governed by anaerobic adaptation but not anaerobic respiration. *Antimicrob Agents Chemother.* 58, 5775–5783. doi: 10.1128/AAC.02793-14

Huerta-Cepas, J., Forslund, K., Coelho, L. P., Szklarczyk, D., Jensen, L. J., von Mering, C., et al. (2017). Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. *Mol. Biol. Evol.* 34, 2115–2122. doi: 10.1093/molbev/msx148

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernandez-Plaza, A., Forslund, S. K., Cook, H., et al. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* 47, D309–D314. doi: 10.1093/nar/gky1085

Igwaran, A., and Okoh, A. I. (2019). Human campylobacteriosis: a public health concern of global importance. *Heliyon* 5:e02814. doi: 10.1016/j.heliyon.2019.e02814

Iovine, N. M. (2013). Resistance mechanisms in *Campylobacter jejuni*. *Virulence* 4, 230–240. doi: 10.4161/viru.23753

Jaen, K. E., Velazquez, D., Delvigne, F., Sigala, J. C., and Lara, A. R. (2019). Engineering *E. coli* for improved microaerobic pDNA production. *Bioprocess Biosyst. Eng.* 42, 1457–1466. doi: 10.1007/s00449-019-02142-5

Jayaraman, R. (2008). Bacterial persistence: some new insights into an old phenomenon. *J. Biosci.* 33, 795–805. doi: 10.1007/s12038-008-0099-3

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* 230, 13–18. doi: 10.1016/S0378-1097(03)00856-5

Kim, J. S., Lee, E. J., and Kim, Y. J. (2014). Inactivation of *Campylobacter jejuni* with dielectric barrier discharge plasma using air and nitrogen gases. *Foodborne Pathog. Dis.* 11, 645–651. doi: 10.1089/fpd.2013.1732

Kim, Y., Wang, X., Ma, Q., Zhang, X. S., and Wood, T. K. (2009). Toxin-antitoxin systems in *Escherichia coli* influence biofilm formation through YjgK (TabA) and fimbriae. *J. Bacteriol.* 191, 1258–1267. doi: 10.1128/JB.01465-08

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry (Mosc)* 70, 267–274. doi: 10.1007/s10541-005-0111-6

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* 5, 48–56. doi: 10.1038/nrmicro1557

Lewis, K. (2008). Multidrug tolerance of biofilms and persister cells. *Curr. Topics Microbiol. Immunol.* 322, 107–131. doi: 10.1007/978-3-540-75418-3_6

Ligowska, M., Cohn, M. T., Stabler, R. A., Wren, B. W., and Brondsted, L. (2011). Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int. J. Food Microbiol.* 145 (Suppl. 1), S111–S115. doi: 10.1016/j.ijfoodmicro.2010.08.027

Lobritz, M. A., Belenky, P., Porter, C. B., Gutierrez, A., Yang, J. H., Schwarz, E. G., et al. (2015). Antibiotic efficacy is linked to bacterial cellular respiration. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8173–8180. doi: 10.1073/pnas.1509743112

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23:795. doi: 10.3390/molecules23040795

Murphy, C., Carroll, C., and Jordan, K. N. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Oh, E., McMullen, L., and Jeon, B. (2015). Impact of oxidative stress defense on bacterial survival and morphological change in *Campylobacter jejuni* under aerobic conditions. *Front. Microbiol.* 6:295. doi: 10.3389/fmicb.2015.00295

Ovsepian, A., Larsen, M. H., Vegge, C. S., and Ingmer, H. (2020). Ciprofloxacin-induced persister-cells in *Campylobacter jejuni*. *Microbiology.* 166, 849–853. doi: 10.1099/mic.0.000953

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Pittman, M. S., Elvers, K. T., Lee, L., Jones, M. A., Poole, R. K., Park, S. F., et al. (2007). Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. *Mol. Microbiol.* 63, 575–590. doi: 10.1111/j.1365-2958.2006.05532.x

Poock, S. R., Leach, E. R., Moir, J. W., Cole, J. A., and Richardson, D. J. (2002). Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of *Escherichia coli*. *J. Biol. Chem.* 277, 23664–23669. doi: 10.1074/jbc.M200731200

Rossler, E., Signorini, M. L., Romero-Scharpen, A., Soto, L. P., Berisvil, A., Zimmermann, J. A., et al. (2019). Meta-analysis of the prevalence of thermotolerant *Campylobacter* in food-producing animals worldwide. *Zoonoses Public Health* 66, 359–369. doi: 10.1111/zph.12558

Sacher, J. C., Flint, A., Butcher, J., Blasdel, B., Reynolds, H. M., Lavigne, R., et al. (2018). Transcriptomic analysis of the *Campylobacter jejuni* response to T4-like phage NCTC 12673 infection. *Viruses* 10:332. doi: 10.3390/v10060332

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Sellars, M. J., Hall, S. J., and Kelly, D. J. (2002). Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. *J. Bacteriol.* 184, 4187–4196. doi: 10.1128/JB.184.15.4187-4196.2002

Shaku, M., Ealand, C., Matlhabe, O., Lala, R., and Kana, B. D. (2020). Peptidoglycan biosynthesis and remodeling revisited.

Adv. Appl. Microbiol. 112, 67–103. doi: 10.1016/bs.aambs.2020.04.001

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:3. doi: 10.2202/1544-6115.1027

Taylor, A. J., and Kelly, D. J. (2019). The function, biogenesis and regulation of the electron transport chains in *Campylobacter jejuni*: new insights into the bioenergetics of a major food-borne pathogen. *Adv. Microb. Physiol.* 74, 239–329. doi: 10.1016/bs.ampbs.2019.02.003

Ting, L., Cowley, M. J., Hoon, S. L., Guilhaus, M., Raftery, M. J., and Cavicchioli, R. (2009). Normalization and statistical analysis of quantitative proteomics data generated by metabolic labeling. *Mol. Cell Proteomics* 8, 2227–2242. doi: 10.1074/mcp.M800462-MCP200

Vega, N. M., Allison, K. R., Khalil, A. S., and Collins, J. J. (2012). Signaling-mediated bacterial persister formation. *Nat. Chem. Biol.* 8, 431–433. doi: 10.1038/nchembio.915

Voskuil, M. I., Covey, C. R., and Walter, N. D. (2018). Antibiotic lethality and membrane bioenergetics. *Adv. Microb. Physiol.* 73, 77–122. doi: 10.1016/bs.ampbs.2018.06.002

Wang, T., El Meouche, I., and Dunlop, M. J. (2017). Bacterial persistence induced by salicylate via reactive oxygen species. *Sci. Rep.* 7:43839. doi: 10.1038/srep43839

Weerakoon, D. R., and Olson, J. W. (2008). The *Campylobacter jejuni* NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. *J. Bacteriol.* 190, 915–925. doi: 10.1128/JB.01647-07

Wilmaerts, D., Windels, E. M., Verstraeten, N., and Michiels, J. (2019). General mechanisms leading to persister formation and awakening. *Trends Genet* 35, 401–411. doi: 10.1016/j.tig.2019.03.007

Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., et al. (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26, 1608–1615. doi: 10.1093/bioinformatics/btq249

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Genomic and Phenotypic Characterisation of *Campylobacter jejuni* Isolates From a Waterborne Outbreak

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Campylobacter infections are the leading cause of bacterial gastroenteritis. In Europe, over 246,000 cases are confirmed annually. Infections are often transmitted *via* contaminated food, such as poultry products, but water may be the source of infection as well. The aim of this study was to characterise a selection of *Campylobacter jejuni* human isolates, together with a water isolate, from a waterborne outbreak in Norway in 2019, including human isolates from early, mid-, and late epidemic. The isolates were characterised with whole-genome sequencing, analysing the expression of putative virulence genes and demonstrating the pathogenic potential in an *in vitro* adhesion model using HT-29 cells. All isolates belonged to the multilocus sequence type 1701 and ST45 clonal complex. In the genomic analysis, the water isolate clustered somewhat separately from the human isolates. There was some variation between the human isolates, but the water isolate seemed to display the greatest pathogenic potential, demonstrated by the highest levels of virulence gene expression, adhesion to epithelial cells and IL-8 induction. These results suggest that the water isolate of the study has potential to cause human infections, and that some bacterial changes due to host or environmental adaptation, may occur during a waterborne *Campylobacter* epidemic. This is, to the best of our knowledge, the first study on *C. jejuni* isolates from a waterborne outbreak, including both human isolates and a water isolate, characterised with genomic and phenotypic approaches.

Keywords: *Campylobacter jejuni*, waterborne, whole-genome sequencing (WGS), outbreak, *in vitro* infection model, gene expression, phylogenetic analyses

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INTRODUCTION

According to the World Health Organization (WHO), foodborne illness poses a global public health challenge, as each year 600 million cases of illness is caused by consuming unsafe food or water (World Health Organization, 2015). *Campylobacter* spp., which cause campylobacteriosis, a diarrhoeal disease in humans, is the most frequently reported foodborne illness in the European Union (EU) creating a financial burden of approximately 2.4 billion euro annually [European Food

Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2015]. *C. jejuni* (80%–90%) and *C. coli* (5%–10%) are the two predominant species causing gastrointestinal infections in humans (Blaser, 1997). The most likely source for *Campylobacter* infections is poultry (Skarp et al., 2016), but due to their broad natural reservoir, *Campylobacter* spp. can also be transmitted *via* water. Other known sources include food products, such as unpasteurised milk and contaminated fresh produce (Humphrey et al., 2007). Usually the incubation time ranges between 2 and 5 days (Blaser, 1997). Campylobacteriosis is characterised by symptoms including high fever, headache, nausea, and diarrhoea, which can sometimes be bloody (Blaser, 1997).

Campylobacter may cause waterborne outbreaks, when the water source is faecally contaminated either by runoff of surface water after rainfall or *via* a leakage of a sewage pipe in close proximity to the drinking water pipeline (Thomas et al., 1998). In sparsely populated districts, which are abundant in the Nordic countries, groundwater is often used without treatment (Guzman-Herrador et al., 2015). *Campylobacter* infections show seasonal variation in temperate climates, with an increase of the number of reported cases during the warmer summer months (Nylen et al., 2002). This is equally true for waterborne infections, as people are more likely to spend time outdoors, drink untreated water and use water for recreational purposes (Thomas et al., 1998; Schönberg-Norio et al., 2004). Particularly in the Nordic countries, waterborne outbreaks due to contamination of drinking water by *C. jejuni* are common and comprise about one third of all waterborne outbreaks with known aetiology (Guzman-Herrador et al., 2015). During 1998–2012, *Campylobacter* spp. caused 36 waterborne outbreaks in the Nordic countries, infecting over 7,000 people (Guzman-Herrador et al., 2015). In Norway, waterborne outbreaks are reported every year, with the second most common microorganism involved being *Campylobacter* spp. (Hyllestad et al., 2020). The microbiological analysis of water during an outbreak can be challenging, as the contamination is often a short period, and by the time the outbreak is detected, the contamination episode is over (Hänninen et al., 2003; Hyllestad et al., 2020).

Campylobacteriosis appears to be dependent on several virulence factors involving adhesion, invasion and motility. The adhesion to eukaryotic cells is mediated by several proteins, including the *Campylobacter* adhesion to fibronectin protein (CadF), which binds specifically to fibronectin in the cell membrane (Monteville et al., 2003). *Campylobacter cadF* mutant strains have been reported to have a significant decrease, up to 50%, in cellular adherence (Krause-Gruszczynska et al., 2007). *Campylobacter* invasion-associated markers include *iamA*, which has been proposed to be a significant virulence factor, but its function remains unknown. It has been reported that *iamA* is expressed in the majority of invasive, but only in a minority of non-invasive *Campylobacter* isolates (Carvalho et al., 2001). *Campylobacter* may secrete a cytolethal distending toxin (CDT), which is an AB toxin composed of three subunits encoded by *cdtA*, *cdtB*, and *cdtC*. CDT has been proposed to be a notable virulence factor for *Campylobacter*, as CdtB-negative mutants were shown to remain completely inactive in HeLa cell cytotoxicity assays. Similarly, in an animal study using immunocompromised mice, CdtB-negative *C. jejuni* demonstrated impaired invasiveness into blood, spleen and liver tissues (Purdy et al., 2000). It has been suggested that all three CDT proteins are membrane-associated and required for the induction of pro-inflammatory IL-8 (Hickey et al., 2000).

The exact molecular pathogenesis of *Campylobacter* infections remains unclear, as there has been a lack of suitable *in vivo* models that would elucidate the disease in humans (Bereswill et al., 2011). However, *in vitro* infection models showing interactions between bacteria and epithelial cell lines can be used to study the pathogenic potential of *Campylobacter* to cause human infections. The adherence of the bacteria to the cells and the release of pro-inflammatory chemokines, like IL-8 can be quantified *in vitro* (Skarp et al., 2017). To closer examine the genomic characteristics of *Campylobacter* isolates, whole-genome sequencing (WGS) has emerged as an effective method, which offers a high-resolution to observe even minor genomic differences. WGS can increase the understanding of the evolutionary and epidemiological dynamics of *Campylobacter* infections and has the potential to improve surveillance and outbreak detection (Llarena et al., 2017).

In this study, a selection of *C. jejuni* isolates from a recent Norwegian waterborne outbreak was characterised using three approaches: genomic, transcriptional, and *in vitro*. Human *C. jejuni* isolates chosen for the study were selected based on sampling date, including isolates from early, mid-, and late epidemic. In addition, a water isolate, the assumed source isolate of the outbreak, was characterised alike. The aim of the project was to demonstrate possible changes occurring on a genomic, transcriptional and *in vitro* level throughout the epidemic and examine the pathogenic potential of the isolates using WGS, expression of putative virulence genes and an *in vitro* infection assay with human HT-29 colon cancer cells.

MATERIALS AND METHODS

Preparing the Bacterial Isolates

During June 2019, *Campylobacter* caused a large waterborne outbreak in Askøy, north-west of Bergen, Norway, where more than 2,000 inhabitants became infected. The outbreak was due to contamination of the municipal drinking water system (Paruch et al., 2020). For this study, the *Campylobacter* isolates were received from the Department of Microbiology at Haukeland University Hospital, responsible for analysing the faecal samples from the affected inhabitants in the outbreak area. All faecal samples sent to Haukeland University Hospital were originally analysed with real-time PCR (Gastro Panel, LightMix Modular *Campylobacter*, TIB MOLBIOL, Berlin, Germany) and samples positive for *C. jejuni* were further cultivated on selective agar plates. The *Campylobacter* isolates were kept in −80°C prior to transportation to Uppsala on faecal swabs (Fecal Transwab, Medical wire, Corsham, UK). The anonymised isolates were

chosen for characterisation based on the stage of the epidemic (**Table 1**). In addition, *C. jejuni* strains NCTC 11168 and 81-176 were included for reference in the experiments. The bacteria were streaked on blood agar plates and incubated at 42°C for 48 h in a microaerobic atmosphere (Campygen, Oxoid, Basingstoke, UK). The isolates were kept in −80°C until needed. All the characterisation experiments were performed on isolates cultured directly from −80°C freezer to keep the passage number low.

Genomics

The *C. jejuni* isolates were cultured for 24 h on blood agar plates prior to DNA extraction. DNA was extracted with the Qiagen EZ1 DNA Tissue Kit (Qiagen Sciences, Germantown, MD, USA), and subjected to WGS. The sequencing itself was carried out by the Swedish Veterinary Institute using MiSeq Desktop Sequencer (Illumina, San Diego, CA, USA). The sequences were assembled into contigs using Geneious (version 8.1.9.) with the Mira plugin (version 1.1.1.) and merging contigs were assembled with Geneious. Read mapping of the virulence genes was done with the raw sequence data to confirm presence of the selected genes that were examined in the *in vitro* and transcriptional experiments. The genetic distance was calculated using the BLASTN algorithm in accurate mode in Gegenees 3.1 with the following parameters: fragment size: 200 bp; step size: 100 bp. Then, a heat map comparing core genomes was constructed using a 20% threshold along with the reference strains NCTC 11168 and 81-176. A neighbour-joining tree based on the core genomes was produced using Geneious (version 8.1.9.). The sequences were deposited to the NCBI database under the project PRJNA656683.

RNA Preparation and cDNA Synthesis

The bacterial RNA was extracted using the ISOLATE II RNA Mini kit (Bioline Reagents Ltd, London, UK) according to the manufacturer's protocol, with two DNase I treatments (Ambion by life technologies, Carlsbad, USA): on column and on the final RNA preparation. The concentration of RNA was measured using NanoDrop (NanoDrop ND-1000, Thermo Fisher Scientific). The integrity of RNA was tested by running 0.5 µg RNA on a 1% agarose gel with 3% chlorine. For cDNA synthesis, 500 ng of RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Fisher Scientific). For the qPCR to determine expression of virulence genes, 1 µl of 10× diluted cDNA was used. Further experiments were performed on four biological extraction replicates.

Real-Time qPCR

Real-time qPCRs were run in the BioRad CFX96 cycler using the DyNAmo HS SYBR green mix (Thermo Fisher Scientific) according to the manufacturer's protocol. The virulence gene primers were: F-CGCGTTGATGTAGGAGCTAA and R-GCTC CTACATCTGTTCCTCCA for *cdtB*, F-TGGTTTAGCAGG TGGAGGATATG and R-GTTGAAACCCAATTATGGTT TGCATGA for *cadF*, F- TGGAAGTGGAAAATCCGTCCTT and R- GTGCAGCAAACTGAAAAACCACA for *iamA*. *Campylobacter* 16S rRNA primers (Skarp et al., 2017) were included for normalisation.

In Vitro Cell Adhesion Assay and IL-8 ELISA

The pathogenic potential of the selected epidemic *C. jejuni* isolates to cause infections in humans was investigated using an *in vitro* infection model with human HT-29 (ECACC 91072201) colon cancer epithelial cells as prior described (Nilsson et al., 2017). The HT-29 cells were maintained in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) (Gibco by life technologies, Carlsbad, California, US), 2 mM glutamine, 100 U/ml PEST (penicillin, streptomycin) and 100 µg/ml gentamycin (Swedish Veterinary Institute, Uppsala, Sweden). The cells were incubated at 37°C with 5% CO_2 and were routinely split before reaching approximately 90% confluency, which was determined with microscopy. For the infections, a confluency of 60%–70% was used.

For the adhesion assay, the bacterial suspensions in RPMI 1640 without antibiotics were added to HT-29 cells grown in RPMI 1640 supplemented with 1% FCS to obtain a multiplicity of infection (MOI) of 100. The cells were incubated for 3 h at 37°C. The cells and bacteria were then harvested for downstream analyses. The cells were washed in PBS to remove non-adhered bacteria and lysed (20 mM Tris, pH 7.5, 150 mM NaCl, 0.15% Triton X-100). The lysate was diluted 10× and 100× for qPCR analysis of the 16S rRNA gene together with 10,000× diluted bacteria to determine the adhesion percentage. The qPCR was run with BioRad CFX96 using DyNAmo HS SYBR green mix (Thermo Fisher Scientific) according to the manufacturer's protocol.

To investigate IL-8 induction, the cell media from the infected cells was removed and diluted four times before analysis using IL-8 ELISA (Thermo Fisher Scientific) according to the manufacturer. The *in vitro* infection assay and IL-8 ELISA were performed on six biological replicates.

TABLE 1 | *C. jejuni* isolates included in the study from different stages of the waterborne epidemic.

C. jejuni isolate	Source	Sampling date	Accession number	Number of Contigs	N50
2	human, faecal	09/07/2019	JACLAY000000000	91	34,442
9	human, faecal	03/07/2019	JACLAZ000000000	198	15,431
13	human, faecal	25/06/2019	JACLBA000000000	169	21,191
18	human, faecal	20/06/2019	JACLBB000000000	87	50,383
46	human, faecal	12/06/2019	JACLBC000000000	122	26,009
28	tap water	19/06/2019	JACLBD000000000	441	17,675

RESULTS

This study characterised human isolates and a water isolate from a waterborne outbreak of *C. jejuni*, which occurred in Askøy, an island northwest of Bergen, Norway during the summer of 2019. According to the local health authorities, approximately 2,000 residents fell ill. This was the largest waterborne outbreak of *C. jejuni* to date in Norway (Hyllestad et al., 2020).

Phylogenetic Analysis

There are currently more than 10,000 sequence types (STs) that have been typed according to the multilocus sequence typing (MLST) system (https://pubmlst.org). All the isolates of the present study belonged to the same ST45 clonal complex (CC) and were of the same ST1701. Phylogenomic reconstruction based on fragment alignment showed that human epidemic isolates of *C. jejuni* 2, 9, 13, 18, and 46 were closely clustered together (**Figure 1**). The epidemic water isolate clustered close to the human epidemic isolates, but remained still distinguishably separate. The human epidemic isolates had on average between 98.7% and 99.6% similar core genomes, whereas the water isolate differed from the other epidemic isolates with an average of one to two percent (**Table 2**). Accordingly, a slight branching of the water isolate away from the other epidemic *C. jejuni* isolates could be observed in the neighbour-joining tree, also based on the core genomic similarities (**Figure 1**).

Expression of Putative Virulence Genes

The expression of selected virulence genes (*cadF*, *iamA*, and *cdtB*) was examined with qPCR (**Figure 2**). The presence of all the selected genes was additionally confirmed by read mapping the raw sequence data to virulence gene references. The expression of the genes was demonstrated as fold increase over the reference strain NCTC 11168. The water isolate showed clearly higher expression levels for all the genes analysed as compared to the human epidemic isolates. In comparison to the reference strains, the expression levels of the water isolate were 5–7 fold for all the genes tested. For *cadF*, the human isolates showed in general low expression levels except for the isolate 2 from the late epidemic. The very same human epidemic isolate 2 showed, together with the isolate 46 from early epidemic, higher expression levels than other human epidemic isolates for *iamA*. In general, human epidemic isolates showed higher expression levels for *cdtB* than for *iamA* and *cadF*. Only isolate 18 from the mid-epidemic showed low expression levels for *cdtB*, too.

In Vitro Infection Assay and IL-8 Induction

The pathogenic potential was examined by infecting HT-29 cells with the epidemic isolates followed by measuring bacterial adherence and the induced IL-8 response. The IL-8 induction results were expressed as fold increase over uninfected (mock) cells, while adherence was calculated as a percentage of the

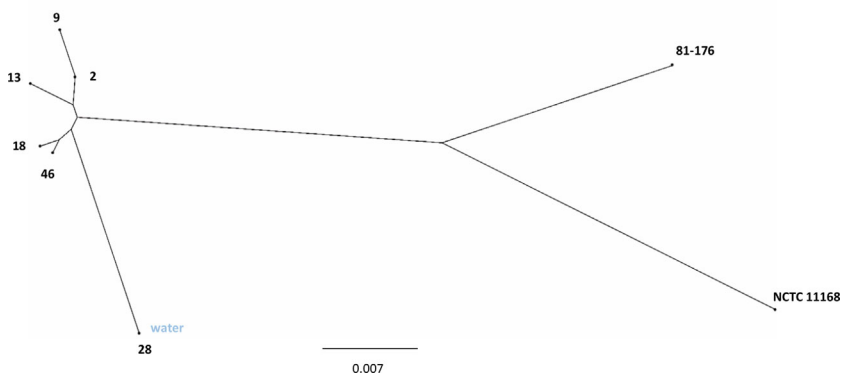

FIGURE 1 | Phylogenetic analysis of *C. jejuni* waterborne outbreak isolates. Neighbour-joining tree based on core genomic similarity displaying the *C. jejuni* human epidemic isolates (2, 9, 13, 18, 46) with the *C. jejuni* water epidemic isolate (28) as well as *C. jejuni* reference strains NCTC 11168 and 81-176.

TABLE 2 | Genomic core similarity of *C. jejuni* isolates.

Isolate	NCTC 11168	81-176	2	9	13	18	46	28
NCTC 11168	100	94.8	93	92.4	92.7	93.1	92.9	92.7
81-176	94.8	100	94.2	93.5	93.8	94.2	94	93.8
2 (late)	93.3	94.4	100	98.7	99.1	99.6	99.4	99.1
9 (late)	93.4	94.5	99.8	100	99.4	99.8	99.7	99.4
13 (mid)	93.2	94.4	99.7	98.9	100	99.7	99.6	99.2
18 (mid)	93.3	94.4	99.6	98.7	99.1	100	99.5	99.1
46 (early)	93.4	94.4	99.6	98.8	99.2	99.7	100	99.2
28 (water)	92	93	98	97.2	97.5	97.9	97.9	100

Heat-plot illustrating the average genomic core similarities between the waterborne outbreak human isolates (2, 9, 13, 18, 46) and water isolate (28) along with C. jejuni reference strains NCTC 11168 and 81-176. The scores were calculated using a threshold value of 20% for the normalised BLAST-score to exclude genetic material not included in the core genome. Green represents a high similarity and lighter green/yellow represents a lower similarity.

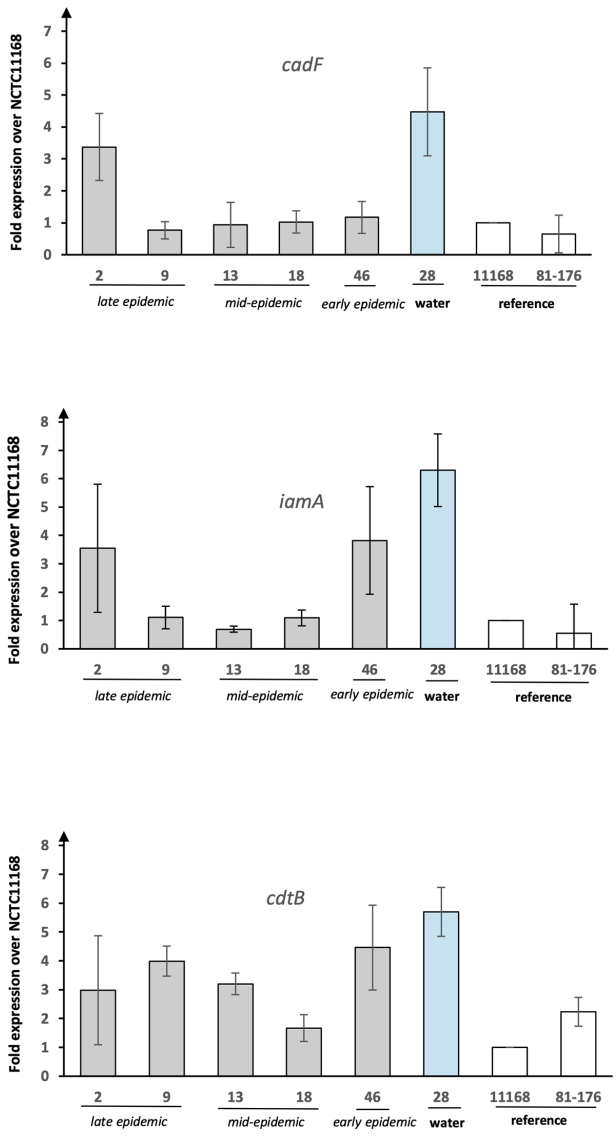

FIGURE 2 | The expression of putative virulence genes from *C. jejuni* waterborne epidemic isolates and two reference strains. Results for the genes *cadF*, *iamA*, and *cdtB* shown as fold increase over reference strain NCTC 11168. Mean values of four biological replicates with error bars indicating standard deviations are shown.

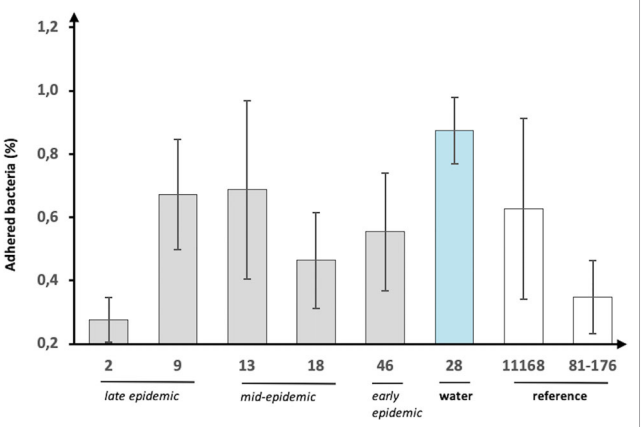

FIGURE 3 | Adherence of *C. jejuni* waterborne epidemic isolates and two reference strains to HT-29 cells. The adherence (%) shown as percentage of the starting inoculum. Mean values of six biological replicates with error bars indicating standard deviations are shown.

starting inoculum. All the isolates adhered to the HT-29 cells but there was substantial variation between the epidemic isolates (**Figure 3**). Notably, the water isolate showed the highest adherence with almost 1% while the human isolates ranged between approximately 0.3–0.7%. Conversely, human isolate 2, which was from the late epidemic, had the lowest adherence to the cells with 0.28% only. In general, there was no correlation between the sampling date (early, mid-, or late epidemic) and the adhesion level observed.

IL-8 responses also showed variation between the epidemic isolates, but the water isolate had the highest induction of IL-8, with a 7× induction rate over the mock (**Supplementary Figure 1**). There was no correlation between the date of sampling (early, mid-, or late epidemic) and the IL-8 level observed. Isolates 13 and 46 were the only isolates that induced a lower IL-8 response, but had higher adherence compared to the 81-176 reference strain.

DISCUSSION

Here, a selection of epidemiologically related *C. jejuni* isolates from a 2019 waterborne outbreak in Norway, including human isolates from early, mid-, and late epidemic, and an assumed source isolate from water, were characterised. The results obtained from the genomic analyses supported the epidemiological relatedness, but transcriptional and *in vitro* infection experiments showed interesting differences between the human epidemic isolates and the water isolate. In the phylogenetic analysis, the water isolate clustered somewhat separately from the closely clustered human isolates, and when the expression levels of selected virulence genes were analysed, the water isolate displayed the highest expression levels. In addition, when the pathogenic potential of the epidemic isolates was further analysed in an *in vitro* infection model with HT-29 cells, the water isolate demonstrated evidently higher adherence to the epithelial cells and induction of IL-8.

WGS offers the highest degree of resolution of the typing methods available and is rapidly replacing the traditional molecular methods, such as MLST, in the investigation of *Campylobacter* outbreaks (Llarena et al., 2017). Indeed, the use of WGS-based typing may change the epidemiological understanding of campylobacteriosis, as a study conducted in Denmark suggested, that a large proportion of *Campylobacter* infections are not sporadic, but rather consist of clusters of human infections (Joensen et al., 2020).

In a Finnish study, WGS analysis of *C. jejuni* isolates, which originated from a waterborne outbreak and had been originally

characterised using pulsed-field gel electrophoresis (PFGE), revealed some genomic differences among the isolates when re-examined (Revez et al., 2014). According to the results of the aforementioned study, the waterborne outbreak had possibly been caused by at least two closely related *C. jejuni* isolates (Revez et al., 2014). This study, in addition to ours, emphasises the high-resolution discriminatory power offered by WGS, which not only leads to an exact source identification in epidemic investigations, but also allows the detection of minor genomic differences between *Campylobacter* outbreak isolates. Whether the minor changes in outbreak isolates occur during human infections, or at another unknown stage of the outbreak, or if the findings actually refer to the presence of several source isolates, cannot always be answered. Although genomic changes detected by WGS have been shown to occur in *C. jejuni* during human infection (Revez et al., 2013), it cannot be entirely excluded that the genomic variation among the outbreak-related isolates observed in the present study could have been due to an original mixture of several *C. jejuni* isolates. Recently, the potential source of contamination for the outbreak of the current study was described as being of equine faecal origin. This was determined by DNA-based faecal source tracking, which pointed to possible cracks in the drinking water pool (Paruch et al., 2020).

The outbreak isolates of the present study were all of the same ST1701 which belongs to the ST45CC. ST45CC is one of the most common lineages of *C. jejuni*, consisting of generalists frequently detected from various hosts (Dearlove et al., 2016). *C. jejuni i*solates belonging to ST45CC have been reported to be abundant in especially chickens and wild birds (Sheppard et al., 2009). Interestingly, a strikingly analogous *C. jejuni* water isolate originating from a waterborne epidemic in Finland (Revez et al., 2014) was detected. This particular water isolate belonged to ST45, the founder ST within ST45CC, and shared almost 98% core genome similarity to the *C. jejuni* water isolate of the present study (data not shown) although isolated almost 20 years prior the Norwegian isolate described here. It remains to be studied if certain lineages within ST45CC are more successful than others in causing waterborne outbreaks.

All of the *C. jejuni* epidemic isolates tested were able to adhere to and induce an IL-8 response in the HT-29 cell line and thus showed pathogenic potential to cause infection in humans. However, there was variation between the epidemic isolates in their pathogenic potential and interestingly, the water isolate demonstrated the highest adhesion level to the epithelial cells as well as the highest induction rate of IL-8. We have recently studied *C. jejuni* and *C. coli* water isolates in order to better understand characteristics needed for water survival and the potential to cause waterborne infections. We have identified both genomic and phenotypic differences between *C. jejuni* and *C. coli* water isolates (Nilsson et al., 2017; Nilsson et al., 2018b) and suggested that certain *Campylobacter* isolates could have the potential to survive better in water (Nilsson et al., 2018a). However, the water isolates studied prior were not known to be associated with any human infections. In fact, the *C. jejuni* water isolates in the previous study had considerably lower levels of IL-8 induction and cellular adherence to HT-29 cells compared to the NCTC 11168 reference strain (Nilsson et al., 2017), which was also used in the present study. Interestingly, in the current study, the assumed source isolate of the waterborne outbreak was highlighted both in the *in vitro* infection assays and virulence gene expression analyses showing the highest pathogenic potential *in vitro* as compared to the human epidemic isolates and the reference strains. Thus, in addition to the earlier investigations showing the epidemiological relatedness of the water isolate in time, location and source, the present findings further support its role in the outbreak.

In the present study, we had the opportunity to study the genomic, transcriptional and phenotypic variation within epidemiologically related *C. jejuni* isolates from a recent waterborne outbreak. The study offers insight into the similarities, but also the differences between the outbreak isolates and highlights the pathogenic potential of the water isolate. To our current knowledge, this combination of genomic and phenotypic approaches in analyses of human and water outbreak isolates is unique.

DATA AVAILABILITY STATEMENT

The sequence data used in this study can be found online at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA656683.

AUTHOR CONTRIBUTIONS

Conceptualisation: HR. Methodology: CJ and RK. Validation: CJ and HR. Formal analysis: ED and RK. Investigation: ED. Resources: ME and HR. Writing – original draft preparation: ED. Writing –review and editing: ED, ME, RK, and HR. Supervision: RK and HR. Project administration: HR. Funding Acquisition: HR. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.594856/full#supplementary-material

REFERENCES

Bereswill, S., Fischer, A., Plickert, R., Haag, L.-M., Otto, B., Kühl, A. A., et al. (2011). Novel murine infection models provide deep insights into the "ménage à trois" of Campylobacter jejuni, microbiota and host innate immunity. *PloS One* 6, e20953. doi: 10.1371/journal.pone.0020953

Blaser, M. J. (1997). Epidemiologic and Clinical Features of Campylobacter jejuni Infections. *J. Infect. Dis.* 176, S103–S105. doi: 10.1086/513780

Carvalho, A. C., Ruiz-Palacios, G. M., Ramos-Cervantes, P., Cervantes, L. E., Jiang, X., and Pickering, L. K. (2001). Molecular characterization of invasive and noninvasive Campylobacter jejuni and Campylobacter coli isolates. *J. Clin. Microbiol.* 39, 1353–1359. doi: 10.1128/JCM.39.4.1353-1359.2001

Dearlove, B. L., Cody, A. J., Pascoe, B., Méric, G., Wilson, D. J., and Sheppard, S. K. (2016). Rapid host switching in generalist Campylobacter strains erodes the signal for tracing human infections. *ISME J.* 10, 721–729. doi: 10.1038/ismej.2015.149

European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (2015). *Campylobacter*. Available at: https://www.efsa.europa.eu/en/topics/topic/campylobacter (Accessed July 7, 2020).

Guzman-Herrador, B., Carlander, A., Ethelberg, S., Freiesleben de Blasio, B., Kuusi, M., Lund, V., et al. (2015). Waterborne outbreaks in the Nordic countries 1998 to 2012. *Euro Surveill.* 20, 37–46. doi: 10.2807/1560-7917.es2015.20.24.21160

Hänninen, M.-L., Haajanen, H., Pummi, T., Wermundsen, K., Katila, M.-L., Sarkkinen, H., et al. (2003). Detection and Typing of Campylobacter Jejuni and Campylobacter Coli and Analysis of Indicator Organisms in Three Waterborne Outbreaks in Finland. *Appl. Environ. Microbiol.* 69, 1391–1396. doi: 10.1128/AEM.69.3.1391-1396.2003

Hickey, T. E., McVeigh, A. L., Scott, D. A., Michielutti, R. E., Bixby, A., Carroll, S. A., et al. (2000). Campylobacter jejuni cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect. Immun.* 68, 6535–6541. doi: 10.1128/iai.68.12.6535-6541.2000

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

Hyllestad, S., Iversen, A., MacDonald, E., Amato, E., Borge, B. Å. S., Bøe, A., et al. (2020). Large waterborne Campylobacter outbreak: use of multiple approaches to investigate contamination of the drinking water supply system, Norway, June 2019. *Euro Surveill.* 25, 2000011. doi: 10.2807/1560-7917.ES.2020.25.35.2000011

Joensen, K. G., Kiil, K., Gantzhorn, M. R., Nauerby, B., Engberg, J., Holt, H. M., et al. (2020). Whole-Genome Sequencing to Detect Numerous Campylobacter jejuni Outbreaks and Match Patient Isolates to Sources, Denmark 2015–2017. *Emerg. Infect. Dis. J.* 26, 523. doi: 10.3201/eid2603.190947

Krause-Gruszczynska, M., van Alphen, L. B., Oyarzabal, O. A., Alter, T., Hänel, I., Schliephake, A., et al. (2007). Expression patterns and role of the CadF protein in Campylobacter jejuni and Campylobacter coli. *FEMS Microbiol. Lett.* 274, 9–16. doi: 10.1111/j.1574-6968.2007.00802.x

Llarena, A.-K., Taboada, E., and Rossi, M. (2017). Whole-Genome Sequencing in Epidemiology of Campylobacter jejuni Infections. *J. Clin. Microbiol.* 55, 1269–1275. doi: 10.1128/JCM.00017-17

Monteville, M. R., Yoon, J. E., and Konkel, M. E. (2003). Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology* 149, 153–165. doi: 10.1099/mic.0.25820-0

Nilsson, A., Johansson, C., Skarp, A., Kaden, R., Engstrand, L., and Rautelin, H. (2017). Genomic and phenotypic characteristics of Swedish C. jejuni water isolates. *PloS One* 12, e0189222. doi: 10.1371/journal.pone.0189222

Nilsson, A., Johansson, C., Skarp, A., Kaden, R., Bertilsson, S., and Rautelin, H. (2018a). Survival of Campylobacter jejuni and Campylobacter coli water isolates in lake and well water. *APMIS* 126, 762–770. doi: 10.1111/apm.12879

Nilsson, A., Skarp, A., Johansson, C., Kaden, R., Engstrand, L., and Rautelin, H. (2018b). Characterization of Swedish Campylobacter coli clade 2 and clade 3 water isolates. *Microbiologyopen* 7, e00583. doi: 10.1002/mbo3.583

Nylen, G., Dunstan, F., Palmer, S. R., Andersson, Y., Bager, F., Cowden, J., et al. (2002). The seasonal distribution of campylobacter infection in nine European countries and New Zealand. *Epidemiol. Infect.* 128, 383–390. doi: 10.1017/S0950268802006830

Paruch, L., Paruch, A. M., and Sørheim, R. (2020). DNA-based faecal source tracking of contaminated drinking water causing a large Campylobacter outbreak in Norway 2019. *Int. J. Hyg. Environ. Health* 224, 113420. doi: 10.1016/j.ijheh.2019.113420

Purdy, D., Buswell, C. M., Hodgson, A. E., McAlpine, K., Henderson, I., and Leach, S. A. (2000). Characterisation of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni. *J. Med. Microbiol.* 49, 473–479. doi: 10.1099/0022-1317-49-5-473

Revez, J., Schott, T., Llarena, A.-K., Rossi, M., and Hänninen, M.-L. (2013). Genetic heterogeneity of Campylobacter jejuni NCTC 11168 upon human infection. *Infect. Genet. Evol.* 16, 305–309. doi: 10.1016/j.meegid.2013.03.009

Revez, J., Llarena, A.-K., Schott, T., Kuusi, M., Hakkinen, M., Kivistö, R., et al. (2014). Genome analysis of Campylobacter jejuni strains isolated from a waterborne outbreak. *BMC Genomics* 15, 768. doi: 10.1186/1471-2164-15-768

Schönberg-Norio, D., Takkinen, J., Hänninen, M.-L., Katila, M.-L., Kaukoranta, S.-S., Mattila, L., et al. (2004). Swimming and Campylobacter Infections1. *Emerg. Infect. Dis.* 10, 1474–1477. doi: 10.3201/eid1008.030924

Sheppard, S. K., Dallas, J. F., MacRae, M., McCarthy, N. D., Sproston, E. L., Gormley, F. J., et al. (2009). Campylobacter genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6. *Int. J. Food Microbiol.* 134, 96–103. doi: 10.1016/j.ijfoodmicro.2009.02.010

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

Skarp, C. P. A., Akinrinade, O., Kaden, R., Johansson, C., and Rautelin, H. (2017). Accessory genetic content in Campylobacter jejuni ST21CC isolates from feces and blood. *Int. J. Med. Microbiol.* 307, 233–240. doi: 10.1016/j.ijmm.2017.04.001

Thomas, C., Gibson, H., Hill, D. J., and Mabey, M. (1998). Campylobacter epidemiology: an aquatic perspective. *J. Appl. Microbiol.* 85, 168S–177S. doi: 10.1111/j.1365-2672.1998.tb05296.x

World Health Organization (2015). *WHO estimates of the global burden of foodborne diseases* (Geneva, Switzerland: World Health Organization).

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Adhesion, Biofilm Formation, and *luxS* Sequencing of *Campylobacter jejuni* Isolated From Water in the Czech Republic

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The microaerophilic pathogen *Campylobacter jejuni* is a leading bacterial cause of human gastroenteritis in developed countries. Even though it has a reputation as a fastidious organism, *C. jejuni* is widespread and can be easily isolated from various animals, food, and environmental sources. It is suggested that an ability to form biofilms is probably necessary for the survival of *C. jejuni* under harsh environmental conditions. The first step required for successful biofilm formation is adhesion to a suitable surface. Therefore, in this work, the degree of adhesion was evaluated, followed by characterization and quantification of biofilms using confocal laser scanning microscopy (CLSM). A total of 15 isolates of *C. jejuni* were used in the experiments (12 isolates from surface and waste waters, 1 human clinical, 1 food and 1 ACTT BAA-2151 collection strain, all samples originated from the Czech Republic). Regardless of the sample origin, all *C. jejuni* isolates were able to adhere to the polystyrene surface within 30 min, with the number of attached cells increasing with the time of incubation. The resulting data showed that all isolates were able to form complex voluminous biofilms after 24 h of cultivation. The average amount of biovolume ranged from 3.59×10^6 μm^3 to 17.50×10^6 μm^3 in isolates obtained from different sources of water, 16.79×10^6 μm^3 in the food isolate and 10.92×10^6 μm^3 in the collection strain. However, the highest amount of biomass was produced by the human clinical isolate (25.48×10^6 μm^3). Similar to the quantity, the architecture of the biofilms also differed, from a rugged flat monolayer of cells to large clustered structures. Further, all isolates were tested for the presence of the *luxS* gene, as the *luxS*/AI-2 (autoinducer-2) quorum sensing pathway has been previously connected with enhanced biofilm formation. Two isolates originated from surface waters did not possess the *luxS* gene. These isolates formed thinner and sparser biofilms lacking the presence of significant clusters. However, the ability to adhere to the surface was preserved. The sequencing of the *luxS*-containing fragments shown a high similarity of the *luxS* gene among the isolates.

Keywords: *Campylobacter jejuni*, biofilm, adhesion, *luxS*, foodborne pathogen, confocal laser scanning microscopy, water

INTRODUCTION

Campylobacteriosis is an infection caused by *Campylobacter* spp., which is considered one of the main causes of foodborne gastrointestinal bacterial infections worldwide (Allos, 2001). According to the latest EFSA report, in total 246,571 cases of campylobacteriosis were confirmed in 36 EU countries in 2018. Interestingly, the Czech Republic is among the countries with the highest incidence of the disease (215.8 cases per 100,000 inhabitants) (EFSA and ECDC, 2019). The most common species causing the human infection is *Campylobacter jejuni* (Kaakoush et al., 2015). Symptoms associated with the infection usually last two to five days and include diarrhea, vomiting, and abdominal pain (Black et al., 1988). The disease is usually self-limiting, but sometimes can result in serious autoimmune diseases, such as Guillain-Barré and Miller-Fischer syndromes, and reactive arthritis (Salloway et al., 1996; Ang et al., 2001; Pope et al., 2007).

Although campylobacteriosis belongs to bacterial zoonoses and is related to consumption of raw or undercooked meat especially from poultry and drinking of unpasteurized milk (Blaser et al., 1983; Humphrey, 1986; Sahin et al., 2001; Zimmer et al., 2003), the disease can also be disseminated also through the environment, in particular through contaminated water (Carter et al., 1987; Obiri-Danso and Jones, 1999; Daczkowska-Kozon and Brzostek-Nowakowska, 2001; Hörman et al., 2004).

It is known, that *C. jejuni* can survive in untreated or inadequately treated aquatic environments, including wells and groundwater (Stanley et al., 1998). Typically, contamination occurs directly through feces of wild animals or livestock, through wastewater from farms, slaughterhouses, manure, and even as a result of heavy rain (Sacks et al., 1986; Eberhart-Phillips et al., 1997; Clark et al., 2003). Therefore, it has been suggested that its survival in the water systems of animal husbandry facilities and animal-processing units contributes to the infection of animals, and cross-contamination of animal carcasses (Humphrey and Beckett, 1987; Pearson et al., 1993). Thus, the survival of *C. jejuni* in the aquatic environment is important both directly and indirectly in the occurrence of human diseases. There were several reports of how the water from the environment may pose a source of outbreaks of campylobacteriosis and in almost all cases, well or drinking water was contaminated with surface or wastewater (Gubbels et al., 2012; Bartholomew et al., 2014; Pedati et al., 2019).

Compared to many other foodborne pathogens, *C. jejuni* is demanding on environmental conditions, it multiplies under a microaerobic atmosphere (5% oxygen, 10% carbon dioxide and 85% nitrogen) at a temperature ranging between 37°C and 42°C (Park, 2002). Theoretically, these properties make *C. jejuni* incapable of existing outside the host in a natural aerobic environment (Park, 2002; Nguyen et al., 2012), but paradoxically, it not only survives in foods that are subjected to difficult processing conditions (preservation, temperature changes, stress, different pH), but can also be transmitted through natural sources (Klancnik et al., 2009).

Judging by the published research, one of the main strategies that *C. jejuni* uses to survive in the environment is the ability to attach to surfaces and form biofilms (Chmielewski and Frank, 2003). Biofilms are commonly defined as adherent microbial cells embedded within a matrix of extracellular polymeric substances (Costerton et al., 1995; Donlan and Costerton, 2002). It is known, that *C. jejuni* can adhere to both various inert surfaces (e.g. stainless steel, fiberglass, coverslips, nitrocellulose membranes, various plastics) and biotic surfaces (animal and human intestinal cell lines) (Pogacar et al., 2009; Sulaeman et al., 2012; Pogačar et al., 2015). Cell adhesion precedes the formation of biofilms, which represent a protection mechanism against environmental stresses, antimicrobial agents, and the host's immune response (Hall-Stoodley et al., 2004; Blanpain-Avet et al., 2011). *C. jejuni* can also form biofilms on various abiotic surfaces commonly used in irrigation systems, such as acrylonitrile butadiene styrene and polyvinyl chloride plastics (Reeser et al., 2007). It also has the ability to form biofilms in water supply systems in livestock complexes and animal processing plants, which can then represent a constant source of infection for both animals and humans (Buswell et al., 1998; Zimmer et al., 2003).

The molecular background of biofilm formation in *Campylobacter* is still not fully understood, although there is evidence that flagella, surface proteins, and quorum sensing represented by S-ribosylhomocysteine lyase (*luxS*) are required to maximize the biofilm formation (Elvers and Park, 2002; Asakura et al., 2007; Kalmokoff et al., 2006; Kim et al., 2015). Several studies have already demonstrated that this gene is involved in a variety of physiological pathways in *C. jejuni*, including motility, autoagglutination, flagellar expression, oxidative stress, and animal colonization. It was also shown that *luxS*-deficient mutants form significantly fewer biofilms (Elvers and Park, 2002; Reeser et al., 2007; Šimunović et al., 2020).

Previous studies of *Campylobacter* spp. biofilms focused mainly on cultivation under standard laboratory conditions or under artificial stress (Reuter et al., 2010; Oh et al., 2016; Melo et al., 2017). Biofilms were mostly characterized by semiquantitative analysis using crystal violet, which generally provides a comparative characteristic of different isolates, however, it does not provide information about the biofilm structure (Gunther and Chen, 2009; Teh et al., 2010; Zhong et al., 2020). Studies that described structural elements of biofilms using CLSM (Sanders et al., 2007; Ica et al., 2012; Bronnec et al., 2016), in turn, did not perform a comparative characterization of biofilms formed by isolates with different backgrounds. As far as we know, the experiments were mostly examining isolates originating from various animal, food and clinical samples, but excluded environmental isolates, such as those isolated from water. Therefore, this study is focused on the comparison of isolates obtained from different sources (surface and waste water, food, and clinical samples), with an emphasis on their ability to adhere to a surface and subsequently form a biofilm. All isolates were also tested for the presence and the respective sequence of the *luxS* gene, to confirm its crucial role in biofilm development.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

All *C. jejuni* isolates obtained from the environment (7 from the surface water, 5 from sewage, 1 form meat sample and 2 clinical human isolates) were collected from surface and waste water within the whole Czech Republic in the period of 2018 to 2019 (**Table 1**) were stored at −80°C in 20% glycerol with 80% Brain Heart Infusion (BHI; Oxoid, UK). They were routinely grown on Karmali agar (Oxoid, UK) at 42°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) for 24 h in a multi-gas incubator MCO-18M (Sanyo, Japan).

Biofilm Formation Assay

All biofilms were produced in 96-well polystyrene microtiter plate with μClear® bottom (thickness of 190 ± 5 μm; Greiner Bio-one, Germany) under static conditions according to Turonova et al. (2015), with certain modifications mentioned below. Briefly, *C. jejuni* was grown 48 h on the Karmali agar, the cells were then resuspended in Mueller-Hinton Broth (MHB) to reach $OD_{600\ nm}$ = 0.8 ± 0.1. Resulting bacterial suspension was inoculated into sterile 96-well plates (250 μl per well) in technical triplicates and incubated at 42°C at microaerobic atmosphere for 2.5 h to allow the cells to adhere to the bottom of the well. After that, each bacterial suspension was carefully replaced with 250 μl of fresh sterile MHB. After 24 h of incubation at 42°C, all wells were carefully washed 3 times with sterile physiological solution. At last, the wells containing 150 μl of physiological solution were stained by adding 50 μl of 5 μM Syto 9 (Invitrogen, USA) directly into the wells. The experiments were carried out in three independent biological replicates and contained a control of potential bacterial contamination (wells containing sterile medium).

TABLE 1 | C. *jejuni* isolates used in this study.

Name	Origin	Source	The presence of the *luxS* gene
Cj5648P	Water	Pond, 2019	Yes
Cj5643P		Pond, 2019	Yes
Cj5683P		Pond, 2019	No
Cj5715P		Pond, 2019	Yes
Cj5654P		Pond, 2019	Yes
Cj5653P		Pond, 2018	No
Cj5650P		Pond, 2019	Yes
Cj5640W		Outlet of a wastewater treatment plant, 2019	Yes
Cj5623W		Outlet of a wastewater treatment plant, 2019	Yes
Cj5689W		Outlet of a wastewater treatment plant, 2019	Yes
Cj5629W		Outlet of a wastewater treatment plant, 2019	Yes
Cj5716W		Outlet of a wastewater treatment plant, 2018	Yes
Cj1M	Food	Butcher shop, 2019	Yes
Cj5718C	Clinical	Hospital, 2019	Yes
Cj81176		ATCC Collection (BAA-2151), originally from outbreak	Yes

Confocal Laser Scanning Microscopy (CLSM)

The biofilm images were acquired with Olympus IX81F- ZDC2 (Olympus, Japan) confocal scanning laser microscope with spinning disc (CLSM), equipped with Ander IQ software (Andor, Belfast, UK) using an objective Clara 10x. All wells were first scanned manually in bright field to observe the biofilm structure, then one representative location was selected for the CLSM analysis. For evaluation of the 3D images of the biofilm structure and its volume, stacks of horizontal planar images with a z-step selected according to the NY Quist sampling (3.57 μm) were recorded in the green channel (excitation 488 nm, emission 525 nm). The single snapshots of 1040 × 1392 pixels representing an area of 670.8 × 897.8 μm were analyzed by the IMARIS × 64 7.6.4 software, resulting in 3D model of the biofilm structure (Biplane, Switzerland). The volume of the model was then used as a parameter for comparison of the amount of the biofilms. The bio-volume corresponds to the total volume of cells and eDNA in the acquired field.

Adhesion Assay

To evaluate the rate of bacterial adhesion, 96- well clear-bottom plates were inoculated with 100 μl of *C. jejuni* suspension in MHB ($OD_{600\ nm}$ = 0.8 ± 0.1). After selected time of incubation (30, 60, 120, 180 min) at 42°C under microaerobic conditions, Syto 9 was added to each well, allowing the cells to stain for 15 min. After the staining, the supernatants containing the non-adhered cells were removed from each well, and the wells were carefully rinsed three times with sterile distilled water and quickly dried in the air. Further, 100 μl of 1.5% low melting agarose (Sigma-Aldrich, USA) was added, in order to fix the bacteria attached to the bottom of the well. After solidification, plates were viewed with the CLSM using a water immersion objective 40x. The adhesion rate of the isolates was evaluated by counting the cells in ten different fields of each well. The experiment was performed in 3 biological and 3 technical replicates.

PCR Confirmation and Cloning of the *luxS* Gene

The genomic DNA of tested isolates was isolated according to the protocol described by He (2011). The presence of the *luxS* gene was confirmed by PCR with primers specific to the inner region of the mentioned gene (primer set 1, **Table 2**). For the purpose of the sequence analysis, approximately 800 bp fragment containing the *luxS* gene was amplified with primer set 2 containing modified adaptors for restriction enzymes NcoI and EcoRI (**Table 2**). Subsequently, amplified fragment was cloned to the pGEM-T easy vector (Promega) *via* technique of the sticky ends. Subsequently, the ligation mixture was transformed into the competent cells of *Escherichia coli* DH5α (NEB, USA) by the routine heat-shock protocol described by Sambrook and Russell (2006). Positive colonies of each sample were selected on Lysogeny agar (LB-A) (Hi-media, India) containing ampicillin (100 μg/ml), X-Gal (40 μg/ml) and IPTG (50 μg/ml) (all from Merck, USA).

TABLE 2 | Primers used in this study.

Primer		Sequence	Size (bp)	Restriction site
Set 1	seq_F	TTGATTTGCGTTTTTGCGTA	222 bp	NA
	seq_R	CTTTCATGGCTGCTTCCCAA	222 bp	NA
Set 2	pGEM_F	CG**CCATGG**GAGCATGAACTTCAAGACCT	800 bp	**NcoI**
	pGEM_R	AC**GAATTC**CAAAGGACGCACTAGATACT	800 bp	**EcoRI**

The restriction sites for NcoI and EcoRI highlighted in bold.

The plasmid containing the fragment of interest of each sample was isolated by the GenElute™ HP Plasmid Miniprep Kit (Merck, USA), sequenced and the data were deposited to the NCBI database.

Statistical Analysis

The data were expressed as the mean ± standard deviation. Statistical analysis was carried out using Statistica 10.0. The significance level was chosen at 95%, consequently, an effect was considered significant if its p-value was lower than 0.05. The association between the ability to adhere and the ability to form biofilm was evaluated by Spearman correlation analysis.

Calculations and graphs were processed using Microsoft Excel 2016.

RESULTS

Adhesion

The ability of cells to adhere to the bottom of the microtiter plate was evaluated by counting the cells stained with Syto 9 after visualization on CLSM. The adhesion of 15 C. *jejuni* isolates was evaluated after 30, 60, 120, and 180 min of incubation at 42°C in MHB under microaerobic conditions. The results showed various adhesion capabilities among the isolates of *C. jejuni* (**Figure 1**). In general, all *C. jejuni* isolates were able to adhere to the surface within 30 min, with the number of cells increasing with the time of incubation. When speaking about the particular isolates, the strongest adhesion ability was observed in the surface water isolate Cj5653P at 120 and 180 min ($p < 0.05$), while the lowest adhesion was observed in the surface water isolate Cj5683P at 120 and 180 min ($p < 0.05$). Statistical analysis (Spearman's rank correlation test) showed a positive correlation adhesion capacity and time of incubation in 7 out of 15 isolates ($p < 0.05$, **Supplementary Table 1**).

Architecture and Quantification of Biofilms

The 15 C. *jejuni* isolates were investigated for static biofilm formation with the selected cultivation protocol. According to the evaluation of CLSM images, all isolates of *C. jejuni* were able to form three-dimensional structures after 24 h of incubation.

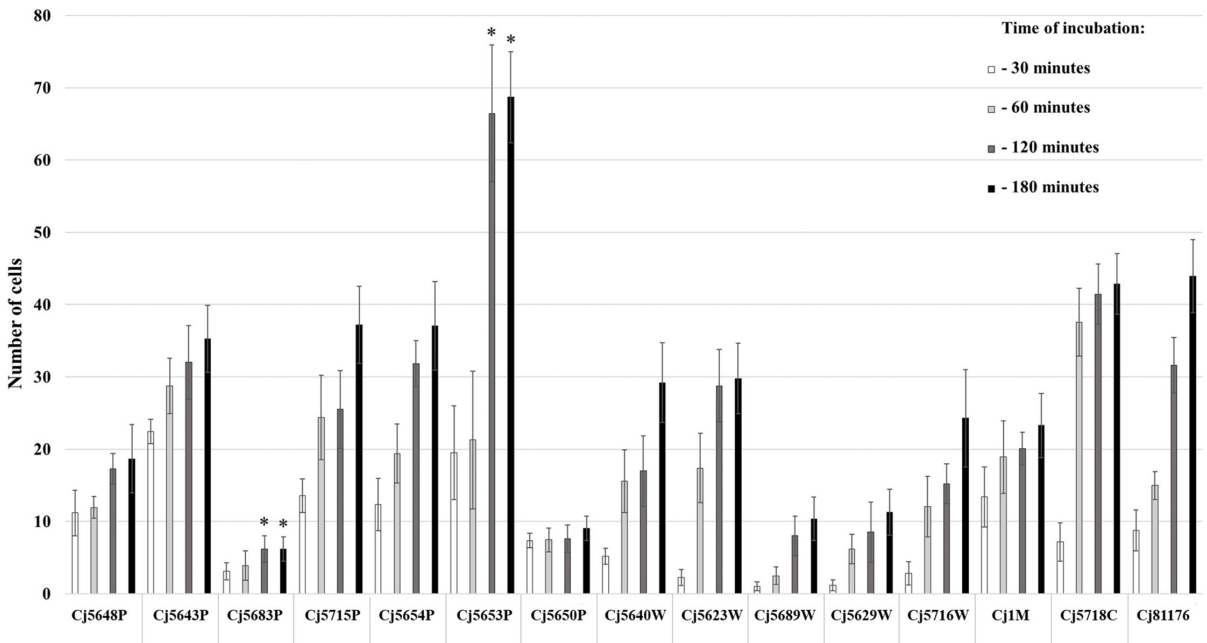

FIGURE 1 | Adhesion capability of *Campylobacter jejuni* isolates measured at four different timepoints of incubation at 42°C under microaerobic atmosphere. The bars represent means of 9 values (triplicate of three independent cultures), the error bars represent standard deviation from the mean; * marks significantly different isolates ($p < 0.05$).

However, the quantity and the architecture of the biofilms of the isolates were diverse (**Figure 2**, **Supplementary Table 2**).

The biofilm architecture ranged from a flat homogeneous layer of cells to complex clustered structures containing hollow voids. Biofilms formed by isolates from pond water displayed as highly structured massive compact clusters. On the contrary, biofilms formed by isolates from waste water had a more homogeneous and continuous structure. Within the collection, only two surface water isolates (Cj5653P and Cj5683W) formed flat biofilms, looking like simple clusters of cells (**Figure 3**). But in general, the visual structure of the water isolates did not differ from the structure of the clinical, food, and collection strains.

When speaking about the biofilm quantity, the average amount of biovolume ranged from 3.59×10^6 μm^3 to 17.50×10^6 μm^3 in isolates isolated from different sources of water, 16.79×10^6 μm^3 in the food isolate and 10.92×10^6 μm^3 in the collection strain. The larger biovolume was produced by the clinical isolate (25.48×10^6 μm^3). The weakest ability to form biofilms was observed in two water isolates: Cj5653P and Cj5683P (3.59×10^6 μm^3 and 4.99×10^6 μm^3), even though the water isolate Cj5653P had the strongest adhesive ability. Correlation analysis of adhesion capacity and biofilm quantity revealed strong positive relationship only in one isolate (Cj5689W) after 30, 120 and 180 min of incubation. In other cases, mostly no correlation was observed. However, six isolates showed negative correlation between the adhesion and biovolume at one timepoint (**Supplementary Table 3**).

Screening and Sequencing of the *luxS* Gene

Since the presence or absence of the *luxS* gene in the genome of *C. jejuni* may affect the ability to form a biofilm, the isolates were tested for a presence of the gene *luxS*, which is responsible for production of the communication molecules AI-2. PCR with the first specific primer set 1 (**Table 2**), which forms a characteristic 222 bp product in the inner region of the *luxS* gene, showed no amplified product in isolates Cj5653P and Cj5683P (**Supplementary Figure 1A**). The results were confirmed by second PCR with primer set 2 (**Table 2**), which bounds to the outer region around the *luxS* and forms characteristic 800 bp product (**Supplementary Figure 1B**). The results of Sanger sequencing of the *luxS*-containing fragments of positive samples showed high rate of similarity among the nucleotide sequences, reaching 95.15% to 99.79% of homology with the collection strain 81 to 176 (**Supplementary Figure 2**). Translation of nucleotide sequence to the amino acid sequence showed several differences (**Table 3**), which could be important for the future studies of the function of the *luxS* gene and the ability to produce the signal molecule. Sequencing data is available in the NCBI database under the following numbers: MT432260, MT432261, MT432262, MT432263, MT432264, MT432265, MT432266, MT432267, MT432268, MT432269, MT432270, MT432271.

DISCUSSION

Due to the high prevalence of human infections caused by *C. jejuni* throughout the world, it is important to understand the ability of this pathogen to persist in the environment and understand the risks it represents to the public health. One of the critical factors ensuring its protection against harsh conditions is its ability to form biofilms (Donlan and Costerton, 2002; Chmielewski and Frank, 2003; Hall-Stoodley et al., 2004).

Although numerous studies have shown that *C. jejuni* can form biofilms on abiotic surfaces, there is very little information concerning environmental isolates, in particular those isolated from water, even though they represent a potential source of infection (Hänninen et al., 2003; Mughini-Gras et al., 2006; Sparks, 2019). Therefore, this work was focused on a

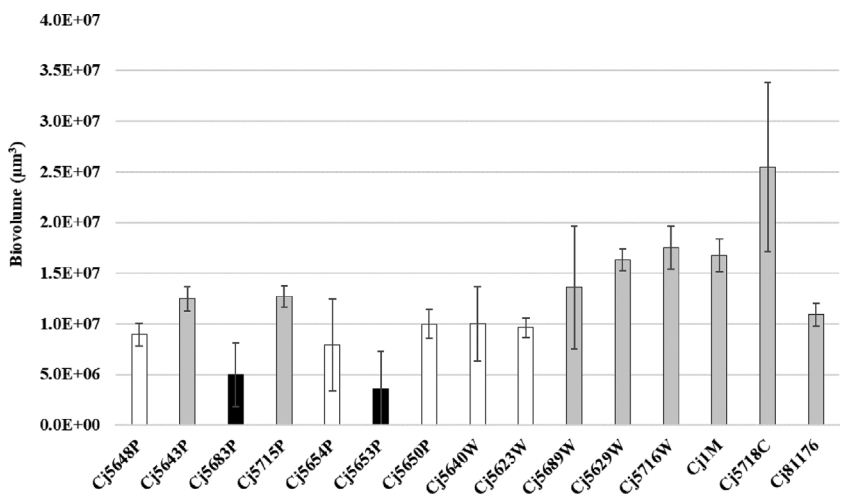

FIGURE 2 | Biofilm biomass quantified by CLSM after the Syto-9 staining. Experiments were performed in triplicate of three independent cultures, the error bars represent standard deviation from the mean. Bar of the same color (white, gray and black) indicates statistically similar values ($p < 0.05$).

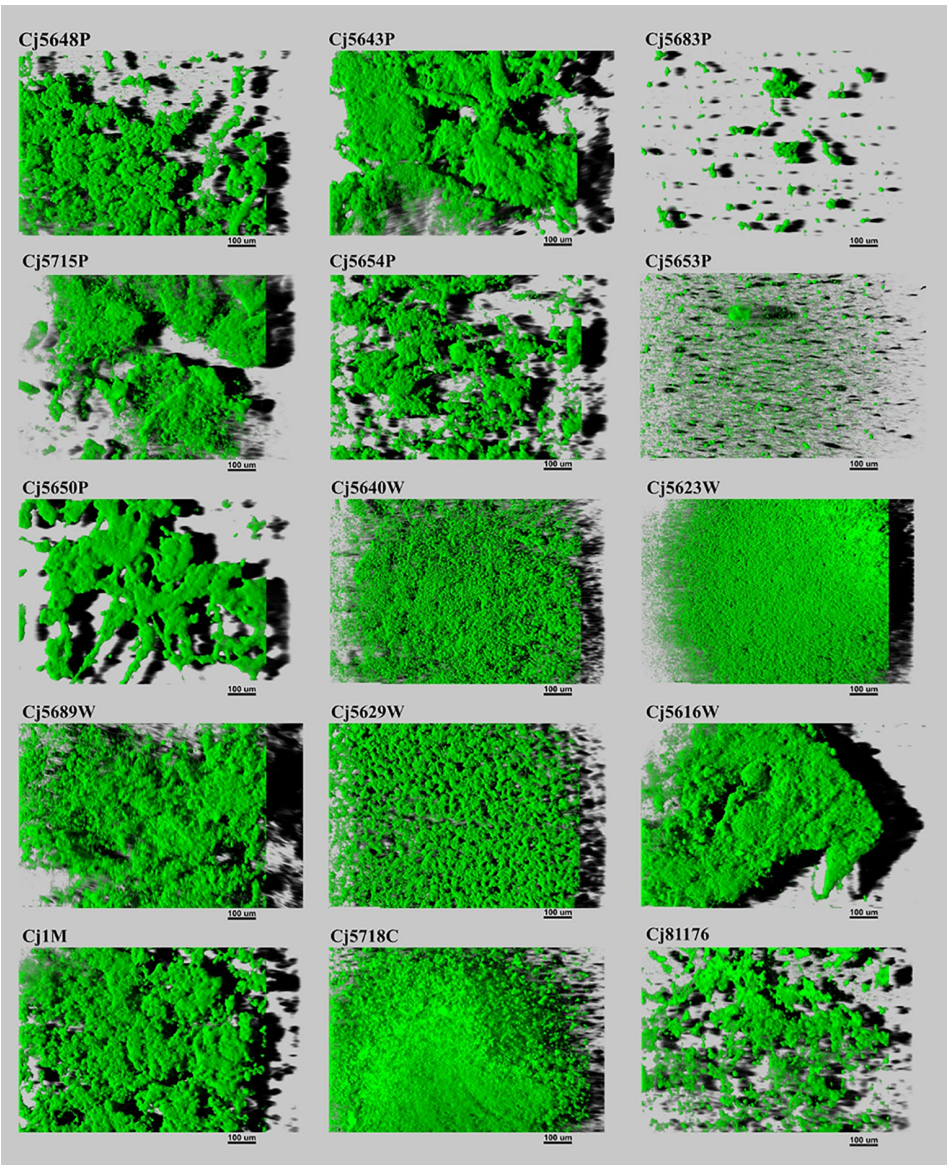

FIGURE 3 | Three-dimensional projections of structures of biofilms obtained from scanning along the z-axis acquired through CLSM. The scale bar represents 100 µm. The CLSM images represent an overhead view of the biofilms formed by 15 isolates of *C. jejuni*, with virtual shadow projection to the right.

comparison of the adhesion and biofilm formation ability of isolates of different origin (surface and waste water, food, and clinical isolates). All tested isolates were able to adhere to the microtiter plate within the first 30 min of incubation, although the numbers of attached cells differed among the isolates. Similar diversity was observed when comparing the volume of the subsequently produced biofilms. Interestingly, even though the adhesion is the first and crucial step of the biofilm formation process, it seems that the adhesion capacity is not directly proportional to the level of the biofilm formation ability, as only one isolate showed positive correlation between the adhesion capacity and the quantity of the biofilm. Moreover, negative correlation was observed in six isolates. However, it is important to mention, that the biofilms were quantified after 24 h of incubation and could, therefore, be in their dispersal phase. To reveal the true relation between the adhesion capacity and biofilm formation ability, further experiments involving measurements of biofilm formation dynamics are needed.

Leaving aside the quantitative analysis, the architecture of the biofilms formed by the tested isolates was also very diverse. The structure of *C. jejuni* biofilms can vary from a monolayer of adherent cells, through flat unstructured multilayers, up to highly structured biomass of clusters containing water channels and voids (Turonova et al., 2015; Bronnec et al., 2016).

TABLE 3 | Differences in LuxS amino acid sequences of isolates as compared to the collection strain *C. jejuni* NCTC 11168.

Isolate	Amino acid variation and their position*
Cj5718C	I→V (100); A→E (106); I→M (154)
Cj5650P	I→V (100); A→E (106); I→M (154)
Cj5689W	I→V (100) A→E (106); I→M (154)
Cj5640W	I→V (100); E→K (105); I→M (154)
Cj5715P	I→V (42, 100); N→D (71); I→M (154); A→E (106)
Cj5716W	L→F (161); I→M (154); A→E (106)
Cj81176	D→N (10); I→V (42); N→D (71); I→V (100); A→E (106); I→M (154)
Cj5648P	A→E (106); I→M (154)
Cj5654P	A→E (106); I→M (154)
Cj5643P	Same as NCTC 11168
Cj5623W	Same as NCTC 11168
Cj5629W	Same as NCTC 11168
Cj1M	Same as NCTC 11168
Cj5653P	NA
Cj5683P	NA

**A, alanine; D, aspartate; E, glutamate; F, phenylalanine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; V, valine.*

The architecture of the biofilm mostly depends on the strains used for the experiments and on the experimental design (Buswell et al., 1998; Joshua et al., 2006). Interestingly, the isolates obtained from the wastewater treatment plant formed more compact biofilms than the isolates from other origins. Their structure was almost carpet-like, with various numbers of small channels and voids. This architecture could be related to the stress that *C. jejuni* cells encounter during the treatment of the water - compact structure with fewer voids means that only a small proportion of the cells is exposed to the chemicals used for the water treatment. Moreover, the affected cells can pass the information about the present danger to the deeper layers of the biofilm *via* quorum sensing, giving the remaining cells enough time to adjust and therefore to ensure the survival of the population.

It is well known that quorum sensing, also known as cell-to-cell signaling, plays a role in biofilm formation (Plummer, 2012). Previous studies reported that *C. jejuni* strains that lack the *luxS* gene responsible for the production of autoinducer-2 molecules had a reduced ability to form static biofilms (Reeser et al., 2007). According to some sources, the distribution of the *luxS* gene within the genus of *C. jejuni* is not uniform and is often missing, especially in environmental isolates (Hepworth et al., 2011). However, in this work *luxS* was absent in only two out of the 12 tested water isolates. The sequence of the gene was relatively well conserved, although the translation to the amino acid sequence revealed several variations, mostly alanine instead of glutamate at position 106, and valine instead of isoleucine at position 100. Interestingly, the strains containing the respective substitutions did not show any similarities in adhesion capacity, biofilm quantity, or the biofilm architecture. Therefore, either the substitution of the amino acids does not cause a functional change of the LuxS enzyme, or it does not influence these particular characteristics of the biofilm formation. Recently, Plummer et al. (2011) described a mutation G92D in *C. jejuni* 81116, which resulted in the loss of production of the AI-2 as well as in decreased the catalytic activity of LuxS in comparison to the wild-type. However, this type of mutation was not detected in among the inspected strains.

The two isolates with missing *luxS* were isolated from surface water (pond) and both produced thin sparse biofilms, lacking the presence of significant clusters (complex interconnected parts of the biomass). Despite the absent *luxS* and reduced biofilm formation ability, the isolates were able to adhere to the surface. Moreover, one of them was marked as the isolate with the highest adhesion ability. These results suggest that the presence or absence of the *luxS* gene itself may have a decisive effect on biofilm formation and clustering ability, but does not affect the adhesion itself. This is in contrast with the data published by Quiñones et al. (2009), who observed reduced adhesion ability in mutants lacking the *luxS* gene. However, the authors used different methodology to assess adhesion capacity.

Overall, this work showed that water isolates of *C. jejuni* can adhere to a surface and subsequently form a spatially structured biofilm. As their adhesion capacity was comparable to the strains of clinical or food origin, they might indeed represent a significant source of contamination in animal husbandry, and as a source of infection in humans. However, further research is needed to evaluate their virulence and persistence in the environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/ MT432260, MT432261, MT432262, MT432263, MT432264, MT432265, MT432266, MT432267, MT432268, MT432269, MT432270, MT432271.

AUTHOR CONTRIBUTIONS

HM, RK, and KD conceived the project. ES and NS provided *C. jejuni* isolates from surface and waste water. ES, MT, and HM designed the experiments, and analyzed and interpreted the data. ES and MT performed the experiments. ES wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Allos, B. M. (2001). *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Dis.* 32 (8), 1201–1206. doi: 10.1086/319760

Ang, C. W., De Klerk, M. A., Endtz, H. P., Jacobs, B. C., Laman, J. D., van der Meché, F. G. A., et al. (2001). Guillain-Barré syndrome- and Miller Fisher syndrome- associated *Campylobacter jejuni* lipopolysaccharides induce anti-GM1 and anti-GQ1b Antibodies in rabbits. *Infect. Immun.* 69 (4), 2462–2469. doi: 10.1128/IAI.69.4.2462-2469.2001

Asakura, H., Yamasaki, M., Yamamoto, S., and Igimi, S. (2007). Deletion of *peb4* gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 275, 278–285. doi: 10.1111/j.1574-6968.2007.00893.x

Bartholomew, N., Brunton, C., Mitchell, P., Williamson, J., and Gilpin, B. (2014). A waterborne outbreak of campylobacteriosis in the South Island of New Zealand due to a failure to implement a multi-barrier approach. *J. Water Health* 12 (3), 555–563. doi: 10.2166/wh.2014.155

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., and Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157 (3), 472–479. doi: 10.1093/infdis/157.3.472

Blanpain-Avet, P., Faille, C., Delaplace, G., and Bénézech, T. (2011). Cell adhesion and related fouling mechanism on a tubular ceramic microfiltration membrane using *Bacillus cereus* spores. *J. Membr. Sci.* 385-386, 200–216. doi: 10.1016/j.memsci.2011.09.041

Blaser, M. J., Wells, J. G., Feldman, R. A., Pollard, R. A., and Allen, J. R. (1983). *Campylobacter enteritis* in the United States. A multicenter study. *Ann. Intern. Med.* 98 (3), 360–365. doi: 10.7326/0003-4819-98-3-360

Bronnec, V., Turoňová, H., Bouju, A., Cruveiller, S., Rodrigues, R., Demnerova, K., et al. (2016). Adhesion, biofilm formation, and genomic features of *Campylobacter jejuni* Bf, an atypical strain able to grow under aerobic conditions. *Front. Microbiol.* 7:1002:1002. doi: 10.3389/fmicb.2016.01002

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T., Marsh, P. D., Keevil, C. W., et al. (1998). Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. *Appl. Environ. Microbiol.* 64 (2), 733–741. doi: 10.1128/AEM.64.2.733-741.1998

Carter, A. M., Pacha, R. E., Clark, G. W., and Williams, E. A. (1987). Seasonal occurrence of *Campylobacter* spp. in surface waters and their correlation with standard indicator bacteria. *Appl. Environ. Microbiol.* 53 (3), 523–526. doi: 10.1128/aem.53.3.523-526.1987

Chmielewski, R. A. N., and Frank, J. F. (2003). Biofilm formation and control in food processing facilities. *Compr. Rev. Food Sci. F.* 2 (1), 22–32. doi: 10.1111/j.1541-4337.2003.tb00012.x

Clark, C. G., Price, L., Ahmed, R., Woodward, D. L., Melito, P. L., Rodgers, F. G., et al. (2003). Characterization of waterborne outbreak-associated *Campylobacter jejuni*, Walkerton, Ontario. *Emerg. Infect. Dis.* 9 (10), 1232–1241. doi: 10.3201/eid0910.020584

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M. (1995). Microbial biofilms. *Annu. Revi. Microbiol.* 49, 711–745. doi: 10.1146/annurev.mi.49.100195.003431

Daczkowska-Kozon, E., and Brzostek-Nowakowska, J. (2001). *Campylobacter* spp. in waters of three main western Pomerania water bodies. *J. Int. J. Hyg. Environ. Health* 203 (5-6), 435–443. doi: 10.1078/1438-4639-00048

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15 (2), 167–193. doi: 10.1128/CMR.15.2.167-193.2002

Eberhart-Phillips, J., Walker, N., Garrett, N., Bell, D., Sinclair, D., Rainger, W., et al. (1997). Campylobacteriosis in New Zealand: results of a case-control study. *J. Epidemiol. Community Health* 51 (6), 686–691. doi: 10.1136/jech.51.6.686

EFSA and ECDC. (2019). The European Union One Health 2018 Zoonoses Report. *EFSA J.* 17 (12). doi: 10.2903/j.efsa.2019.5926

Elvers, K. T., and Park, S. F. (2002). Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule. *Microbiology* 148 (Pt 5), 1475–1481. doi: 10.1099/00221287-148-5-1475

Gubbels, S.-M., Kuhn, K. G., Larsson, J. T., Adelhardt, M., Engberg, J., Ingildsen, P., et al. (2012). A waterborne outbreak with a single clone of *Campylobacter jejuni* in the Danish town of Køge in May 2010. *Scand. J. Infect. Dis.* 44 (8), 586–594. doi: 10.3109/00365548.2012.655773

Gunther, N. W. 4., and Chen, C.-Y. (2009). The biofilm forming potential of bacterial species in the genus *Campylobacter*. *Food Microbiol.* 26 (1), 44–51. doi: 10.1016/j.fm.2008.07.012

Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. doi: 10.1038/nrmicro821

Hänninen, M.-L., Haajanen, H., Pummi, T., Wermundsen, K., Katila, M.-L., Sarkkinen, H., et al. (2003). Detection and typing of *Campylobacter jejuni* and *Campylobacter coli* and analysis of indicator organisms in three waterborne outbreaks in Finland. *Appl. Environ. Microbiol.* 69 (3), 1391–1396. doi: 10.1128/AEM.69.3.1391-1396.2003

He, F. (2011). *E. coli* Genomic DNA Extraction. *Bio-* 101, e97. doi: 10.21769/BioProtoc.97

Hepworth, P. J., Ashelford, K. E., Hinds, J., Gould, K. A., Witney, A. A., Williams, N. J., et al. (2011). Genomic variations define divergence of water/wildlife-associated *Campylobacter jejuni* niche specialists from common clonal complexes. *Environ. Microbiol.* 13 (6), 1549–1560. doi: 10.1111/j.1462-2920.2011.02461.x

Hörman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C. H., Torvela, N., Heikinheimo, A., et al. (2004). *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in southwestern Finland 2000-2001. *Appl. Environ. Microbiol.* 70 (1), 87–95. doi: 10.1128/AEM.70.1.87-95.2004

Humphrey, T. J., and Beckett, P. (1987). *Campylobacter jejuni* in dairy cows and raw milk. *Epidemiol. Infect.* 98 (3), 263–269. doi: 10.1017/s0950268800062014

Humphrey, T. J. (1986). Techniques for the optimum recovery of cold injured *Campylobacter jejuni* from milk or water. *J. Appl. Bacteriol.* 61 (2), 125–132. doi: 10.1111/j.1365-2672.1986.tb04265.x

Ica, T., Caner, V., Istanbullu, O., Nguyen, H. D., Ahmed, B., Call, D. R., et al. (2012). Characterization of mono- and mixed-culture *Campylobacter jejuni* Biofilms. *Appl. Environ. Microbiol.* 78 (4), 1033–1038. doi: 10.1128/AEM.07364-11

Joshua, G. W. P., Guthrie-Irons, C., Karlyshev, A. V., and Wren, B. W. (2006). Biofilm formation in *Campylobacter jejuni*. *Microbiology* 152 (Pt 2), 387–396. doi: 10.1099/mic.0.28358-0

Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28 (3), 687–720. doi: 10.1128/CMR.00006-15

Kalmokoff, M., Lanthier, P., Tremblay, T.-L., Foss, M., Lau, P. C., Sanders, G., et al. (2006). Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J. Bacteriol.* 188 (12), 4312–4320. doi: 10.1128/JB.01975-05

Kim, J.-S., Park, C., and Kim, Y.-J. (2015). Role of *flgA* for flagellar biosynthesis and biofilm formation of *Campylobacter jejuni* NCTC11168. *J. Microbiol. Biotechnol.* 25 (11), 1871–1879. doi: 10.4014/jmb.1504.04080

Klancnik, A., Guzej, B., Jamnik, P., Vucković, D., Abram, M., and Mozina, S. S. (2009). Stress response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. *Res. Microbiol.* 160 (5), 345–352. doi: 10.1016/j.resmic.2009.05.002

Melo, R. T., Mendonça, E. P., Monteiro, G. P., Siqueira, M. C., Pereira, C. B., Peres, P. A. B. M., et al. (2017). Intrinsic and extrinsic aspects on *Campylobacter jejuni* biofilms. *Front. Microbiol.* 8:1332. doi: 10.3389/fmicb.2017.01332

Mughini-Gras, L., Penny, C., Ragimbeau, C., Schets, F. M., Blaak, H., Duim, B., et al. (2006). Quantifying potential sources of surface water contamination with *Campylobacter jejuni* and *Campylobacter. Coli. Water Res.* 101, 36–45. doi: 10.1016/j.watres.2016.05.069

Nguyen, V. T., Fegan, N., Turner, M. S., and Dykes, G. A. (2012). Role of attachment to surfaces on the prevalence and survival of *Campylobacter* through food systems. *J. Food Prot.* 75 (1), 195–206. doi: 10.4315/0362-028X.JFP-11-012

Obiri-Danso, K., and Jones, K. (1999). The effect of a new sewage treatment plant on faecal indicator numbers, campylobacters and bathing water compliance in Morecambe Bay. *J. Appl. Microbiol.* 86 (4), 603–614. doi: 10.1046/j.1365-2672.1999.00703.x

Oh, E., Kim, J.-C., and Jeon, B. (2016). Stimulation of biofilm formation by oxidative stress in *Campylobacter jejuni* under aerobic conditions. *Virulence* 7 (7), 846–851. doi: 10.1080/21505594.2016.1197471

Park, S. F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol.* 74 (3), 177–188. doi: 10.1016/s0168-1605(01)00678-x

Pearson, A. D., Greenwood, M., Healing, T. D., Rollins, D., Shahamat, M., Donaldson, J., et al. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 59 (4), 987–996. doi: 10.1128/AEM.59.4.987-996.1993

Pedati, C., Koirala, S., Safranek, T., Buss, B. F., and Carlson, A. V. (2019). Campylobacteriosis outbreak associated with contaminated municipal water supply- Nebraska 2017. *MMWR Morb. Mortal. Wkly. Rep.* 68 (7), 169–173. doi: 10.15585/mmwr.mm6807a1

Plummer, P., Zhu, J., Akiba, M., Pei, D., and Zhang, Q. (2011). Identification of a key amino acid of LuxS involved in AI-2 production in *Campylobacter jejuni*. *PloS One* 6 (1), e15876. doi: 10.1371/journal.pone.0015876

Plummer, P. J. (2012). *LuxS and* quorum-sensing in *Campylobacter*. *Front. Cell. Infect. Microbiol.* 2, 22. doi: 10.3389/fcimb.2012.00022

Pogacar, M. S., Mihaljević, R. R., Klancnik, A., Brumini, G., Abram, M., and Mozina, S. S. (2009). Survival of stress exposed *Campylobacter jejuni* in the murine macrophage J774 cell line. *Int. J. Food Microbiol.* 129 (1), 68–73. doi: 10.1016/j.ijfoodmicro.2008.11.010

Pogačar, M.Š., Klančnik, A., Bucar, F., Langerholc, T., and Možina, S. S. (2015). *Alpinia katsumadai* extracts inhibit adhesion and invasion of *Campylobacter jejuni* in animal and human foetal small intestine cell lines. *Phytother. Res.* 29 (10), 1585–1589. doi: 10.1002/ptr.5396

Pope, J. E., Krizova, A., Garg, A. X., Thiessen-Philbrook, H., and Ouimet, J. M. (2007). *Campylobacter* reactive arthritis: a systematic review. *Semin. Arthritis Rheumatol.* 37 (1), 48–55. doi: 10.1016/j.semarthrit.2006.12.006

Quiñones, B., Miller, W. G., Bates, A. H., and Mandrell, R. E. (2009). Autoinducer-2 production in *Campylobacter jejuni* contributes to chicken colonization. *Appl. Environ. Microbiol.* 75 (1), 281–285. doi: 10.1128/AEM.01803-08

Reeser, R. J., Medler, R. T., Billington, S. J., Jost, B. H., and Joens, L. A. (2007). Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73 (6), 1908–1913. doi: 10.1128/AEM.00740-06

Reuter, M., Mallett, A., Pearson, B. M., and van Vliet, A. H. M. (2010). Biofilm formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl. Environ. Microbiol.* 76 (7), 2122–2128. doi: 10.1128/AEM.01878-09

Sacks, J. J., Lieb, S., Baldy, L. M., Berta, S., Patton, C. M., White, M. C., et al. (1986). Epidemic campylobacteriosis associated with a community water supply. *Am. J. Public Health* 76 (4), 424–428. doi: 10.2105/ajph.76.4.424

Sahin, O., Zhang, Q., Meitzler, J. C., Harr, B. S., Morishita, T. Y., and Mohan, R. (2001). Prevalence, antigenic specificity, and bactericidal activity of poultry anti-*Campylobacter* maternal antibodies. *Appl. Environ. Microbiol.* 67 (9), 3951–3957. doi: 10.1128/aem.67.9.3951-3957.2001

Salloway, S., Mermel, L. A., Seamans, M., Aspinall, G. O., Nam Shin, J. E., Kurjanczyk, L. A., et al. (1996). Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. *Infect. Immun.* 64 (8), 2945–2949. doi: 10.1128/IAI.64.8.2945-2949.1996

Sambrook, J., and Russell, D. W. (2006). Preparation and transformation of competent *E. coli* using calcium chloride. *CSH Protoc.* (1), pdb.prot3932. doi: 10.1101/pdb.prot3932

Sanders, S. Q., Boothe, D. H., Frank, J. F., and Arnold, J. W. (2007). Culture and detection of *Campylobacter jejuni* within mixed microbial populations of biofilms on stainless steel. *J. Food Prot.* 70 (6), 1379–1385. doi: 10.4315/0362-028x-70.6.1379

Šimunović, K., Ramić, D., Xu, C., and Možina, S. S. (2020). Modulation of *Campylobacter jejuni* motility, adhesion to polystyrene surfaces, and invasion of INT407 cells by quorum- sensing inhibition. *Microorganisms* 8 (1), 104. doi: 10.3390/microorganisms8010104

Sparks, N. H. C. (2019). The role of the water supply system in the infection and control of *Campylobacter in* chicken. *Worlds Poultry Sci. J.* 65 (3), 459–474. doi: 10.1017/S0043933909000324

Stanley, K., Cunningham, R., and Jones, K. (1998). Isolation of *Campylobacter jejuni* from groundwater. *J. Appl. Microbiol.* 85 (1), 187–191. doi: 10.1046/j.1365-2672.1998.00494.x

Sulaeman, S., Hernould, M., Schaumann, A., Coquet, L., Bolla, J.-M., Dé, E., et al. (2012). Enhanced adhesion of *Campylobacter jejuni* to abiotic surfaces is mediated by membrane proteins in oxygen-enriched conditio. *PloS One* 7 (9), e46402. doi: 10.1371/journal.pone.0046402

Teh, K. H., Flint, S., and French, N. (2010). Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations. *Int. J. Food Microbiol.* 143 (3), 118–124. doi: 10.1016/j.ijfoodmicro.2010.07.037

Turonova, H., Briandet, R., Rodrigues, R., Hernould, M., Hayek, N., Stintzi, A., et al. (2015). Biofilm spatial organization by the emerging pathogen *Campylobacter jejuni*: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6, 709. doi: 10.3389/fmicb.2015.00709

Zhong, X., Wu, Q., Zhang, J., Ma, Z., Wang, J., Nie, X., et al. (2020). *Campylobacter jejuni* biofilm formation under aerobic conditions and inhibition by ZnO nanoparticles. *Front. Microbiol.* 11, 207. doi: 10.3389/fmicb.2020.00207

Zimmer, M., Barnhart, H., Idris, U., and Lee, M. D. (2003). Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. *Avian. Dis.* 47 (1), 101–107. doi: 10.1637/0005-2086(2003)047[0101:DOCJSI]2.0.CO;2

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Investigating Major Recurring *Campylobacter jejuni* Lineages in Luxembourg Using Four Core or Whole Genome Sequencing Typing Schemes

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Campylobacter jejuni is the leading cause of bacterial gastroenteritis, which has motivated the monitoring of genetic profiles circulating in Luxembourg since 13 years. From our integrated surveillance using a genotyping strategy based on an extended MLST scheme including *gyrA* and *porA* markers, an unexpected endemic pattern was discovered in the temporal distribution of genotypes. We aimed to test the hypothesis of stable lineages occurrence by implementing whole genome sequencing (WGS) associated with comprehensive and internationally validated schemes. This pilot study assessed four WGS-based typing schemes to classify a panel of 108 strains previously identified as recurrent or sporadic profiles using this in-house typing system. The strain collection included four common lineages in human infection (N = 67) initially identified from recurrent combination of ST-*gyrA*-*porA* alleles also detected in non-human samples: veterinary (N = 19), food (N = 20), and environmental (N = 2) sources. An additional set of 19 strains belonging to sporadic profiles completed the tested panel. All the strains were processed by WGS by using Illumina technologies and by applying stringent criteria for filtering sequencing data; we ensure robustness in our genomic comparison. Four typing schemes were applied to classify the strains: (i) the cgMLST SeqSphere+ scheme of 637 loci, (ii) the cgMLST Oxford scheme of 1,343 loci, (iii) the cgMLST INNUENDO scheme of 678 loci, and (iv) the wgMLST INNUENDO scheme of 2,795 loci. A high concordance between the typing schemes was determined by comparing the calculated adjusted Wallace coefficients. After quality control and analyses with these four typing schemes, 60 strains were confirmed as members of the four recurrent lineages regardless of the method used (N = 32, 12, 7, and 9, respectively). Our results indicate that, regardless of the typing scheme used, epidemic or endemic signals were detected as reflected by lineage B (ST2254-*gyrA*9-*porA*1) in 2014 or lineage A (ST19-*gyrA*8-*porA*7), respectively. These findings support the clonal expansion of stable genomes in

Campylobacter population exhibiting a multi-host profile and accounting for the majority of clinical strains isolated over a decade. Such recurring genotypes suggest persistence in reservoirs, sources or environment, emphasizing the need to investigate their survival strategy in greater depth.

Keywords: whole genome sequencing, *Campylobacter jejuni*, typing schemes, WGS typing scheme comparison, recurring genotypes, clones, core genome MLST, whole genome MLST

INTRODUCTION

Campylobacter spp. is the leading cause of bacterial foodborne diarrheal disease worldwide (WHO, 2013) and the main zoonotic agent in the European Union (EU) (EFSA and ECDC, 2019). In 2018, the reported EU-wide incidence of campylobacteriosis was 64.1 cases per 100,000 population and Luxembourg had one of the highest rates in Europe (103.8) (EFSA and ECDC, 2019). *Campylobacter* is responsible for a large health and economic burden world-wide with a cost-of-illness of $1.56 billion in the USA (Scharff, 2012; Devleesschauwer et al., 2017) and 8.28 disability adjusted life years (DALYs) per 100,000 population in Europe (Cassini et al., 2018).

More than 80% of cases of campylobacteriosis are caused by *Campylobacter jejuni* and poultry is considered the main reservoir of human infections (Mughini-Gras et al., 2012; Ragimbeau et al., 2014; Mossong et al., 2016; EFSA and ECDC, 2019). Transmission is commonly associated with cross-contamination during handling of raw meat, the consumption of undercooked meat or raw drinking milk (EFSA and ECDC, 2018). *C. jejuni* lives as a commensal bacterium in the gastrointestinal tract of wild and domestic birds and mammals, including cattle and sheep. Environmental transmission routes are less frequently reported, but risks include exposure during outdoor sports, swimming in natural waters or contact with garden soil (Stuart et al., 2010; Ellis-Iversen et al., 2012; Mughini-Gras et al., 2012; Bronowski et al., 2014; Mossong et al., 2016; Kuhn et al., 2018).

Unlike for other foodborne pathogens, molecular surveillance of *C. jejuni* has not been implemented in many European countries as the majority of human infections are thought to be sporadic with a low fatality rate (0.03% in EU in 2017) (EFSA and ECDC, 2019). Nevertheless, due to the high number of reported human cases in the EU, campylobacteriosis ranks third in cause of death behind listeriosis and salmonellosis. In addition, outbreaks caused by *Campylobacter* spp. are increasingly being identified and reported on a regular basis, often linked to consumption of untreated drinking water, raw milk or chicken liver paté (Jakopanec et al., 2008; Revez et al., 2014; Davis et al., 2016; Lahti et al., 2017; Kang et al., 2019; Hyllestad et al., 2020).

The generally high incidence recorded in Luxembourg over the last decade has motivated a national implementation of molecular monitoring of *Campylobacter* circulating in food, farm animals, and environmental waters, as part of an integrated surveillance (Ragimbeau et al., 2008; Berthe et al., 2013; Ragimbeau et al., 2014; Mossong et al., 2016). Monitoring the *C. jejuni* population circulating in a community can function as early warning signals for outbreaks and detect long-term changes in the bacterial population, such as emerging new virulence traits or antimicrobial resistance. Further, monitoring the types of *C. jejuni* in different reservoirs and environments can shed light on the epidemiology of campylobacteriosis in that region.

Initially, genotypes from the molecular monitoring were defined according to an in-house typing system originally developed for the Sanger sequencing method. This typing method consists of the seven housekeeping genes from the Multi Locus Sequence Typing (MLST) method (Maiden et al., 1998; Dingle et al., 2001) combined with allelic profiles from two additional loci: *porA* (Clark et al., 2007) and *gyrA* (Wang et al., 1993). Including *porA* and *gyrA* refines the resolution scale of MLST and creates a reliable extended MLST typing method. The *porA* locus encodes the major outer membrane protein and is highly polymorphic, but stable during human passage and within family outbreaks making it a suitable molecular marker for epidemiologic investigations (Cody et al., 2009). Jay-Russell et al. (2013) supported this finding by utilizing variations in *porA* sequences as a screening tool for discriminating genetically related strains in the situation of a large outbreak (Jay-Russell et al., 2013). Interestingly, specific point mutations within *porA* were identified as markers of hyper virulence for a *C. jejuni* clone causing abortion in ruminants and foodborne disease in humans (Sahin et al., 2012; Wu et al., 2016). A sequence-based *gyrA* method was recently developed and it provides information of isolates in two respects: (i) to distinguish the major nucleotide mutation (C257T) conferring the quinolone resistance (i.e., the peptide shift Thr86Ile), and (ii) to source-track clinical isolates according to a host signature in *gyrA* alleles, potentially predictive of domestic birds as source (Jesse et al., 2006; Ragimbeau et al., 2014). The discriminative power resulting from this extended MLST method indexed on a 9-loci basis is sufficient to define different lineages and human clusters (Dingle et al., 2008; Ragimbeau et al., 2014). This has recently been superseded by whole genome sequencing (WGS).

The advent of Next Generation Sequencing (NGS) technologies has significantly increased the amount of genetic information available for the characterization of bacterial isolates. Comparisons at the genome level are more relevant for defining relationships between isolates at unprecedented resolution while simultaneously allowing the full characterization of the virulome, resistome, and metabolome of the isolate. Phylogenetic approaches based on WGS data rely on calculating genetic

distances based on either SNPs (single nucleotide polymorphism) or allele differences (ADs) [known as core or whole genome MLST (cg/wgMLST)] (ECDC, 2016). Unlike other common food and waterborne bacterial pathogens [*Listeria monocytogenes* (Ragon et al., 2008) or *Salmonella enterica serovar Typhi* (Lan et al., 2009)], *Campylobacter* populations display high genetic diversity likely driven by horizontal genetic exchange (de Boer et al., 2002; Sheppard et al., 2011) and to a lesser extent by chromosomal mutations. As a result, SNP analyses that compare strains at the nucleotide level tend to overestimate genetic exchange events and, consequently, decimate the signals of the *Campylobacter* population structure (Sheppard et al., 2012). After conducting comparative studies between the SNP and the cgMLST approaches for different pathogens, it appears that the gene-by-gene approach is more suitable for identifying lineages with this recombining species (Dangel et al., 2019; Jajou et al., 2019). This gene-by-gene method defines allelic profiles from a set of common loci, known as core genome common to a representative panel of isolates. Including accessory loci, present in only a subsection of genomes and often associated with specific phenotypic traits of interest, improves the discriminatory power of the gene-by-gene analysis (Sheppard et al., 2012). For WGS analysis of *C. jejuni* and *C. coli*, several typing schemes have been developed, including two cgMLST schemes; a commercial cgMLST schema containing 637 loci from the SeqSphere+ software (Ridom GmbH, Münster, Germany; www.cgMLST.org) and the Oxford cgMLST schema with 1,343 loci (Cody et al., 2017). Two wgMLST schemes were also defined for *C. jejuni/coli* within the SeqSphere+ software (including the cgMLST and 958 accessory loci) and by the Oxford University (1,643 loci) (Cody et al., 2013). Moreover, two typing schemes were developed specifically for *C. jejuni*: a cgMLST (678 loci) and a wgMLST (2,795 loci) from the INNUENDO platform (Llarena et al., 2018). The method-dependent definition of a WGS-based genotype underlines the need for an international nomenclature to improve communication in outbreak investigation and in surveillance.

Through vigilant surveillance and molecular subtyping with extended MLST, we discovered an unexpected endemic pattern in the temporal distribution of genotypes associated with human infection over several years. The aim of this study was to investigate if these strains were indeed clonal by applying a higher resolution typing method, namely the WGS gene-by-gene approach. We simultaneously assessed the concordance between the four different typing schemes developed for *Campylobacter* spp. and their ability to separate closely related strains.

MATERIALS AND METHODS

Strain Selection

Five thousand *C. jejuni* isolates, from human and non-human sources collected in Luxembourg between 2006 and 2018, were inspected. Years 2009 and 2010 were not included as no molecular surveillance data were available. Genotypic data associated with this collection included extended MLST profiles indexed on nine loci: 7 targets of MLST (Dingle et al., 2001), the partial sequence of *gyrA* (Ragimbeau et al., 2014), and the Sequence Variable Region of *porA* (Campylobacter MOMP database; Dingle et al., 2008). The nomenclature for displaying the results of this extended MLST was defined as follows: sequence type (ST), *gyrA* (allele number), and *porA* (allele number). For example, the combination of alleles including ST19 associated with *gyrA* allele number 8 and *porA* allele number 7 is displayed as follows: ST 19-8-7.

From these, a panel of strains with identical ST-*gyrA*-*porA* profiles over four successive years was selected, including some strains with one allele variation in either the *gyrA* or *porA* loci. Care was taken to achieve a representative strain collection from all available sources (clinical, food, animal, and environmental) and years (between 2006 and 2018). Finally, we also selected a control panel of "sporadic" isolates from patients lacking a recent travel history, i.e., only domestic cases, and whose ST-*gyrA*-*porA* profile occurred only once between 2011 and 2018. This control panel was used as outgroup.

Culture, DNA Extraction, Library Preparation, and WGS

All isolates were stored in −80°C in FBP medium (a combination of ferrous sulfate, sodium metabisulfite, sodium pyruvate and glycerol) (Gorman and Adley, 2004). For each strain, a loopful of frozen culture was spread on chocolate PolyVitex plates (BioMerieux, Marcy-l'Etoile, France) and incubated under micro-aerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h. Then, a subculture of one colony was made again on chocolate PolyVitex agar, and incubated 16 h in the above-mentioned conditions. DNA was extracted with the DNA QIAamp Mini Kit (Qiagen, The Netherlands) according to the manufacturer's instructions. DNA was quantified with the Qubit 2.0 Fluorometer (Invitrogen, Belgium) and the Qubit® dsDNA BR Assay Kit (Life Technologies, Belgium). The DNA concentration was adjusted to be within the range of 30 to 170 µg/ml for subsequent sequencing. Libraries were prepared using the Nextera™ DNA Flex Library Prep Kit or the Nextera™ XT DNA Library Preparation Kit and sequenced on the MiSeq or the MiniSeq platforms achieving either 150- or 250-bp paired-end reads. All chemistry and instrumentations are supplied by Illumina, San Diego, CA, USA. Sequenced raw reads have been uploaded to ENA and are available under the accession project number PRJEB40465.

Genome Assembly and Quality Control (QC) Criteria

For the cgMLSTs SeqSphere+ and Oxford, the paired-end raw read data were *de novo* assembled using Velvet Optimizer v.1.1.04 implemented in Ridom SeqSphere+ v6.1 (Ridom GmbH, Münster, Germany) (Jünemann et al., 2013). Velvet Optimizer was run with automatic determination of the coverage cut-off and minimum contig length and only assemblies with >30x coverage, 1.6Mb ± 10% bp in size and maximum number of 150 contigs were included in the downstream analysis (Zerbino and Birney, 2008; Cody et al., 2017). For the cgMLST INNUENDO and wgMLST

INNUENDO, the raw data were assembled into contigs using the INNUca pipeline v. 4.2.1 with default settings (Machado et al. 2017). Only profiles with no more than 2% of missing loci in either cgMLST were included in the comparative study.

WGS-Based Typing Schemes for Genome Comparison

cgMLST and Accessory Schemes in SeqSphere+

For SeqSphere+, an *ad hoc* cgMLST scheme (N = 637 loci) for *C. jejuni/C. coli* developed by the commercial firm Ridom SeqSphere+ and publicly available at www.cgMLST.org was used. Details of the material and methods used for defining this typing scheme were kindly provided by Prof. Dr. Harmsen (**Supplementary Data S1**). The cgMLST scheme consisted of 637 genes (https://www.cgmlst.org/ncs/schema/145039/locus/). Using genomic data from previously described local outbreaks, a Complex Type (CT) threshold of thirteen was defined to give guidance for delineation of possibly related from not-related genomes (Mellmann et al., 2004). In addition, cgMLST (v1.3) was merged with a screening of the alleles of the accessory genes (N = 958). Altogether, the combined typing wgMLST scheme targets 1,595 loci and the nomenclature remain the same as in the cgMLST analyses with the definition of CTs, solely based on core genome analyses, with a cluster alert of 13.

cgMLST Oxford Scheme

Cody et al. (2017) designed a cgMLST scheme composed of 1,343 loci, available as an open-access and web-accessible analyses online (PubMLST - Campylobacter Sequence Typing; Jolley et al., 2018). The system assigns a unique profile ID from each isolate sequences submitted. Clustering to identify groups can be performed by selecting a threshold empirically chosen (depending on the discrimination power needed). However for this study, the scheme was implemented in SeqSphere+ for comparing strains by using an in-house nomenclature.

cgMLST and wgMLST INNUENDO Schemes

The cgMLST and wgMLST schemes from INNUENDO include 678 and 2,795 loci, respectively, and are publicly available at Zenodo (https://zenodo.org/record/1322564#.X5l_4IhKg2y, Rossi et al., 2018). The cgMLST and wgMLST profiles of the INNUca assembled genomes produced in this study were called using chewBBACA suite (v 2.0.17.1) (Silva et al., 2018). The achieved cgMLST profiles were added to the cgMLST allelic profiles of the 6,526 *C. jejuni* genomes of the INNUENDO dataset, which is also available at Zenodo (Allele_Profiles/Cjejuni_cgMLST_alleleProfiles.tsv, https://zenodo.org/record/1322564#.X5l_4IhKg2y, Rossi et al., 2018). Minimum Spanning Trees (MST) and goeBURST distances were calculated using the goeBURST Full MST algorithm implemented in PHYLOViZ 2.0, and used to define L1:L2:L3 profiles for the cgMLST at 4, 59, and 292 loci variance (Feil et al., 2004; Francisco et al., 2009; Francisco et al., 2012; Nascimento et al., 2017; Llarena et al., 2018). This classification system is hierarchical: L1 is the level representing the highest resolution with a threshold of 4 and it is applied for outbreak detection and investigation, L2 is the intermediate level and is used for long-term longitudinal monitoring. L3 is defined as the level with the highest concordance with the seven-gene MLST classification (Llarena et al., 2018). The wgMLST INNUENDO defines genotypes based on the combination of alleles from the 2,795 loci; no rules were initially developed for clustering isolates with similar profiles.

Comparison of the Targets Included in Each cgMLST Schemes

To crosslink loci with different naming conventions across the four typing schemes, we compared the allele sequences in a pairwise manner. Allele sequences for cgMLST SeqSphere+ were downloaded from https://www.cgmlst.org/ncs/schema/145039/. Allele sequences for cgMLST Oxford were downloaded *via* the pubMLST RESTful API (scheme 4) (Jolley et al., 2017). Allele sequences for cgMLST INNUENDO were downloaded from Zenodo (Rossi et al., 2018). We selected the first allele sequence for each loci of the four typing schemes and performed pairwise reciprocal best hit comparison for the three schemes with the rbh function of the MMseqs2 toolkit ver. 11.e1a1c (Mirdita et al., 2019) using nucleotide search including forward and reverse strand, as well as default parameters. Hits with bitscore above 100 were selected and connected across schemes with a custom script in R 3.4.4. (R Core Team, 2018) using the igraph package 1.2.5. (Csárdi and Nepusz, 2006). Sets of matching loci within the three schemes were visualized with the UpSetR 1.4.0. package (Conway et al., 2017).

Typing System Concordance

The adjusted Wallace coefficient (AWC) (Wallace, 1983; Severiano et al., 2011) was used to estimate the concordance between the different typing schemes in classifying strains (Pinto et al., 2008) by the online Comparing Partitions tool (http://www.comparingpartitions.info), using the strain panel (**Supplementary Data S2**). The degree of equivalence is reflected by AWC. It indicates the probability that two strains with the same type by one method are also categorized into the same type by another method.

Detection of wgMLST Targets Shared by Recurrent Lineages

To determine the overlap of detected wgMLST INNUENDO targets, the allelic profiles of all strains were compared. We extracted lists of targets that appeared at least once within each of the respective lineages in the collection of strains to determine and visualize overlapping and unique sets with a webtool (http://www.molbiotools.com/listcompare.html).

Cluster Analyses

In SeqSphere+, *Campylobacter* isolates are classified in CTs in which the first CT assigned chronologically is definitively fixed in

the database and referred to as the CT founder (Ridom SeqSphere+, 2013). In contrast, the goeBURST algorithm produces a hierarchical classification with the gene-by-gene approach and aims to predict the founder of a clonal complex based on the allele frequency in the dataset. It assumes that the ancestral genotype is the predominant one, which subsequently generates variants. To deduct and visualize, the possible evolutionary relationships between strains, the goeBURST algorithm and its expansion to generate a complete MST implemented in PHYLOViZ 2.0 was used for the cgMLSTs SeqSphere+, INNUENDO, Oxford, the cgMLST SeqSphere+ combined with the accessory targets and the wgMLST INNUENDO (https://online2.phyloviz.net/index) (Feil et al., 2004; Nascimento et al., 2017).

The dynamic shared-genome based approach was performed on the MST generated for the cgMLST Oxford and the wgMLST INNUENDO in order to determine a clustering threshold. Genomic clusters were determined according to the definition of goeBURST groups, based on allelic differences ranging from 0.5 to 1% (Llarena et al., 2018). The in-house nomenclature for displaying the results of cgMLST Oxford and wgMLST INNUENDO were defined as follows: Ox+number and wg +number. For example, the Ox profile number 10 and the wg profile number 8 are displayed as follows: Ox10 and wg8, respectively. The wgMLST profiles were used in the dynamic shared-genome based approach for the comparison and only to increase resolution for clustering strains.

RESULTS

Recurrent Extended MLST Profiles in Campylobacteriosis

By focusing solely on human clinical isolates from our historical collection (N = 3,000), we identified approximately one hundred distinct ST-*gyrA*-*porA* combinations. Two-thirds (N = 2,010) of the human strains in the collection belong to 108 main combinations (**Supplementary Data S3**). Four lineages (ST19-8-7, ST2254-9-1, ST464-8-1678, and ST6175-9-1625, hereafter referred to as lineage A, B, C, and D, respectively) were selected due to the high number of strains (N ≥ 45) and their frequency in human infection over time (**Figure 1** and **Supplementary Data S3**). Some minor variations were accepted in *gyrA* and *porA* alleles: three variants of *porA* and one of *gyrA* in lineage A, and one variant of *porA* in lineage B (**Table 1**). In lineage A, the variation of *gyrA* alleles (*gyrA*1 instead of *gyrA*8) leads to the loss of the quinolone resistance (Wang et al., 1993; Payot et al., 2006). Concerning the *porA* variations, two are linked to deletions in lineage A and two to a non-synonymous mutation (one in lineage A and one in lineage B, respectively). Lineage A has appeared regularly after 2005, with an average of five strains per year and up to 23 in 2012, while 68% of all the strains belonging to lineage B were gathered in a peak in 2014 (**Figure 1**). For lineage C, strains displayed the same combination of alleles and occurred once in August 2008 and then reemerged from July 2014 to January 2018 (**Figure 1**). For lineage D, strains were characterized by the same allele combination (**Table 1**) and occurred once in June

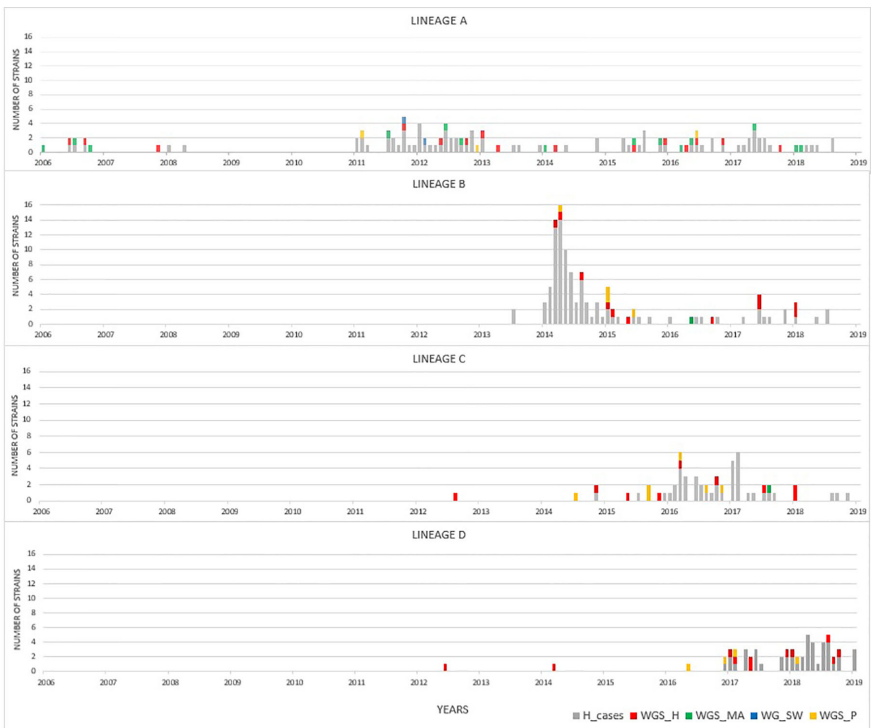

FIGURE 1 | Distribution of strains occurrence for lineages A to D over time. Clinical strains of the laboratory collection are displayed in gray (extended MLST typing). Colors represent to source of selected isolates that were analyzed by WGS: human (red), cattle and sheep (green), poultry (yellow), and surface water (blue) samples.

TABLE 1 | Distribution of main lineages, extended MLST, and variant of the strain collection.

Lineage	Main combination	Variants	Human	Poultry	Ruminants	Environmental	Total
A	ST 19 – *gyrA* 8 – *porA* 7	ST 19 – *gyrA* 8 – *porA* 582	13	3	14	2	37
		ST 19 – *gyrA* 8 – *porA* 2070	1	1	1		
		ST 19 – *gyrA* 8 – *porA* 2068	1				
		ST 19 – *gyrA* 1 – *porA* 7	1				
B	ST 2254 – *gyrA* 9 – *porA* 1	ST 2254 – *gyrA* 9 – *porA* 275	10	4	1	0	16
			1				
C	ST 464 – *gyrA* 8 – *porA* 1678		10	8	1	0	19
D	ST 6175 – *gyrA* 9 – *porA* 1628		12	4	1	0	17
Total			49	20	18	2	89

ST, Sequence Type.

2012, once in March 2014 and then regularly, from May 2016 to October 2018 (**Figure 1**).

Selection of a Strain Panel

Overall, the selected panel included strains from various sources as the four lineages occurring in human infections were also detected in non-human samples. Altogether, the collection included isolates from human (N = 67), poultry (N = 21), and ruminant (N = 18). To complete the panel, two strains from environmental sources (surface waters) assigned to lineage A were added (**Table 1**). A total of 108 strains was selected for the strain panel and subjected to WGS. To achieve equal distribution of strains over the study period, strains belonging to lineage A (N = 37 of 70), lineage B (N = 16 of 97), lineage C (N = 19 of 45), and lineage D (N = 17 of 58) were selected. In addition, 19 strains with a unique ST-*gyrA*-*porA* combination were included in the panel as an outgroup (**Supplementary Data S2**).

The acquired assemblies varied between 35× and 120× in depth of coverage and 1 to 150 contigs, associated with a percentage of good targets ranging from 98.6% to 99.8% (mean value = 99.3%) for cgMLST SeqSphere+ and from 98.0% to 99.3% (mean value = 98.4%) for the cgMLST Oxford. According to the quality criteria defined above (see Methods 2.3) as well as those of the INNUca pipeline, 15 genomes were discarded (14 with SeqSphere+ and 1 with INNUENDO criteria; 4, 1, 4 and 7 genomes were removed from lineages A, B, C and D, respectively). Consequently, genomes of 93 strains were included in the downstream analysis (**Supplementary Data S2**).

Comparison of the Loci Included in the Different Schemes

As the number of loci selected for the core genome varies between the schemes, we compared the respective sequences to assess the number of shared loci. We compared allele sequences by reciprocal best hits. All schemes shared 432 loci, constituting the majority of targets in cgMLST SeqSphere+ and cgMLST INNUENDO with 68% and 64% of targets respectively (**Figure 2**). The majority of targets that differed between cgMLST SeqSphere+ and cgMLST INNUENDO was present in cgMLST Oxford. The wgMLST INNUENDO had an additional 1,775 loci not present in any of the other three cgMLST schemas (**Supplementary Data S4**). The mean size of targets included in each cgMLST typing scheme ranges from 93 to 4,553 bp and the complete lists of targets are provided in **Supplementary Data S4**.

Gene-by-Gene WGS Analysis

With the dynamic shared-genome based approach using 1% allelic differences, thresholds of 11 and 9 AD were defined to classify the strains by the cgMLST Oxford and the wgMLST INNUENDO scheme respectively (**Table 2**). The number of partitions, or clusters, obtained with the different methods was very close: 28 for extended MLST, 22 for cgMLST SeqSphere+, 26 for cgMLST Oxford, and 24 for cgMLST INNUENDO. The largest number of partitions (N = 32) was obtained with the wgMLST INNUENDO analysis (**Supplementary Data S2**). From this pan-genome analysis including 2,795 targets, an average of 974 loci was detected in each lineage, with 870 loci shared between the four lineages (**Figure 3**).

For the analysis of unique combinations, all sporadic strains were classified distinctly by the typing schemes, with one exception regarding two strains that were classified in the same CT (CT 1639) with cgMLST SeqSphere+, in the same profile L1:L2:L3 (66:81:1) with cgMLST INNUENDO and in the same profile with wgMLST INNUENDO (wg30). The allelic profiles for the strains generated by all typing methods were clustered and visualized in PHYLOViZ online tool, in which all five typing schemes achieved very similar unrooted MSTs (**Figure 4**) (PHYLOViZ Online).

Lineage A (ST19-8-7, N = 34) had very limited genetic diversity according to our gene-by-gene WGS analyses. The cgMLST SeqSphere+ assigned all strains to the same CT (CT 82), as did the cgMLST Oxford scheme: Ox1 (**Table 3**). On the contrary, the cgMLST INNUENDO divided lineage A in two groups, of which the majority (33/34) were of the same L1:L2:L3 profile (1:9:1). The 34th strain had a different genotype at L1 level (2695:9:1). Thirty-two of 34 lineage A strains had an identical wgMLST INNUENDO profile: wg1, while two strains had a deviating wgMLST profile (wg5 and wg6). The strains belonging to profile wg1 were collected over a wide timespan (2006–2018) and a range of sources (human, veterinary, or environmental sources).

Lineage B (ST2254-9-1) had low genetic variability according to the cg/wgMLST analyses: altogether, 15 of 16 strains had a similar cgMLST SeqSphere+ (CT 51), cgMLST Oxford (Ox2), and cgMLST INNUENDO (19:49:4) profiles. The increased resolution offered by the wgMLST INNUENDO divided the

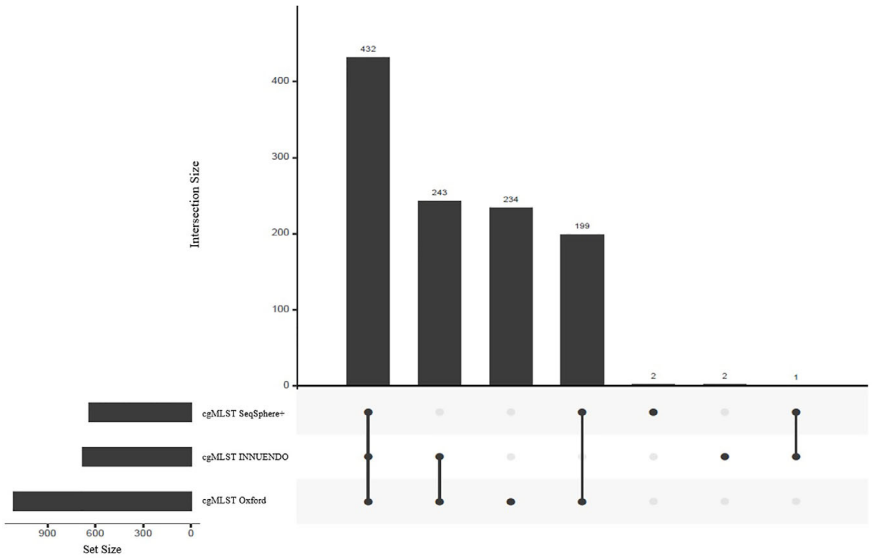

FIGURE 2 | Shared targets between the three compared schemes: cgMLST SeqSphere+ (637 targets), cgMLST INNUENDO (678 targets) and cgMLST Oxford (1,343 targets) highlighted as set sizes. The central bars represent the number of shared or unique targets in or between the different schemes. The points below define the members of the respective sets. For example, 432 targets are present in all three cgMLSTs (SeqSphere+, Oxford, and INNUENDO) and 243 targets are present in both the cgMLSTs Oxford and INNUENDO but not in the cgMLST SeqSphere+. For an overview of shared targets, also refer to **Supplementary Data S4**.

TABLE 2 | Characteristics of the different typing schemes to analyze WGS data from *C. jejuni*.

Typing scheme	Number of targets	Cluster Alert distance*
Extended MLST	9	1
cgMLST SeqSphere+	637	13
cgMLST Oxford	1,343	11
cgMLST INNUENDO	678	L1: 4, L2: 59, and L3: 292
wgMLST INNUENDO	2,795	9

**The cluster alert distance is defined by a threshold value corresponding to the maximum number of different alleles between strains belonging to the same cluster.*
MLST, Multi Locus Sequence Typing; cg, core genome; wg, whole genome.

strains in three types: 75% of the strains were of wg2 while the remaining quarter was divided between wg7 and wg8. The strains belonging to the genotype wg2 were isolated from 2014 to 2018 and from diverse sources (**Table 4**).

Lineage C (ST464-8-1678) was more variable than A and B: all 15 strains were of the CT 75 and the 29:70:7 according to the cgMLST SeqSphere+ and cgMLST INNUENDO, respectively. Contrary to this, the cgMLST Oxford split the panel into three: Ox3, Ox5, and Ox6 (**Table 5**). The wgMLST INNUENDO discriminated six different genotypes collected from diverse range of sources between 2014 and 2017 (**Table 5**).

For lineage D (ST6175-9-1625), all the 10 strains were gathered by the cgMLST SeqSphere+ in the same CT (CT 543), while with the cgMLSTs Oxford and INNUENDO and the wgMLST INNUENDO, one strain had a different profile from the others. The strains were isolated between 2017 and 2018 and from diverse sources (**Table 6**).

Concordance Between the Typing Methods

This analysis was performed on the 93 strains selected in the panel (Methods 3.2 and **Supplementary Data S2**). The cgMLST INNUENDO, the cgMLST Oxford, and the wgMLST INNUENDO had an AWC of 1.000 to the cgMLST SeqSphere+ schema, meaning that all strains clustering together using one of these three typing schemes are also classified together with the cgMLST SeqSphere+. The cgMLST Oxford had an AWC of 0.948 with the cgMLST INNUENDO and, conversely, the cgMLST INNUENDO had an AWC of 0.956 with cgMLST Oxford; 95% of the strains are clustered similarly using either cgMLSTs Oxford and INNUENDO. The majority (93.7% and 94.5%) of the strains that clustered with the cgMLST SeqSphere+ schema were also grouped by the cgMLST INNUENDO and the cgMLST Oxford, respectively. The wgMLST INNUENDO bundled 94.0% of the strains in a similar manner as the cgMLST INNUENDO and 99.8% as the cgMLST Oxford (**Table 7**).

DISCUSSION

From our long-term surveillance of campylobacteriosis at national scale, our data suggested the presence of recurring genotypes defined by an extended MLST method indexing 9-loci over a 13-year period. This study investigated the relationship of a collection of isolates classified in four commonly identified lineages in Luxembourg at genome level. The aim was to assess the potential occurrence of stable genomes through the concordance of different

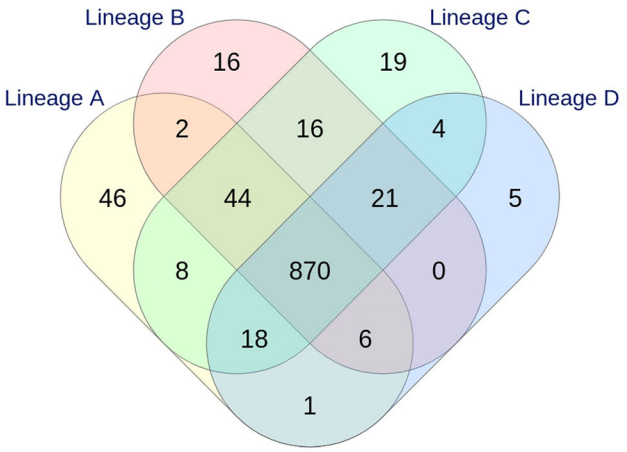

FIGURE 3 | Venn diagram showing the relationship between the loci identified in the four lineages by wgMLST INNUENDO analysis (2,795 targets). A total of 995, 975, 1,000, and 925 targets were detected in lineages A, B, C, and D, respectively.

WGS-based typing schemes exploring and comparing isolates at the core genome level (cgMLSTs from SeqSphere+, Oxford, and INNUENDO) or at the pan genome scale (wgMLST INNUENDO).

Our findings suggested that the genetic population structure of *Campylobacter jejuni* is partly composed of clonal expansion of some genotypes that persist over a long period spanning up to 13 years. Contrary to the epidemic curve commonly detected in case of foodborne outbreaks, stable genetic lineages of this pathogen could emerge to observable frequency through an endemic pattern, causing human infections on a regular basis throughout the country. In order to delineate these lineages with more confidence, efforts were focused on (i) comparing data with a robust design, and (ii) defining cut-offs values aligned with previously published data from genetic variability of the species as well as pre-established threshold for the different typing schemes tested.

Our first concern was to avoid biases generated due to low quality in the raw data and/or assemblies by establishing defined criteria before applying the gene-by-gene approach (Clark et al., 2016; Cody et al., 2017; Llarena et al., 2018; Besser et al., 2019). Quality filtering is a key prerequisite for faithful comparison of genomic data and applied criteria should be clearly stated in all WGS related reports. In their studies, Cody et al. (2013) and Kovanen et al. (2014) implemented a quality threshold in filtering the length of the reads with fixed criteria before the assembly. In 2017, Cody et al. applied a maximum of 150 contigs covering at least 95% of cgMLST targets (Cody et al., 2017), whereas the INNUENDO pipeline included a QC step requiring an assembled depth of coverage of 30x associated with at least 98% of scheme targets found in the cgMLST analyses (Llarena et al., 2018). The Draft Standard of International Standardization Organization (ISO/DIS 23418) suggests a depth of coverage of at least 20x for Illumina short-read raw data and 95% of the read lengths should be over 120 bp (International Organization for Standardization) depending on the application. In our analyses, we implemented stringent criteria for quality filtering to ensure robustness and minimizing potential biases related to missing targets generated by poor quality sequencing data.

While a core genome of a bacterial species is expected to consist of a conserved panel of functional genes (also properly called housekeeping genes), mostly present in the genomes of interest and essential to the microorganism, the cgMLST of the three tested methods included a different number of loci. This discrepancy resulted from a more or less stringent definition of the core genome applied to a panel of reference genome varying in size and quality. It is also noteworthy that the cgMLST INNUENDO schema was specifically determined from the *C. jejuni* species while the two others have included some *C. coli* genomes to create their schemes. In summary, SeqSphere+ and INNUENDO selected targets present in at least 90% of the complete genomes (N = 12) or in 99.9% of draft genomes (N = 6,526), respectively (Llarena et al., 2018). The cgMLST Oxford was built from loci occurring in 95% of the *Campylobacter* sp. reference panel (N = 2,472) to take into consideration variation in sequence quality and applied algorithms (Cody et al., 2017). They proposed a more relaxed core genome definition as some isolates may contain mutations, leading to the reduction of the core genome size as more isolates are selected, and that analyses conducted on incomplete draft genomes might constitute a source of missing data (Cody et al., 2017). The finalized cgMLST Oxford scheme represents thus 82% of the reference genome NCTC11168, which places this typing scheme as an intermediate between a core genome and a whole genome MLST scheme with a total of 1,343 loci vs. 637/678 for the two others. A sample-set independent approach was recently proposed to select a conserved-sequence genome as a novel core genome methodology to address this issue (Van Aggelen et al., 2019).

Further, the locus definition is different in the various schemes as well as allele calling algorithms. In the so-called gene-by-gene approach, a locus does not necessarily correspond to the complete coding sequence of a gene but can constitute a specific region. Thus, each schema includes target sequences of varying length ranging from 100 bp to several kb. Surprisingly, the sizes distributions of the targets in the three cgMLSTs tested are very similar with approximately: 21% below 500 bp, 40% ranging from 500 to 1,000 bp, 26% ranging between 1,000 and 1,500 bp, and 13% above 1,500 bp. Except for the SeqSphere+ commercial platform, the design of allele-calling pipelines from the two others WGS-based schemes were published (Jolley et al., 2018; Silva et al., 2018). Both define alleles from sequence assemblies but perform a search by using nucleotide or translated sequences with BLASTN or BLASTP queries and by using "exemplar alleles" as reference or all alleles already recorded in the database. The procedure differs mainly when new sequences display no exact match with known alleles. However, both pipelines validate the nucleotide sequence after translation of DNA codons and include a threshold in percentage sequence identity and length.

A predefined allele distance threshold allows assignment of an unique identifier to genomes displaying a high level of similarities in their cg/wgMLST profiles. The cut-off distance

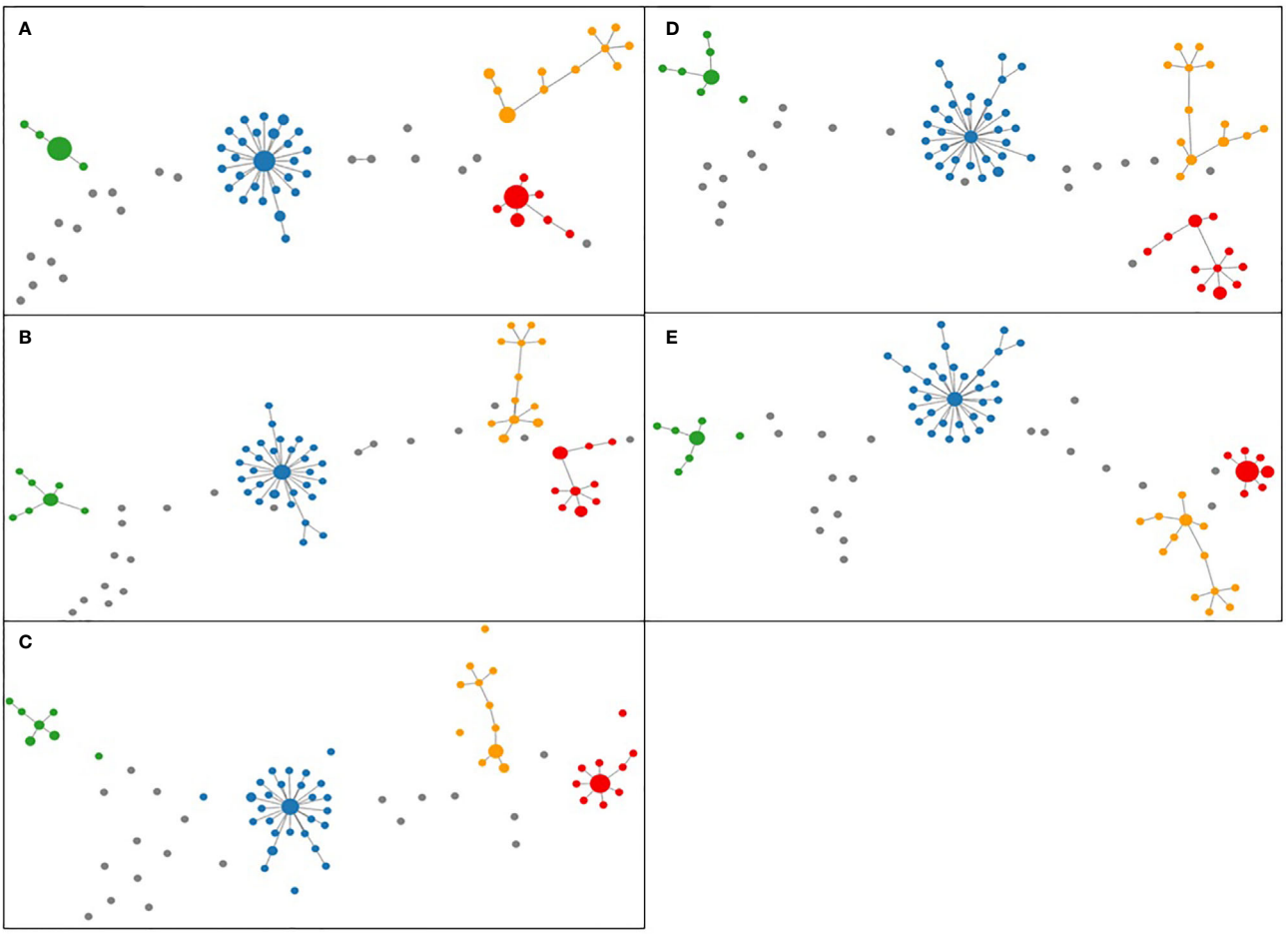

FIGURE 4 | Minimum Spanning Trees generated using PHYLOViZ for **(A)** cgMLST SeqSphere+ (cut-off: 13), **(B)** cgMLST and accessory targets SeqSphere+ (cut-off: 13), **(C)** cgMLST INNUENDO (cut-off: 4), **(D)** cgMLST Oxford (cut-off defined by dynamic core analysis: 11) and **(E)** wgMLST INNUENDO (cut-off defined by dynamic core analysis: 9) analyses on tool. Lineage A is displayed in blue, lineage B in red, lineage C in orange, and lineage D in green and unique combinations in gray.

value for distinguishing clusters is expressed as a number of ADs and is species or even lineage-specific. To calibrate this value, a test population commonly includes clonal outbreak strains as well as non-epidemiologically linked outgroups. Therefore, the established thresholds are based on strains collected over a relatively short period, and may thus not be appropriate for long-term surveillance. Genomic variations linked to insufficient sequencing quality and microevolutions generated during the gut passage are taken into account for classifying strains (Cody et al., 2013; Revez et al., 2013; Thomas et al., 2014; Barker et al., 2020). For instance, Cody et al. (2013) observed between 3 to 14 loci differences (of 1,643 loci in total) in *Campylobacter* sp., during human gut passage, mainly restricted to insertions and deletions in homopolymeric tracts in contingency loci regulating phase variations of surface structures (Jerome et al., 2011; Barker et al., 2020). To classify related-genomes, Cody et al. (2013) tested two methods: a hierarchical approach based on an increasing number of loci in order to detect closely related isolates and a pairwise comparison based on 1,026 loci shared by the 379 C. *jejuni* genomes analyzed. Their results lead to the conclusion that the hierarchical approach is better suited to examine isolates epidemiologically related, while pairwise comparisons are preferable for the identification of outbreaks without initial suspicion (Cody et al., 2013). We assessed genomic clusters in our WGS data with goeBURST and we found that defined low cut-off values ranging from 6 to 11 AD and from 5 to 9 AD were appropriated to classify profiles generated with cgMLST Oxford and wgMLST INNUENDO schemes, respectively. By utilizing our newly established thresholds, the classification was consistent with the ones created by cgMLST methods that use a predefined threshold like SeqSphere+ (AD = 13 of 637 targets) and cgMLST INNUENDO (AD = 4 of 678 targets).

Overall, a high concordance in clustering strains was observed between the three cgMLST typing schemes, although congruence is higher between the cgMLSTs Oxford and INNUENDO schemes (predictive of each other in 95% of the cases) compared to the SeqSphere+ scheme. This was not expected, at first glance, as cgMLST schemes from SeqSphere+ and

TABLE 3 | Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage A.

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp001	MA	2005	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp005	H	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp003	H	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp004	MA	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp006	H	2007	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp002	MA	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp009	H	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp010	MA	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp008	SW	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp014	H	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp012	MA	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp011	SW	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp015	MA	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp016	V	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp018	H	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp017	H	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp021	MA	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp019	H	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp020	MA	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp023	MA	2015	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp029	MA	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp028	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp027	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp032	V	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp031	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp035	H	2017	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp036	MA	2018	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp037	MA	2018	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp024	MA	2015	19 – 8 – 582	82	Ox1	1:9:1	wg1
Camp025	H	2015	19 – 8 – 2068	82	Ox1	1:9:1	wg1
Camp022	H	2015	19 – 8 – 2070	82	Ox1	1:9:1	wg1
Camp013	H	2012	19 – 8 – 7	82	Ox1	2695:9:1	wg1
Camp030	V	2016	19 – 8 – 7	82	Ox1	1:9:1	wg5
Camp034	H	2017	19 – 1 – 7	82	Ox11	1:9:1	wg6

In the column Isolation Source, H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry, and SW to surface waters.
ST, Sequence Type; CT, Complex Type; MLST, Multi Locus Sequence Typing; cg, core genome; wg, whole genome.

INNUENDO have a close number of targets (637 vs. 678 targets, respectively) and share 68% of loci. The concordance between the cgMLST schemes Oxford and INNUENDO, both defined from a large collection of strains, suggests a more representative and stably defined core genome. It is noteworthy that in this study, the added value of the number of loci in the cgMLST Oxford cannot be truly attributed on its discriminative power as the datasets contain several clonal population. A largest test population, reflecting the genetic diversity within the *C. jejuni* species, would have been more appropriate for evaluating the resolution of the different typing schemes. As expected, cgMLST profiles could not be mapped with confidence to the wgMLST INNUENDO profiles including a significant larger number of targets. Differences in the accessory genome composition or in the allelic variations could explain these discrepancies. As all the lineages selected for this study originated from various hosts, it could be interesting to further investigate on a possible link between accessory genomes and niche adaption (Woodcock et al., 2017).

The clonality signal appearing through the concordance of the different typing schemes in classifying strains supports the idea of stability of these clones over time and sources. Two independent studies introduced the concept of monomorphic genotypes for *C. jejuni* within the generalist lineages Clonal Complex (CC) ST-21 (Wu et al., 2016) and ST-45 (Llarena et al., 2016). The first study investigated the genetic basis responsible for the hyper virulence of a known clone named "sheep abortion" (clone SA, ST-8), causing foodborne illnesses in human and ruminant abortion (Wu et al., 2016). The second study explored the population structure of the generalist ST-45-CC, overrepresented in human cases in Finland (Llarena et al., 2016). Considering another field, clonal expansion linked to the acquisition of antibiotic resistance has also already been highlighted in *Campylobacter* (Wimalarathna et al., 2013). Observing stable genotypes in *Campylobacter jejuni* over time are in accordance with these results, hypothesizing that predominant clonal evolution is a major adaptive evolutionary strategy in microbial pathogens (Tibayrenc and Ayala, 2017).

In our study, the best example for stable genome over time is lineage A (ST19-*gyrA*8-*porA*7) as its recurrence occurs over more than a decade, although at a low level, representing an average of 13.4% of human cases per year (data not shown).

TABLE 4 | Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage B.

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp038	H	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp041	V	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp040	H	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp045	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp043	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp046	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp042	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp047	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp044	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp048	H	2016	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp051	H	2017	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp052	H	2018	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp049	MA	2016	2254 – 9 – 1	51	Ox2	19:49:4	wg7
Camp053	H	2018	2254 – 9 – 1	51	Ox2	19:49:4	wg7
Camp050	H	2017	2254 – 9 – 275	51	Ox2	19:49:4	wg8

In the column Isolation Source, H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry, and SW to surface waters.
ST, Sequence Type; CT, Complex Type; MLST, Multi Locus Sequence Typing; cg, core genome; wg, whole genome.

TABLE 5 | Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage C.

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp059	V	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp058	H	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp067	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp065	H	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp064	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp069	MA	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp063	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp060	H	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg9
Camp070	H	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg10
Camp055	V	2014	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp056	H	2014	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp066	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp068	H	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp054	H	2012	464 – 8 – 1678	75	Ox5	29:70:7	wg12
Camp072	H	2018	464 – 8 – 1678	75	Ox6	29:70:7	wg13

In the column Isolation Source, H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry, and SW to surface waters.
ST, Sequence Type; CT, Complex Type; MLST, Multi Locus Sequence Typing; cg, core genome; wg, whole genome.

TABLE 6 | Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage D.

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp082	V	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp083	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp081	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp084	MA	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp080	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp085	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp086	V	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp087	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp089	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp088	H	2018	6175 – 9 – 1625	543	Ox7	2724:68:27	wg14

In the column Isolation Source, H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry and SW to surface waters.
ST, Sequence Type; CT, Complex Type; MLST, Multi Locus Sequence Typing; cg, core genome; wg, whole genome.

TABLE 7 | Adjusted Wallace coefficients values (CI 95%) for typing schemes comparison.

	Extended MLST	cgMLST SeqSphere+	cgMLST INNUENDO	cgMLST Oxford	wgMLST INNUENDO
Extended MLST		1.000 (1.000–1.000)	0.931 (0.832–1.000)	0.935 (0.876–0.994)	0.757 (0.647–0.867)
cgMLST SeqSphere+	0.795 (0.637–0.953)		0.937 (0.843–1.000)	0.945 (0.895–0.994)	0.728 (0.596–0.859)
cgMLST INNUENDO	0.790 (0.630–0.951)	1.000 (1.000–1.000)		0.956 (0.909–1.000)	0.729 (0.597–0.862)
cgMLST Oxford	0.787 (0.622–0.952)	1.000 (1.000–1.000)	0.948 (0.852–1.000)		0.769 (0.634–0.903)
wgMLST INNUENDO	0.829 (0.658–0.997)	1.000 (1.000–1.000)	0.940 (0.828–1.000)	0.998 (0.996–1.000)	

Thirty-two strains of 34 from diverse sources (human, cattle and sheep, poultry, and environmental samples) were gathered in the same genetic profile at the whole genome level. This result reflects that this lineage is likely derived from one common ancestor, which thereafter disseminated broadly to a variety of mammals and birds, clearly demonstrating an ability to disperse in the environment and adapt to different ecological niches. Thus, the question of the environmental transmission routes arises, particularly concerning animal reservoirs such as poultry and ruminants that could contribute to water contamination (Mughini-Gras et al., 2016). Persistent strains have already been identified, mainly in poultry farms and in milk, and it would be interesting to link lineage A with other contamination sources such as insects, rodents, drinking water, or the surrounding environment (Kudirkienė et al., 2010; Perez-Boto et al., 2012; Rauber-Würfel et al., 2019; Jaakkonen et al., 2020).

The lineage B (ST2254-*gyrA*9-*porA*1) arose unexpectedly from our national surveillance with an epidemic curve between March and April 2014 (>70 campylobacteriosis cases). Interestingly, after this episode, clinical isolates of this lineage were still collected but at a much lower frequency during the following four years. To put things into context, this particular ST was singular in 2014 and by querying the pubmlst.org database (Jolley et al., 2018); only a dozen strains had been recorded at that time including two from poultry origin. Interestingly, the same "clone" was finally isolated in the framework of the official controls conducted by the state veterinary laboratory in Luxembourg and supported chicken as a possible source of this outbreak. In molecular epidemiology, the expression "clone" generally refers to a set of independently isolated microbial organisms that have similar genotypic traits as a results of a shared common ancestor (Van Belkum et al., 2007). The analysis using different typing schemes gathered 80% of the tested strains from lineage B in the same CT, whereas only 50% of isolates from lineage C formed a cluster. These data support the occurrence of the most large-scale outbreak caused by *C. jejuni* ever identified in Luxembourg and linked to chicken imported from neighboring countries, as the local production is negligible. Two years after the epidemic episode, this clone was isolated from a bovine source for the first time, while the remainder of lineage B was mainly isolated from poultry. The extent of ecological niches suggests that strains from lineage B were able to cross ecological barriers and disseminate in the environment with a generalist profile (Sheppard et al., 2014).

Lineage C (ST464-*gyrA*8-*porA*1678) displayed two micro-epidemic peaks: one in March 2016 and a second in January and February 2017. Since then, its incidence has been low with less than 10 human cases per year since March 2017 and we observed a first sample of bovine source isolated in August 2017. An average of two human cases per month from December 2016 to May 2018 indicates the profile of an emerging clone tending to have an endemic profile. Notably, this lineage displays the *gyrA* allele 8, one of the nucleotide allele in *C. jejuni* containing the C257T mutation (i.e., the peptide shift Thr86Ile) which confers quinolone resistance (Ragimbeau et al., 2014). Indeed, dispersion of antimicrobial resistant lineages due to positive selection was previously described for bacterial pathogens, such as uropathogenic *Escherichia coli* (ST 131 for example) (Totsika et al., 2011; Yamaji et al., 2018) and *C. jejuni* (ST 464 for instance) (Cha et al., 2016). For lineage D (ST6175-*gyrA*9-*porA*1625), the first isolate was identified in 2012 from a human infection, then in 2014 and at the beginning of 2016. A link with poultry source was observed.

Whatever the typing scheme used, clear signals appeared in our molecular surveillance for identifying an outbreak (lineage B in 2014) or the phenomenon of recurrent clones, which cause of more than 50% of human infections in Luxembourg. This study provides new insights for the genomic surveillance of *Campylobacter* infections. Through the exploration of the large collection of data that we have initiated 15 years ago, we seek to demonstrate the strong interest in monitoring genotypes causing gastroenteritis in the sense that campylobacteriosis is not only of sporadic nature. A recent study based on collected WGS data in Denmark also supported these findings (Joensen et al., 2018).

Molecular surveillance of foodborne pathogens is currently implemented for *Salmonella* (Dangel et al., 2019), *Listeria* (Van Walle et al., 2018), and VTEC (Joensen et al., 2014) at the EU level (ECDC, 2019) and in the USA (Ribot et al., 2019). For *C. jejuni*, such monitoring in routine is hindered by the absence of a validated scheme at international level and the lack of evidence for the spread possibility of cross-border genotypes. The presence of recurring genotypes highlights the possible long-term existing of stable clones representing a risk factor of geographic spread that needs to be investigated further. Like for the acquisition of antibiotic resistance, persistent strains may have acquired specific phenotypic traits to adapt to other hosts or disperse in the environment. Habituation to ambient air (Rodrigues et al., 2015; Rodrigues et al., 2016), adhesion to inert surface (Sulaeman et al., 2010; Oh et al., 2016) and

biofilm formation (Reuter et al., 2010; Turonova et al., 2015) could contribute to the survival strategies of *C. jejuni* in the environment. In the future, studying the phenotypic traits of recurrent clones and their relationship to spatiotemporal persistence would broaden our understanding on *Campylobacter* adaptation and its transmission to humans.

DATA AVAILABILITY STATEMENT

Sequenced raw reads have been uploaded to ENA and are available under the accession project number PRJEB40465.

AUTHOR CONTRIBUTIONS

CR conceived, designed, and overseen the study. OT contributed to data analyses, data organization, and the conception of the paper. MN and CR were involved in data acquisition. A-KL performed all INNUENDO related analyses. MH conducted the targets comparison analysis. CP and SL provided environmental and veterinary samples, respectively. JM advised on bioinformatics analysis and revised the manuscript. MN wrote the initial draft of the manuscript. All co-authors critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.608020/full#supplementary-material

REFERENCES

Barker, C. R., Painset, A., Swift, C., Jenkins, C., Godbole, G., Maiden, M. C. J., et al. (2020). Microevolution of Campylobacter jejuni during long-term infection in an immunocompromised host. *Sci. Rep.* 10, 10109. doi: 10.1038/s41598-020-66771-7

Berthe, F., Hugas, M., and Makela, P. (2013). Integrating surveillance of animal health, food pathogens and foodborne disease in the European Union. *Rev. Off. Int. Epizoot.* 32, 521–528. doi: 10.20506/rst.32.2.2243

Besser, J. M., Carleton, H. A., Trees, E., Stroika, S. G., Hise, K., Wise, M., et al. (2019). Interpretation of whole-genome sequencing for enteric disease surveillance and outbreak investigation. *Foodborne Pathog. Dis.* 16, 504–512. doi: 10.1089/fpd.2019.2650

Bronowski, C., James, C. E., and Winstanley, C. (2014). Role of environmental survival in transmission of Campylobacter jejuni. *FEMS Microbiol. Lett.* 356, 8–19. doi: 10.1111/1574-6968.12488

Campylobacter MOMP database. Available at: https://pubmlst.org/campylobacter/info/porA_method.shtml (Accessed April 15, 2020).

Cassini, A., Colzani, E., Pini, A., Mangen, M.-J. J., Plass, D., McDonald, S. A., et al. (2018). Impact of infectious diseases on population health using incidence-based disability-adjusted life years (DALYs): results from the burden of communicable diseases in Europe study, European Union and European Economic Area countrieto 2013. *Euro. Surveill* 23, 17-00454. doi: 10.2807/1560-7917.ES.2018.23.16.17-00454

Cha, W., Mosci, R., Wengert, S. L., Singh, P., Newton, D. W., Salimnia, H., et al. (2016). Antimicrobial susceptibility profiles of human Campylobacter jejuni isolates and association with phylogenetic lineages. *Front. Microbiol.* 7, 589. doi: 10.3389/fmicb.2016.00589

Clark, C. G., Beeston, A., Bryden, L., Wang, G., Barton, C., Cuff, W., et al. (2007). Phylogenetic relationships of Campylobacter jejuni based on porA sequences. *Can. J. Microbiol.* 53, 27–38. doi: 10.1139/w06-099

Clark, C. G., Berry, C., Walker, M., Petkau, A., Barker, D. O. R., Guan, C., et al. (2016). Genomic insights from whole genome sequencing of four clonal outbreak Campylobacter jejuni assessed within the global C. jejuni population. *BMC Genomics* 17, 990. doi: 10.1186/s12864-016-3340-8

Cody, A. J., Maiden, M. J. C., and Dingle, K. E. (2009). Genetic diversity and stability of the porA allele as a genetic marker in human Campylobacter infection. *Microbiology* 155, 4145–4154. doi: 10.1099/mic.0.031047-0

Cody, A. J., McCarthy, N. D., Jansen van Rensburg, M., Isinkaye, T., Bentley, S. D., Parkhill, J., et al. (2013). Real-time genomic epidemiological evaluation of human Campylobacter isolates by use of whole-genome multilocus sequence typing. *J. Clin. Microbiol.* 51, 2526–2534. doi: 10.1128/JCM.00066-13

Cody, A. J., Bray, J. E., Jolley, K. A., McCarthy, N. D., and Maiden, M. C. J. (2017). Core genome multilocus sequence typing scheme for stable, comparative analyses of Campylobacter jejuni and C. coli human disease isolates. *J. Clin. Microbiol.* 55, 2086–2097. doi: 10.1128/JCM.00080-17

Conway, J. R., Lex, A., and Gehlenborg, N. (2017). UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* 33, 2938–2940. doi: 10.1093/bioinformatics/btx364

Csárdi, G., and Nepusz, T. (2006). The igraph software package for complex network research. *InterJ. Complex Syst* 1965, 1–9. Available at: https://www.semanticscholar.org/paper/The-igraph-software-package-for-complex-network-Cs%C3%A1rdi-Nepusz/1d2744b83519657f5f2610698a8ddd177ced4f5c [Accessed September 17, 2020].

Dangel, A., Berger, A., Messelhäußer, U., Konrad, R., Hörmansdorfer, S., Ackermann, N., et al. (2019). Genetic diversity and delineation of Salmonella Agona outbreak strains by next generation sequencing, Bavaria, Germanto 2018. *Euro. Surveill.* 24, 1800303. doi: 10.2807/1560-7917.ES.2019.24.18.1800303

Davis, K. R., Dunn, A. C., Burnett, C., McCullough, L., Dimond, M., Wagner, J., et al. (2016). Campylobacter jejuni infections associated with raw milk consumption - Uta. *MMWR Morb. Mortal. Wkly. Rep.* 65, 301–305. doi: 10.15585/mmwr.mm6512a1

de Boer, P., Wagenaar, J. A., Achterberg, R. P., van Putten, J. P. M., Schouls, L. M., and Duim, B. (2002). Generation of Campylobacter jejuni genetic diversity in vivo. *Mol. Microbiol.* 44, 351–359. doi: 10.1046/j.1365-2958.2002.02930.x

Devleesschauwer, B., Bouwknegt, M., Mangen, M.-J. J., and Havelaar, A. H. (2017). "Chapter 2 - Health and economic burden of Campylobacter," in

Campylobacter. Ed. G. Klein (London, UK: Academic Press), 27–40. doi: 10.1016/B978-0-12-803623-5.00002-2

Dingle, K. E., Colles, F. M., Wareing, D. R. A., Ure, R., Fox, A. J., Bolton, F. E., et al. (2001). Multilocus sequence typing system for Campylobacter jejuni. *J. Clin. Microbiol.* 39, 14–23. doi: 10.1128/JCM.39.1.14-23.2001

Dingle, K. E., McCarthy, N. D., Cody, A. J., Peto, T. E. A., and Maiden, M. C. J. (2008). Extended sequence typing of Campylobacter spp., United Kingdom. *Emerg. Infect. Dis.* 14, 1620–1622. doi: 10.3201/eid1410.071109

ECDC (2016).Expert opinion on whole genome sequencing for public health surveillance. In: *European Centre for Disease Prevention and Control*. Available at: http://ecdc.europa.eu/en/publications-data/expert-opinion-whole-genome-sequencing-public-health-surveillance (Accessed August 9, 2018).

ECDC (2019).ECDC strategic framework for the integration of molecular and genomic typing into European surveillance and multi-country outbreak investigations. In: *European Centre for Disease Prevention and Control*. Available at: https://www.ecdc.europa.eu/en/publications-data/ecdc-strategic-framework-integration-molecular-and-genomic-typing-european (Accessed November 15, 2019).

EFSA and ECDC (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA J.* 16, 5500. doi: 10.2903/j.efsa.2018.5500

EFSA and ECDC (2019). The European Union One Health 2018 zoonoses report. *EFSA J.* 17, e05926. doi: 10.2903/j.efsa.2019.5926

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

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., and Spratt, B. G. (2004). eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* 186, 1518–1530. doi: 10.1128/JB.186.5.1518-1530.2004

Francisco, A. P., Bugalho, M., Ramirez, M., and Carriço, J. A. (2009). Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. *BMC Bioinf.* 10, 152. doi: 10.1186/1471-2105-10-152

Francisco, A. P., Vaz, C., Monteiro, P. T., Melo-Cristino, J., Ramirez, M., and Carriço, J. A. (2012). PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. *BMC Bioinf.* 13, 87. doi: 10.1186/1471-2105-13-87

Gorman,, and Adley, (2004). An evaluation of five preservation techniques and conventional freezing temperatures of –20°C and –85°C for long-term preservation of Campylobacter jejuni. *Lett. Appl. Microbiol.* 38, 306–310. doi: 10.1111/j.1472-765X.2004.01490.x

Hyllestad, S., Iversen, A., MacDonald, E., Amato, E., Borge, B.Å.S., Bøe, A., et al. (2020). Large waterborne Campylobacter outbreak: use of multiple approaches to investigate contamination of the drinking water supply system, Norway, June 2019. *Eurosurveillance* 25, 2000011. doi: 10.2807/1560-7917.ES.2020.25.35.2000011

International Organization for Standardization Draft standard of International Standardization Organization. Available at: https://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/07/55/75509.html (Accessed April 27, 2020). ISO/CD 23418). ISO.

Jaakkonen, A., Kivistö, R., Aarnio, M., Kalekivi, J., and Hakkinen, M. (2020). Persistent contamination of raw milk by Campylobacter jejuni ST-883. *PloS One* 15, e0231810. doi: 10.1371/journal.pone.0231810

Jajou, R., Kohl, T. A., Walker, T., Norman, A., Cirillo, D. M., Tagliani, E., et al. (2019). Towards standardisation: comparison of five whole genome sequencing (WGS) analysis pipelines for detection of epidemiologically linked tuberculosis cases. *Euro. Surveill.* 24, 1900130. doi: 10.2807/1560-7917.ES.2019.24.50.1900130

Jakopanec, I., Borgen, K., Vold, L., Lund, H., Forseth, T., Hannula, R., et al. (2008). A large waterborne outbreak of campylobacteriosis in Norway: the need to focus on distribution system safety. *BMC Infect. Dis.* 8, 128. doi: 10.1186/1471-2334-8-128

Jay-Russell, M. T., Mandrell, R. E., Yuan, J., Bates, A., Manalac, R., Mohle-Boetani, J., et al. (2013). Using major outer membrane protein typing as an epidemiological tool to investigate outbreaks caused by milk-borne Campylobacter jejuni isolates in California. *J. Clin. Microbiol.* 51, 195–201. doi: 10.1128/JCM.01845-12

Jerome, J. P., Bell, J. A., Plovanich-Jones, A. E., Barrick, J. E., Brown, C. T., and Mansfield, L. S. (2011). Standing genetic variation in contingency loci drives the rapid adaptation of Campylobacter jejuni to a novel host. *PloS One* 6, e16399. doi: 10.1371/journal.pone.0016399

Jesse, T. W., Englen, M. D., Pittenger-Alley, L. G., and Fedorka-Cray, P. J. (2006). Two distinct mutations in gyrA lead to ciprofloxacin and nalidixic acid resistance in Campylobacter coli and Campylobacter jejuni isolated from chickens and beef cattle*. *J. Appl. Microbiol.* 100, 682–688. doi: 10.1111/j.1365-2672.2005.02796.x

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., et al. (2014). Real-Time Whole-Genome Sequencing for Routine Typing, Surveillance, and Outbreak Detection of Verotoxigenic Escherichia coli. *J. Clin. Microbiol.* 52, 1501–1510. doi: 10.1128/JCM.03617-13

Joensen, K. G., Kuhn, K. G., Müller, L., Björkman, J. T., Torpdahl, M., Engberg, J., et al. (2018). Whole-genome sequencing of Campylobacter jejuni isolated from Danish routine human stool samples reveals surprising degree of clustering. *Clin. Microbiol. Infect.* 24, 201.e5–201.e8. doi: 10.1016/j.cmi.2017.07.026

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2017). A RESTful application programming interface for the PubMLST molecular typing and genome databases. *Database (Oxford)* 2017, bax060. doi: 10.1093/database/bax060

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* 3, 124. doi: 10.12688/wellcomeopenres.14826.1

Jünemann, S., Sedlazeck, F. J., Prior, K., Albersmeier, A., John, U., Kalinowski, J., et al. (2013). Updating benchtop sequencing performance comparison. *Nat. Biotechnol.* 31, 294–296. doi: 10.1038/nbt.2522

Kang, C. R., Bang, J. H., and Cho, S.II (2019). Campylobacter jejuni foodborne infection associated with cross-contamination: outbreak in Seoul in 2017. *Infect. Chemother.* 51, 21–27. doi: 10.3947/ic.2019.51.1.21

Kovanen, S. M., Kivistö, R. L., Rossi, M., Schott, T., Kärkkäinen, U.-M., Tuuminen, T., et al. (2014). Multilocus Sequence Typing (MLST) and Whole-Genome MLST of Campylobacter jejuni isolates from human infections in three districts during a seasonal peak in Finland. *J. Clin. Microbiol.* 52, 4147–4154. doi: 10.1128/JCM.01959-14

Kudirkienė, E., Malakauskas, M., Malakauskas, A., Bojesen, A. M., and Olsen, J. E. (2010). Demonstration of persistent strains of Campylobacter jejuni within broiler farms over a 1-year period in Lithuania. *J. Appl. Microbiol.* 108, 868–877. doi: 10.1111/j.1365-2672.2009.04490.x

Kuhn, K. G., Nielsen, E. M., Mølbak, K., and Ethelberg, S. (2018). Determinants of sporadic Campylobacter infections in Denmark: a nationwide case-control study among children and young adults. *Clin. Epidemiol.* 10, 1695–1707. doi: 10.2147/CLEP.S177141

Lahti, E., Löfdahl, M., Ågren, J., Hansson, I., and Olsson Engvall, E. (2017). Confirmation of a campylobacteriosis outbreak associated with chicken liver pâté using PFGE and WGS. *Zoonoses Public Health* 64, 14–20. doi: 10.1111/zph.12272

Lan, R., Reeves, P. R., and Octavia, S. (2009). Population structure, origins and evolution of major Salmonella enterica clones. *Infect. Genet. Evol.* 9, 996–1005. doi: 10.1016/j.meegid.2009.04.011

Llarena, A.-K., Zhang, J., Vehkala, M., Välimäki, N., Hakkinen, M., Hänninen, M.-L., et al. (2016). Monomorphic genotypes within a generalist lineage of Campylobacter jejuni show signs of global dispersion. *Microb. Genom.* 2, e000088. doi: 10.1099/mgen.0.000088

Llarena, A.-K., Ribeiro-Gonçalves, B. F., Silva, D. N., Halkilahti, J., Machado, M. P., Silva, M. S. D., et al. (2018). INNUENDO: a cross-sectoral platform for the integration of genomics in the surveillance of food-borne pathogens. *EFSA Support. Public.* 15, 1498E. doi: 10.2903/sp.efsa.2018.EN-1498

Machado, M. P., Halkilahti, J., Jaakkonen, A., Silva, D. N., Mendes, I., Nalbantoglu, Y., et al. (2017) *INNUca GitHub*. Available at: https://github.com/B-UMMI/INNUca.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., et al. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3140–3145. doi: 10.1073/pnas.95.6.3140

Mellmann, A., Mosters, J., Bartelt, E., Roggentin, P., Ammon, A., Friedrich, A. W, et al. (2004). Sequence-based typing of flaB is a more stable screening tool than typing of flaA for monitoring of Campylobacter populations. *J. Clin. Microbiol.* 42, 4840–4842. doi: 10.1128/JCM.42.10.4840-4842.2004

Mirdita, M., Steinegger, M., and Söding, J. (2019). MMseqs2 desktop and local web server app for fast, interactive sequence searches. *Bioinformatics* 35, 2856–2858. doi: 10.1093/bioinformatics/bty1057

Mossong, J., Mughini-Gras, L., Penny, C., Devaux, A., Olinger, C., Losch, S., et al. (2016). Human campylobacteriosis in Luxembour–2013: a case-control study combined with multilocus sequence typing for source attribution and risk factor analysis. *Sci. Rep.* 6, 20939. doi: 10.1038/srep20939

Mughini-Gras, L., Smid, J. H., Wagenaar, J. A., de Boer, A. G., Havelaar, A. H., Friesema, I. H. M., et al. (2012). Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. *PloS One* 7, e42599. doi: 10.1371/journal.pone.0042599

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

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A. P., Carriço, J. A., and Vaz, C. (2017). PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* 33, 128–129. doi: 10.1093/bioinformatics/btw582

Oh, E., Kim, J.-C., and Jeon, B. (2016). Stimulation of biofilm formation by oxidative stress in Campylobacter jejuni under aerobic conditions. *Virulence* 7, 846–851. doi: 10.1080/21505594.2016.1197471

Payot, S., Bolla, J.-M., Corcoran, D., Fanning, S., Mégraud, F., and Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in Campylobacter spp. *Microbes Infect.* 8, 1967–1971. doi: 10.1016/j.micinf.2005.12.032

Perez-Boto, D., Garcia-Peña, F. J., Abad-Moreno, J. C., and Echeita, M. A. (2012). Dynamics of populations of Campylobacter jejuni in two grandparent broiler breeder farms: persistent vs. transient strains. *Vet. Microbiol.* 159, 204–211. doi: 10.1016/j.vetmic.2012.03.042

PHYLOViZ. Available at: http://online2.phyloviz.net/index (Accessed September 14, 2020).

Pinto, F. R., Melo-Cristino, J., and Ramirez, M. (2008). A confidence interval for the Wallace coefficient of concordance and its application to microbial typing methods. *PloS One* 3, e3696. doi: 10.1371/journal.pone.0003696

PubMLST *Campylobacter Sequence Typing*. Available at: https://pubmlst.org/campylobacter/ (Accessed September 18, 2020).

R Core Team (2018). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: https://www.r-project.org/ (Accessed June 30, 2020).

Ragimbeau, C., Schneider, F., Losch, S., Even, J., and Mossong, J. (2008). Multilocus sequence typing, pulsed-field gel electrophoresis, and fla short variable region typing of clonal complexes of Campylobacter jejuni strains of human, bovine, and poultry origins in Luxembourg. *Appl. Environ. Microbiol.* 74, 7715–7722. doi: 10.1128/AEM.00865-08

Ragimbeau, C., Colin, S., Devaux, A., Decruyenaere, F., Cauchie, H.-M., Losch, S., et al. (2014). Investigating the host specificity of Campylobacter jejuni and Campylobacter coli by sequencing gyrase subunit A. *BMC Microbiol.* 14, 205. doi: 10.1186/s12866-014-0205-7

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., et al. (2008). A new perspective on Listeria monocytogenes evolution. *PloS Pathog.* 4, e1000146. doi: 10.1371/journal.ppat.1000146

Rauber-Würfel, S., de, F., Voss-Rech, D., dos Santos Pozza, J., Coldebella, A., Santiago Silva, V., et al. (2019). Population dynamics of thermotolerant Campylobacter in broilers reared on reused litter. *Foodborne Pathog. Dis.* 16, 738–743. doi: 10.1089/fpd.2019.2645

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

Revez, J., Schott, T., Llarena, A.-K., Rossi, M., and Hänninen, M.-L. (2013). Genetic heterogeneity of Campylobacter jejuni NCTC 11168 upon human infection. *Infect. Genet. Evol.* 16, 305–309. doi: 10.1016/j.meegid.2013.03.009

Revez, J., Llarena, A.-K., Schott, T., Kuusi, M., Hakkinen, M., Kivistö, R., et al. (2014). Genome analysis of Campylobacter jejuni strains isolated from a waterborne outbreak. *BMC Genomics* 15, 768. doi: 10.1186/1471-2164-15-768

Ribot, E. M., Freeman, M., Hise, K. B., and Gerner-Smidt, P. (2019). PulseNet: entering the age of next-generation sequencing. *Foodborne Pathog. Dis.* 16, 451–456. doi: 10.1089/fpd.2019.2634

Ridom SeqSphere+ (2013). *Ridom SeqSphere+ Documentation - Task Procedure*. Available at: https://www.ridom.de/seqsphere/ug/v60/Task_Procedure.html#Cluster-Alert_Settings (Accessed April 14, 2020).

Rodrigues, R. C., Pocheron, A.-L., Hernould, M., Haddad, N., Tresse, O., and Cappelier, J.-M. (2015). Description of Campylobacter jejuni Bf, an atypical aero-tolerant strain. *Gut Pathog.* 7, 30. doi: 10.1186/s13099-015-0077-x

Rodrigues, R. C., Haddad, N., Chevret, D., Cappelier, J.-M., and Tresse, O. (2016). Comparison of proteomics profiles of Campylobacter jejuni strain Bf under microaerobic and aerobic conditions. *Front. Microbiol.* 7, 1596. doi: 10.3389/fmicb.2016.01596

Rossi, M., Santos Da Silva, M., Ribeiro-Gonçalves, B. F., Silva, D. N., Machado, Oleastro, M., et al. (2018). INNUENDO whole genome and core genome MLST schemas and datasets for Campylobacter jejuni. (Version 1.0) (Data set). *Zenodo*. doi: 10.5281/zenodo.1322564

Sahin, O., Fitzgerald, C., Stroika, S., Zhao, S., Sippy, R. J., Kwan, P., et al. (2012). Molecular evidence for zoonotic transmission of an emergent, highly pathogenic Campylobacter jejuni clone in the United States. *J. Clin. Microbiol.* 50, 680–687. doi: 10.1128/JCM.06167-11

Scharff, R. L. (2012). Economic burden from health losses due to foodborne illness in the United States. *J. Food Prot.* 75, 123–131. doi: 10.4315/0362-028X.JFP-11-058

Severiano, A., Pinto, F. R., Ramirez, M., and Carriço, J. A. (2011). Adjusted Wallace coefficient as a measure of congruence between typing methods. *J. Clin. Microbiol.* 49, 3997–4000. doi: 10.1128/JCM.00624-11

Sheppard, S. K., McCarthy, N. D., Jolley, K. A., and Maiden, M. C. J. (2011). Introgression in the genus Campylobacter: generation and spread of mosaic alleles. *Microbiology* 157, 1066–1074. doi: 10.1099/mic.0.045153-0

Sheppard, S. K., Jolley, K. A., and Maiden, M. C. J. (2012). A gene-by-gene approach to bacterial population genomics: whole genome MLST of Campylobacter. *Genes (Basel)* 3, 261–277. doi: 10.3390/genes3020261

Sheppard, S. K., Cheng, L., Méric, G., de Haan, C. P. A., Llarena, A.-K., Marttinen, P., et al. (2014). Cryptic ecology among host generalist Campylobacter jejuni in domestic animals. *Mol. Ecol.* 23, 2442–2451. doi: 10.1111/mec.12742

Silva, M., Machado, M. P., Silva, D. N., Rossi, M., Moran-Gilad, J., Santos, S., et al. (2018). chewBBACA: a complete suite for gene-by-gene schema creation and strain identification. *Microb. Genom.* 4, e000166. doi: 10.1099/mgen.0.000166

Stuart, T. L., Sandhu, J., Stirling, R., Corder, J., Ellis, A., Misa, P., et al. (2010). Campylobacteriosis outbreak associated with ingestion of mud during a mountain bike race. *Epidemiol. Infect.* 138, 1695–1703. doi: 10.1017/S095026881000049X

Sulaeman, S., Bihan, G. L., Rossero, A., Federighi, M., Dé, E., and Tresse, O. (2010). Comparison between the biofilm initiation of Campylobacter jejuni and Campylobacter coli strains to an inert surface using BioFilm Ring Test®. *J. Appl. Microbiol.* 108, 1303–1312. doi: 10.1111/j.1365-2672.2009.04534.x

Thomas, D. K., Lone, A. G., Selinger, L. B., Taboada, E. N., Uwiera, R. R. E., Abbott, D. W., et al. (2014). Comparative variation within the genome of Campylobacter jejuni NCTC 11168 in human and murine hosts. *PloS One* 9, e88229. doi: 10.1371/journal.pone.0088229

Tibayrenc, M., and Ayala, F. J. (2017). "Chapter Six - Is predominant clonal evolution a common evolutionary adaptation to parasitism in pathogenic parasitic protozoa, fungi, bacteria, and viruses?," in *Advances in Parasitology*. Eds. D. Rollinson and J. R. Stothard (San Diego, United States: Academic Press), 243–325. doi: 10.1016/bs.apar.2016.08.007

Totsika, M., Beatson, S. A., Sarkar, S., Phan, M.-D., Petty, N. K., Bachmann, N., et al. (2011). Insights into a multidrug resistant Escherichia coli pathogen of the globally disseminated ST131 lineage: genome analysis and virulence mechanisms. *PloS One* 6, e26578. doi: 10.1371/journal.pone.0026578

Turonova, H., Briandet, R., Rodrigues, R., Hernould, M., Hayek, N., Stintzi, A., et al. (2015). Biofilm spatial organization by the emerging pathogen Campylobacter jejuni: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6, 709. doi: 10.3389/fmicb.2015.00709

Van Aggelen, H., Kolde, R., Chamarthi, H., Loving, J., Fan, Y., Fallon, J. T., et al. (2019). A core genome approach that enables prospective and dynamic monitoring of infectious outbreaks. *Sci. Rep.* 9, 7808. doi: 10.1038/s41598-019-44189-0

Van Belkum, A. V., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., et al. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin. Microbiol. Infect.* 13, 1–46. doi: 10.1111/j.1469-0691.2007.01786.x

Van Walle, I., Björkman, J. T., Cormican, M., Dallman, T., Mossong, J., Moura, A., et al. (2018). Retrospective validation of whole genome sequencing-enhanced

surveillance of listeriosis in Europto 2015. *Euro. Surveill* 23, 1700798. doi: 10.2807/1560-7917.ES.2018.23.33.1700798

Wallace, D. L. (1983). A method for comparing two hierarchical clusterings. *J. Am. Stat. Assoc.* 78, 569–576. doi: 10.2307/2288118

Wang, Y., Huang, W. M., and Taylor, D. E. (1993). Cloning and nucleotide sequence of the Campylobacter jejuni gyrA gene and characterization of quinolone resistance mutations. *Antimicrob. Agents Chemother.* 37, 457–463. doi: 10.1128/AAC.37.3.457

WHO (2013). The global view of campylobacteriosis. *WHO*. Available at: https://www.who.int/foodsafety/publications/campylobacteriosis/en/ (Accessed November 11, 2019).

Wimalarathna, H. M., Richardson, J. F., Lawson, A. J., Elson, R., Meldrum, R., Little, C. L., et al. (2013). Widespread acquisition of antimicrobial resistance among Campylobacter isolates from UK retail poultry and evidence for clonal expansion of resistant lineages. *BMC Microbiol.* 13, 160. doi: 10.1186/1471-2180-13-160

Woodcock, D. J., Krusche, P., Strachan, N. J. C., Forbes, K. J., Cohan, F. M., Méric, G., et al. (2017). Genomic plasticity and rapid host switching can promote the evolution of generalism: a case study in the zoonotic pathogen Campylobacter. *Sci. Rep.* 7, 9650. doi: 10.1038/s41598-017-09483-9

Wu, Z., Periaswamy, B., Sahin, O., Yaeger, M., Plummer, P., Zhai, W., et al. (2016). Point mutations in the major outer membrane protein drive hypervirulence of a rapidly expanding clone of Campylobacter jejuni. *Proc. Natl. Acad. Sci. U. S. A.* 113, 10690–10695. doi: 10.1073/pnas.1605869113

Yamaji, R., Rubin, J., Thys, E., Friedman, C. R., and Riley, L. W. (2018). Persistent pandemic lineages of uropathogenic Escherichia coli in a college community from 1999 to 2017. *J. Clin. Microbiol.* 56, e01834-17. doi: 10.1128/JCM.01834-17

Zerbino, D. R., and Birney, E. (2008). Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* 18, 821–829. doi: 10.1101/gr.074492.107

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REVIEW
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Survival and Control of *Campylobacter* in Poultry Production Environment

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Campylobacter species are Gram-negative, motile, and non–spore-forming bacteria with a unique helical shape that changes to filamentous or coccoid as an adaptive response to environmental stresses. The relatively small genome (1.6 Mbp) of *Campylobacter* with unique cellular and molecular physiology is only understood to a limited extent. The overall strict requirement of this fastidious microorganism to be either isolated or cultivated in the laboratory settings make itself to appear as a weak survivor and/or an easy target to be inactivated in the surrounding environment of poultry farms, such as soil, water source, dust, surfaces and air. The survival of this obligate microaerobic bacterium from poultry farms to slaughterhouses and the final poultry products indicates that *Campylobacter* has several adaptive responses and/or environmental niches throughout the poultry production chain. Many of these adaptive responses remain puzzles. No single control method is yet known to fully address *Campylobacter* contamination in the poultry industry and new intervention strategies are required. The aim of this review article is to discuss the transmission, survival, and adaptation of *Campylobacter* species in the poultry production environments. Some approved and novel control methods against *Campylobacter* species throughout the poultry production chain will also be discussed.

Keywords: *Campylobacter*, survival, control, poultry-processing plants, poultry farms

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INTRODUCTION

The name of *Campylobacter* [kam′′pə-lo-bak′tər] originally came from the ancient Greek meaning curved rod where kampylos means curved and baktron means rod. However, the unique shape of *Campylobacter* looks more like a spiral or helical one rather than a curved rod shape. *Campylobacter* can change its shape into filamentous or coccoid to adapt to the stressful conditions (Gaynor et al., 2005; Tresse et al., 2017). It was first isolated from a sheep abortion case and classified as a *Vibrio*-like bacterium (McFadyean and Stockman, 1913; Skirrow, 2006) and then renamed as *Campylobacter* after showing a clear different taxonomy profile from the *Vibrio* species. *Campylobacter* bacteria are very diverse microorganisms not only on the species levels but also on the subspecies and strain levels (Gaynor et al., 2005; Vidal et al., 2016). Diversity includes differences in genetic and phenotypic characteristics as well as growth requirement, which may

explain their presence in different hosts or ecological niches including different poultry and wild birds. Some *Campylobacter* species are flagellated with a single polar flagellum or bipolar flagella (*e.g.*, *C. jejuni*, *C. coli C. concisus* and *C. showae*), while fewer species (*e.g.*, *C. hominis* and *C. ureolyticus*) have no flagellum (Man, 2011).

Emerging *Campylobacter* bacteria are species that have been identified recently to cause illnesses (Kaakoush et al., 2015). They include *C. concisus, C. curvus, C. fetus, C. gracilis, C. mucosalis, C. pinnipediorum, C. rectus, C. showae, C. sputorum, C. lari, C. ureolyticus, C. upsaliensis*, and *C. volucris*. The clinical importance and pathogenicity of emerging *Campylobacter* species have been reviewed (Man, 2011; Costa and Iraola, 2019). Available evidence showed that they could attach and invade human epithelial cells, alter intestinal barrier integrity, avoid host immune response, secrete toxins and invade macrophages. In contrast, the actual contribution of emerging *Campylobacter* species to campylobacteriosis is still not clear because available cultivation methods including hydrogen-enhanced microaerobic and anaerobic conditions failed to successfully grow these microbes under the laboratory condition (Kaakoush et al., 2015). This is due to several reasons including the slow growing nature of some fastidious species or individual strains, growth inhibition by antibiotics added in selective media, limited hydrogen source, presence of competitive microorganisms, and/or difficulties in identifying some *Campylobacter* species due to their morphological diversities. Nevertheless, hydrogen enhancement (generally 3–7%) in the microaerobic condition improved the detection of *C. concisus* from 0.03% to 1.92% (Casanova et al., 2015). Symptoms of *C. concisus* infections and other *Campylobacter* bacterial infections are usually milder than that with C. jejuni and *C. coli*. However, emerging *Campylobacter* species are also important and require better isolation techniques for their detection and diagnosis. A previous report showed that infections of *C. concisus* and *C. fetus* were more common than infections of *C. jejuni* and *C. coli* in the elderly (68.4 years old) than young adults of 28.6 years old on average (Bessède et al., 2014). In conclusion, although *C. jejuni* and *C. coli* remain the leading cause of campylobacteriosis, more effective detection methods are required for a better understanding of how emerging *Campylobacter* bacteria evolve in the environment, transmit to agri-food systems, and contribute to campylobacteriosis.

Available evidence suggests that campylobacteriosis incidence has been rising in both developed and developing countries in the recent years (Kaakoush et al., 2015). The size of *Campylobacter* outbreaks in different countries ranged from 10 to 100 cases between 2007 and 2013 (Kaakoush et al., 2015). Poultry and untreated water were the most reported sources of *Campylobacter* outbreaks. The number of *Campylobacter* cases in different countries within the same region can vary significantly. This is not only due to the unreported cases but also limited sensitivity of detection methods, population size and composition, variation in public health standards, intervention strategies, surveillance systems, food safety practices, and the prevalence of *Campylobacter* in natural reservoirs in different regions. The epidemiological data from Asia, Africa, and the Middle East shows that *Campylobacter* infection is prevalent in this region although the data is incomplete. The total number of *Campylobacter* infections in Canada was estimated to be about 145,350 cases per year (Thomas et al., 2013). British Columbia (BC) had an annual *Campylobacter* infection rate of 37.74 cases per 100,000 people (1,818 cases) in 2017 (BC Center for Disease Control, 2017). In comparison, Japan had a rate of 1,512 cases per 100,000 people (Kubota et al., 2011) and New Zealand had a rate of 161.5 per 100,000 people (Sears et al., 2011) within the last decade. In USA, the surveillance system, new regulations, and control strategies have contributed to the decline of several foodborne pathogens including *Salmonella*, *Listeria*, and *E. coli* O157:H7 from 2006 to 2014, but not *Campylobacter* and *Vibrio* (Crim et al., 2014). Altogether, both individual cases and outbreaks of campylobacteriosis are generally prevalent around the world.

Several risk factors that can lead to *Campylobacter* infections include traveling or person-to-person transmission, contact with animals, and consumption of contaminated food or water. Meta-analysis data suggest that international and domestic traveling was the most critical risk factor of *Campylobacter* infections, followed by the consumption of uncooked chicken meat, environmental exposure, and direct contact with the farm animals. A Canadian report showed that campylobacteriosis was responsible for the highest number of causes of travel-related diseases [123/446 cases (27.57%)] from 2005 to 2009 (Ravel et al., 2010). In addition, overlapping exists between risk factors. For example, travel-related diseases are frequently linked to the consumption of contaminated foods (Kaakoush et al., 2015). Although traveling abroad contributes to the overall *Campylobacter* transmission, the spread of antibiotic-resistant *Campylobacter* strains between countries and continents through international agri-food trade is also a considerable public health concern (Mughini-Gras et al., 2014).

THE UNIQUE PHYSIOLOGY OF *CAMPYLOBACTER*

Campylobacter species are not only unique in their shape, but they also have a relatively small genome with unique cellular and molecular physiology compared to other foodborne pathogens. The first whole-genome sequencing analysis of *C. jejuni* (NCTC11168 strain) showed that the genome (1.6 Mbp) has uniquely a limited number of repeated sequences and no insertion or phage associated regions (Parkhill et al., 2000). Other reports showed that *C. jejuni* lacks the regulator *rpoS* (starvation/stationary phase sigma factor) and their stationary-phase cultures are ununiformed dynamic populations unlike most of other bacteria (Kelly et al., 2001). This could be a survival strategy that *C. jejuni* uses to reduce its starvation stress during the stationary phase at least in some strains. Although the existence of stationary phase in *C. jejuni* is elusive, a transition from exponential to stationary phase was observed in *C. jejuni* populations with a number of changes in

the transcriptomic and proteomic profiles between the two phases (Turonova et al., 2017). These data also suggest that the pleiotropic regulator *cosR* gene acts as a negative autoregulator and is alternative to *rpoS* gene in *C. jejuni* during the stationary phase of growth. In addition, *C. jejuni* is an asaccharolytic bacterium (*i.e.*, unable to break down carbohydrate for energy) due to the absence of some key glycolytic enzymes [*e.g.*, glucokinase (GIK) and phosphofructokinase (Pfk)] that involved in the functional Embden-Meyerhof-Parnas glycolysis pathway (Tresse et al., 2017). *Campylobacter* is also a chemo-organotrophic bacterium that oxidizes the chemical bonds in amino acids or intermediate molecules of tricarboxylic acid (Krebs) cycle as their energy and carbon source. Moreover, *C. jejuni* uses gluconeogenesis fueled by amino acids to generate glucose from non-carbohydrate sources. The Entner-Doudoroff (ED) pathway is used in bacteria for synthesizing pyruvate from extracellular glucose. A complete group of genes encoding ED pathway was identified in some rare *C. jejuni* and *C. coli* isolates (Vegge et al., 2016). Interestingly, this gene set increased the survival and biofilm formation in *Campylobacter*. Altogether, *C. jejuni* lacks many important stress response genes, but has developed different mechanisms to adapt to and survive in the new environmental and/or under stress conditions.

Campylobacter species have many unique growth requirements that can limit but not eliminate their prevalence outside warm-blooded hosts in foods and/or food environments. Most *Campylobacter* bacteria grow optimally at either 42°C (chicken body temperature) or 37°C (human body temperature), but none of them can grow below 30°C (Park, 1996). The growth rate of most other bacteria reduces gradually near their minimum growth temperature unlike *Campylobacter* that suddenly stops to grow below 30°C (Hazeleger et al., 1998). No growth adaptation of *C. jejuni* was observed below 30°C. This raises the question of how different the metabolic activity of *Campylobacter* is below and above the minimum growth temperature. This question will be answered below according to several reports about the survival of *Campylobacter* in food and food-related conditions. Moreover, *Campylobacter* is unable to survive under the ambient oxygen level due to several combined reasons (Mace et al., 2015). These include (i) limited tolerance against reactive oxygen species (ROS), (ii) incompetence of producing adequate antioxidant enzymes, (iii) low respiratory rate, and (iv) presence of oxygen-labile essential enzymes (Velayudhan et al., 2004). A few enzymes present in *Campylobacter* are believed to play a critical role in protecting the cells from oxygen tension. These include catalase, glutathione reductase, glutathione synthetase, peroxidase, and superoxide dismutase (Keener et al., 2004).

HUMAN INFECTIONS

Campylobacter is documented in 2019 to be the leading foodborne pathogen associated with the consumption of animal-source food products worldwide (Li et al., 2019). Classical symptoms of *Campylobacter* infections (called campylobacteriosis) include fever, severe watery or bloody diarrhea, cramps, and weight loss for 6 days on average in humans (Véron and Chatelain, 1973; Kaakoush et al., 2015). Most infections are self-limiting and do not require medical therapy other than hydration and electrolyte balance (Acheson and Allos, 2001). Antibiotic treatment is only applied either in severe cases or to immunocompromised individuals. *C. jejuni* and *C. coli* are the major causes of campylobacteriosis in humans (Kaakoush et al., 2015). Several studies showed that infections of both *C. jejuni* and *C. coli* occur more frequently during the summer than other seasons (Nielsen et al., 2013; Bessède et al., 2014). *C. jejuni* infection is greater than *C. coli* in many countries, but *C. coli* is also an important species and reported to be the second most contributor to campylobacteriosis after *C. jejuni*. In fact, a comparison study of patients infected with either *C. jejuni* or *C. coli* showed that slightly older patients (34.6 compared to 27.5 years old) have a greater risk of being infected with *C. coli* than *C. jejuni* (Bessède et al., 2014). Campylobacteriosis has also been linked to a range of gastrointestinal conditions, such as inflammatory bowel diseases (IBD), periodontitis, esophageal disease, functional gastrointestinal disorders, celiac disease, and colon cancer in humans (Véron and Chatelain, 1973; Kaakoush et al., 2015). *C. jejuni* infections may lead to autoimmune disorders known as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome. According to an infection study of 111 volunteers, *C. jejuni* dosage correlated with colonization rate, but not with the development of illnesses (Black et al., 1988). The infectious dose to develop campylobacteriosis varied depending on immunity and health status of the individuals. Only 800 *Campylobacter* cells were able to cause diarrhea to some volunteers, while other data showed that campylobacteriosis was developed with a dose as low as 360 cells (Hara-Kudo and Takatori, 2011). Several genes, proteins and components of *C. jejuni* are involved in different virulence factors (**Table 1**).

SURVIVAL IN FOOD AND FOOD-RELATED CONDITIONS

Campylobacter is sensitive to food and food processing-related stresses. It is more sensitive to heat treatment compared to other foodborne pathogens. For example, the *D*-value of *E. coli* is five times higher than that of *C. jejuni* at 55°C (Rusin et al., 1997). Simply freezing at −15°C could reduce *C. jejuni* count by 3 log CFU/g in ground beef (Stern and Kotula, 1982). Desiccation at room temperature inactivated *Campylobacter* within a few days (Doyle and Roman, 1982b). *Campylobacter* cannot survive for a long period of time on food contact surfaces, such as cutting boards, countertops, equipment or kitchen utensils. In contrast, *Campylobacter* can remain viable on fresh foods, such as ground beef (Stern and Kotula, 1982), fresh produce (Kärenlampi and Hänninen, 2004), fresh chicken (Blankenship and Craven, 1982), and milk (Doyle and Roman, 1982b) during the entire shelf life up to 3 weeks. In addition, the combination of these wet and cold refrigeration conditions of fresh foods assists *Campylobacter* in

TABLE 1 | Examples of some important virulence factors and their roles in *C. jejuni*.

Virulence factors	*C. jejuni* gene, protein, or component	Role	References
Stress response	*CosR*	Oxidative stress response regulator	(Hwang et al., 2011)
	GroESL	Heat shock operon	(Thies et al., 1999)
	DnaJ	Heat shock protein	(Konkel et al., 1998)
	Lon protease	Heat shock protein	(Thies et al., 1999)
	RacR-RacS	Regulate temperature during growth and colonization	(Brás et al., 1999)
Motility and chemotaxis	fliA	Flagellar (sigma 28) → regulates the transcription of flagellar genes	(Jagannathan et al., 2001)
	ropN	Flagellar (sigma 54) → regulates the transcription of flagellar genes	(Jagannathan et al., 2001)
	FlgR,S	Regulate the flagellum protein biosynthesis	(Hendrixson, 2006)
	CheA, B, R, W, V, Y	Chemotaxis signal transduction (Che) proteins network.	(Chandrashekhar et al., 2017)
	CheY	Response regulator used for flagellar rotation	(Yao et al., 1997)
Adhesion	RacR-RacS	Persistent colonization of the gut.	(Van der Stel et al., 2015)
	LOS	Involved in adherence and display molecular mimicry of neuronal ganglioside →	(Young et al., 2007)
	CadF	Guillain-Barré syndrome Fibronectin-binding outer membrane protein	(Konkel et al., 1997)
Invasion	Flagellum	Non-flagellated mutants are less invasive.	(Konkel et al., 1999b)
	LOS	Lipopolysaccharide → Involved in invasion	(Grant et al., 1993)
	CPS	Capsular polysaccharide → Involved in invasion	(Karlyshev and Wren, 2001)
	Cia	Invasive antigens	(Rivera-Amill et al., 2001)
Secretion	pVir	Plasmid found in some *C. jejuni* isolates led to type IV secretion system.	(Bacon et al., 2002)
	CiaB	Play role in invasion and type III secretion system	(Konkel et al., 1999a; Konkel et al., 1999b)
Toxins	CdtA, B, C (Cytolethal distending toxin)	Cell distension, cell cycle block and DNA damage → Cell death.	(Lara-Tejero and Galán, 2001)

surviving on dry surfaces for a few weeks instead of a few days (Doyle and Roman, 1982a; Stern and Kotula, 1982).

Refrigeration is one of the most common food preservation methods either used alone or in combination with other antimicrobial strategies or food preservation methods such as the addition of preservatives, irradiation, or modification of atmosphere. *Campylobacter* grows in a limited temperature range compared to other food microorganisms (**Figure 1**). The growth rates of the majority of microorganisms drop to the minimum or stop at refrigeration temperatures. However, fewer pathogenic and spoilage bacteria can grow from a few cells to a large number (*e.g.*, psychrotrophic bacteria) and cause serious food poisonings (Chan and Wiedmann, 2008) or spoilage recall incidents that can be associated with food loss and negative impact on the economy (Pothakos et al., 2014). *Pseudomonas* species (Chouliara et al., 2007; Zhang et al., 2012; Al-Nehlawi et al., 2013), lactic acid bacteria (Chouliara et al., 2007; Doulgeraki et al., 2012; Zhang et al., 2012), and *Brochothrix thermosphacta* (Chouliara et al., 2007; Zhang et al., 2012) are considered as the most problematic spoilage psychrotrophic bacteria in poultry meat. In contrast, *Campylobacter* and *Salmonella* are the most causes of human gastroenteritis due to poultry meat consumption (Rouger et al., 2017). *Campylobacter* in poultry is ranked as the leading pathogen-food combination to cause health risks and negatively impacts the economy (Batz et al., 2012). Kaakoush and others reported that poultry consumption was the most cause of campylobacteriosis outbreaks between 2007 and 2013 (Kaakoush et al., 2015). A more recent report showed that 28 campylobacteriosis outbreaks were linked to the consumption of chicken livers in USA between 2000 and 2016 (Lanier et al., 2018). Up to 90% of commercially available chicken meat in different regions has been identified to be contaminated by *Campylobacter* at ~log 4 CFU/carcass (Willis and Murray, 1997; Jorgensen et al., 2002; Walker et al., 2019).

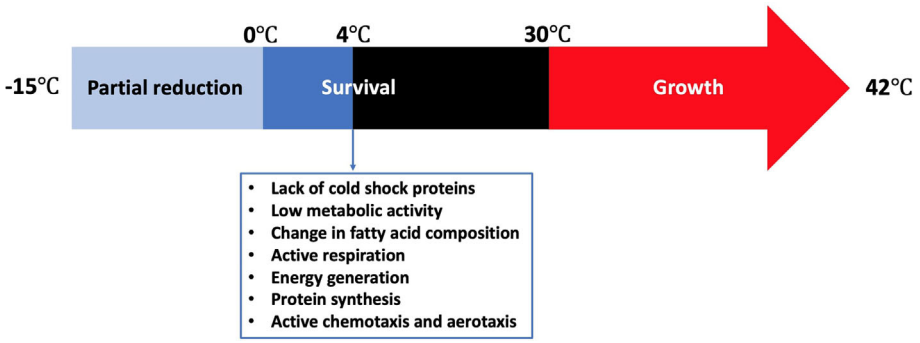

FIGURE 1 | Temperature range for the survival of *Campylobacter* and its stress response at 4°C.

Cold stress response of *Campylobacter* is significantly different from other common foodborne pathogens. Although *Campylobacter* lacks cold shock proteins, this microbe can still be active during the shelf life of different refrigerated foods or during the winter season in the agro-ecosystem (Murphy et al., 2006). Hazeleger and others compared the changes in fatty acid composition of the membrane of coccoid-shaped *Campylobacter* cells with that of the spiral-shaped cells incubated at 4°C (Hazeleger et al., 1995). The change in the fatty acid composition in both groups was similar. In contrast, a significant change in the composition of fatty acids occurred when the cells were incubated either at 12°C or 25°C. This included a significant increase in the percentage of 16:0 and 18:0 fatty acids and a significant decline in the percentage of 14:0, 16:1 and 19:0 fatty acids. The same group reported in another study that the vital processes of *C. jejuni* including cellular respiration, catalase activity, energy generation, and protein synthesis were still be functional at 4°C, which was far below the minimum growth temperature at 30°C (Hazeleger et al., 1998). The total amount of ATP (*i.e.*, produced + consumed) as indicated by the respiration rate at 4°C was only 5% of that at 40°C, suggesting that *C. jejuni* has a relatively low metabolic activity at low temperatures. However, the concentration of the produced ATP at 4°C was almost 50% of that at 40°C. Physiological functions such as chemotaxis and aerotaxis were similarly observed at 4, 20, and 40°C, indicating that *C. jejuni* could normally move toward substrates even below 30°C. The effect of cold exposure (*i.e.*, 6°C for 24 h) on the thermal tolerance (*i.e.*, 56°C) of *C. jejuni* was compared with that of *E. coli* K-12 (Hughes et al., 2009). *C. jejuni* was more tolerant than *E. coli* K-12 to thermal treatment as the ratio of the unsaturated to saturated fatty acids did not change after cold exposure, which was different from that of *E. coli* K-12. In conclusion, *Campylobacter* not only remains viable at low temperatures but also maintains sufficient metabolic activity to survive and move to the favorable places even in the absence of cold shock proteins.

CAMPYLOBACTER IN POULTRY FARMS

Transmission into Poultry Farms

Poultry has been considered as the major source of food-related transmission of *Campylobacter* species to humans since the early years of poultry industry (Skirrow, 1977). *C. jejuni* is a common commensal microorganism in chicken microbiome (Hendrixson and DiRita, 2004; Awad et al., 2016; Ijaz et al., 2018). Poultry is also a reservoir of other *Campylobacter* species including *C. lari*, *C. upsaliensis*, and *C. concisus* (Kaakoush et al., 2014). This bacterium usually transmits horizontally from different environmental sources to flocks (Sahin et al., 2002; Kaakoush et al., 2015). It was reported that *Campylobacter* species are usually abundant in the surrounding environment of poultry farms, such as soil, water source, dust, surfaces and air (Ellis-Iversen et al., 2012). Animal feed and/or drinking water can transmit *Campylobacter* from the environment to poultry farms. Farmers and farm visitors who carry *Campylobacter* can also transmit this microbe to poultry farms. Several studies isolated *Campylobacter* from wild bird feces around poultry houses, suggesting that wild birds contributed to the transmission of this microbe into the poultry houses (Craven et al., 2000; Hiett et al., 2002). For example, a molecular subtype analysis showed that 12 *Campylobacter* strains isolated from the broiler flocks were closely related to a strain isolated from wild bird feces identified in the same farm environment (Hiett et al., 2002).

Other organisms including flies, insects, amoebae, yeasts and molds have been found to be also important routes of horizontal transmission of *Campylobacter* into poultry houses (Axelsson-Olsson et al., 2005; Newell et al., 2011). The presence of *Campylobacter* cells with amoebae, yeasts and molds allow them to survive longer. A lesser mealworm beetle and their larvae (*Alphitobius diaperunus*) were identified as important carriers of *C. jejuni* in the poultry facilities. They could transmit *C. jejuni* not only within batches but also cross-contaminate flocks in the successive rearing cycles (Hazeleger et al., 2008). In addition, microbial eukaryotes may act as a reservoir of *Campylobacter* in the environment. For example, numerous *C. jejuni* strains are able to invade, replicate, and remain viable inside an amoeba host (*i.e., Acanthamoeba polyphaga*) (Axelsson-Olsson et al., 2005). Since eukaryotes are usually prevalent in both drinking water systems and microbial biofilms on farms (Snelling et al., 2006), it is highly possible that infected eukaryotes contribute to *C. jejuni* transmission to poultry infrastructure.

There has been a long controversy about whether *Campylobacter* can be transmitted vertically from one generation of poultry to the other (Cox et al., 2012). One study including 60,000 progeny parent breeders identified a lack of evidence for vertical transmission of *Campylobacter* to chickens (Callicott et al., 2006). All chickens used in the study were hatched from eggs of *Campylobacter*-colonized grandparent flocks. However, egg passage can lead to the transmission of fecal bacteria including *Campylobacter* and subsequently contaminate the shell, shell membrane, and albumen of newly laid and fertile eggs (Cox et al., 2012). This can lead to *Campylobacter* ingestion after the chicks emerge from their eggs, colonization and spread of *Campylobacter* in poultry houses. In contrast, vertical transmission is well-established in *Salmonella* as they contaminate the egg within the reproductive tract before the shell is formed or penetrate the eggshell and invade the yolk of the post-lay egg (Gast and Beard, 1990; Miyamoto et al., 1997; Yang et al., 2001). In addition, *Salmonella* is the major cause of foodborne outbreaks linked to poultry eggs (Guard-Petter, 2001), while *Campylobacter* egg-associated outbreaks are extremely rare (Finch and Blake, 1985). A systematic review including a primary set of 4,316 references showed that *Campylobacter* was rarely isolated from the internal egg contents (Newell et al., 2011), which was also validated by several on-farm studies (Shanker et al., 1986; Van de Giessen et al., 1992; Pearson et al., 1993; Jacobs-Reitsma, 1995; Jacobs-Reitsma et al., 1995; Petersen et al., 2001; Smith et al., 2004; Callicott et al., 2006; Byrd et al., 2007; Kiess et al., 2007).

Therefore, improving biosecurity systems and applying effective intervention strategies are the key elements to limit the prevalence of *Campylobacter* in broiler farms.

Chicken Colonization of *Campylobacter*

Colonization of *Campylobacter* in farm chickens occurs usually due to horizontal transmission from the environment, such as *via* drinking water or animal feed. Once *Campylobacter* enters the chicken flock, it spreads rapidly and colonizes the intestinal tracts (crap, small intestine, and ceca) of most chickens after one week (Beery et al., 1988; Shanker et al., 1990; Newell et al., 2011). The level of *C. jejuni* inside these niches could be as high as 10^9 cells/gram of intestinal tracts with no symptoms or noticeable harmful effects until slaughtering (Stern et al., 2001). One study reported that *C. jejuni* is not just a commensal bacterium in broiler chickens, but it can cause chronic inflammation, gut tissue damage, and diarrhea (Humphrey et al., 2014). In contrast, four combined and eight individual chicken genotypes showed no difference or negative effect on *C. jejuni* colonization and proliferation regardless of chicken growth rate or breed (Gormley et al., 2014).

Several factors affect chicken colonization by *Campylobacter*. These include chicken strain, *Campylobacter* strain, dosage of viable *Campylobacter* cells, and seasonality (Newell and Fearnley, 2003). Colonization potential of chickens by some *Campylobacter* strains could be enhanced by 1,000-folds (Ringoir and Korolik, 2003) or 10,000-folds (Cawthraw et al., 1996) under *in-vivo* experimental conditions, leading to the challenges to predict the ability of *Campylobacter* wild strains to colonize chicken flocks in the real commercial farms. There is generally a higher rate of colonization in summer than any other time of the year (Humphery et al., 1993). The colonization level (Wallace et al., 1997) and type of strains (Hudson et al., 1999) are also seasonally dependent. Besides high temperature and humidity, poultry houses require more ventilation during summer, which exposes the birds to more *Campylobacter* from the outside environment than any other time of the year (Hudson et al., 1999). Even individually caged birds showed a seasonal variation (increased to the peak in late April) in the fecal excretion of *C. jejuni*, suggesting that the surrounding temperature affects bird colonization even under limited conditions of *C. jejuni* transmission (Doyle, 1984).

Moreover, geographical locations, flock size, and type of the production systems (*i.e.*, organic or conventional) can also influence the colonization of *Campylobacter* in chicken flocks (Newell and Fearnley, 2003). According to a previous study, up to 100% of flock were *Campylobacter*-positive in the case of organic and free-range flocks (Heuer et al., 2001). This is probably due to the exposure to the outside environment and a longer time the birds require to grow to the slaughter size compared to the indoor reared flocks. In the cases where the colonization of *Campylobacter* identified at species level, *C. jejuni* was the leading group by colonizing about 90% of *Campylobacter*-positive birds. The remaining ones were almost equally colonized by *C. coli* and *C. lari* (Uyttendaele et al., 1996). Several studies conducted in Europe suggested that the indoor-grown flocks were primarily colonized by one or two *C. jejuni* strains. Other studies conducted in North America and Australia showed that several *C. jejuni* strains usually colonized the indoor-grown flocks. This might be due to different levels of biosecurity standards in different countries as the incidences of *C. jejuni* colonization can be either due to the exposure to multiple sources consisting of different strains or a single source (*e.g.*, feed or water) consisting of multiple strains. Interestingly, Hald and co-authors reported that *C. jejuni* colonization was higher in a total of 88 randomly selected poultry flocks raised in Danish farms that fed external grains compared to farms that fed home-grown grains (Hald et al., 2000).

Another important factor of chicken colonization is the adaptation capability and response of *Campylobacter* strains to the environmental conditions. For example, Gaynor and others identified a remarkable ability of *C. jejuni* to evolve rapidly during storage, culture, and condition passage (Gaynor et al., 2005). The colonization ability of *C. jejuni* 11168-O strain recognized as an excellent chicken colonizer was compared with *C. jejuni* 11168-GS clone recognized as a poor chicken colonizer after either aerobic or anaerobic incubation. The anaerobic priming of 11168-GS increased its colonization while the aerobic passaging of 11168-O decreased its colonization compared to their original strains.

Some procedures, such as feed withdrawal and transportation, affect the presence of *Campylobacter* in live chickens before their arrival into the poultry-processing plants. Feed withdrawal is a common commercial practice that the farmers remove the animal feeds from poultry houses 3 to 18 h before slaughtering (Byrd et al., 1998). The purpose of this practice is to clear the gastrointestinal tract and reduce the level of fecal materials in the body so as to minimize cross-contamination during poultry processing. Byrd and co-authors showed that feed withdrawal could increase the prevalence of *Campylobacter* in the crops of broiler chickens at the slaughter age (Byrd et al., 1998). *Campylobacter*-positive samples increased on average from 25% to 62.4% before and after feed withdrawal. The limitation of nutrients in the broiler crops might have resulted in a less diverse and competitive microbiota and subsequently enhanced the growth of *Campylobacter*. Transportation from farms to processing plants has been identified as a critical harbor for the transmission and colonization of *Campylobacter* in live birds. This is due to the reuse of contaminated crates for shipping, animal hoarding, and induced-stress during the transportation of live birds from different flocks and/or farms to slaughterhouses (Slader et al., 2002; Newell and Fearnley, 2003). Decontamination methods used for cleaning the reusable shipping crates for transportation was identified to be ineffective (Wedderkopp et al., 2001). Up to 70% *C. jejuni*-negative chickens became colonized after exposure to artificially contaminated shipping crates (Clark and Bueschkens, 1988). Whyte and others demonstrated that poultry overcrowding and stress induced during transportation could extensively increase the shedding of *Campylobacter* in fecal material of broilers and contributed to cross-contamination of their carcasses during processing (Whyte et al., 2001).

Several studies indicated that *C. jejuni* acts as a commensal and a super colonizer in chicken cecal microbiota (Awad et al., 2016; Connerton et al., 2018; Ijaz et al., 2018). Awad and co-authors identified that the microbial communities in the luminal and mucosa gut microbiome shifted in a timely manner during the growth of broiler chickens (Awad et al., 2016). A similar finding was observed when the chicken cecal microbiome was analyzed even without any artificial inoculation of *C. jejuni* (Ijaz et al., 2018). The critical periods for *C. jejuni* colonization ranged from 12 to 28 d of the broiler chicken age (Awad et al., 2016; Ijaz et al., 2018). Another report showed that *C. jejuni* appeared in 6-day old chicken birds (Connerton et al., 2018). The microbiota variation is usually influenced by the diet and microorganisms present in the surrounding environment, feed, and water (Connerton et al., 2018). For all of these reasons, it is quite challenging to inactivate *C. jejuni* once broiler chickens are colonized. Early prevention of *C. jejuni* colonization on poultry farms is very important to avoid further colonization. Although no symptom is associated with colonization of *C. jejuni* and *C. coli* in the broiler chickens, the high mortality rate might reflect the colonization prevalence (Powell et al., 2012).

On-Farm Intervention Strategies Against *Campylobacter*

Early stage on-farm control of *Campylobacter* in broiler chickens has gained increasing attention during the last two decades because *Campylobacter* can effectively colonize chickens from the early days of their lives and remain prevalent at a high level throughout the poultry-processing line (**Table 2**). The potential of different intervention strategies by using vaccination, phage therapy, bacteriocins, probiotics, fatty acids, and essential oils has been investigated. Each strategy has some advantages and disadvantages (**Table 3**). For example, numerous bacteriocins (antimicrobial peptides) produced by commensal bacteria from chicken gut microbiota, such as *Lactobacillus salivarius*, could effectively inactivate *Campylobacter* under both *in-vitro* and *in-vivo* experimental settings (Svetoch and Stern, 2010). Specifically, the L-1077 bacteriocin was able to reduce >4 log CFU/g of *C. jejuni* in the cecal content. In a recent study, oral administration of three types of bacteriocins from *Lactobacillus salivarius* (OR-7) and *Enterococcus faecium* (E-760 and E50-52) were used in broiler chickens to investigate the development of resistance by *C. jejuni* (Mavri and Smole Možina, 2013). CmeABC multidrug efflux pump in *C. jejuni* played an important role in intrinsic and acquired resistance against bacteriocins. Thus, combining bacteriocins with an efflux pump inhibitor might synergistically inactivate *C. jejuni* and prevent the development of antimicrobial resistance.

Several studies have investigated the efficacy of *Campylobacter* phages to either reduce *Campylobacter* count or prevent their colonization in chicken broilers (Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009; Carvalho et al., 2010; Kittler et al., 2013). Some used artificial infections (Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009; Kittler et al., 2013) while others used naturally infected birds (Carrillo et al., 2005). One study used an effective colonizer strain of *C. jejuni* and observed a significant reduction by several phages isolated from the same environment of the bacterial host (Wagenaar et al., 2005). Some phages caused up to 3 log reduction within the first 24 h, while others caused about 1 log reduction for up to 30 days at a high Multiplicity of Infection (MOI). The efficacy of *Campylobacter* phage therapy is not sufficient for a sustainable control of this bacterium. *C. jejuni* strain and phages used in that study were not isolated from representative samples of chicken farms, meats, or feces. Thus, this model cannot be generalized to be used in a wider therapeutic application in farms. However, the same study showed promising results and indicated that the high dose of phages (11 log PFU/ml) did not show any negative impact on the broilers' health.

More studies are required to achieve sustainable benefit of *Campylobacter* phage therapy. The importance of using phages in the form of cocktail was observed in several studies (Wagenaar et al., 2005; El-Shibiny et al., 2009; Carvalho et al., 2010). Many other factors, such as oral administration route (*i.e.* phage delivery) and stability of individual phages, play important roles in the overall efficacy of phage therapy application in chicken broilers (Ushanov et al., 2020). For example, different studies indicated that the addition of phages into drinking water can be more effective than oral gavaging, which is not practical for large commercial scale production (Carrillo et al., 2005; Carvalho et al., 2010; Ushanov et al., 2020). However, such application requires more stable phages than others. Altogether, many studies agreed that phage therapy can be

TABLE 2 | Examples of the average prevalence and load of *Campylobacter* throughout the poultry-processing chain.

Stage	Source	Prevalence (%) and/or average load of *Campylobacter*	Reference
Farm	Broilers	87.5%, 9 log CFU/g of cecal content (n = 50)	(Stern et al., 2001)
	Feces	96.4%, 5.16 log CFU/g of fecal content (n = 948)	(Stern and Robach, 2003)
Transportation	Caecum	6.5 log CFU/g of cecal content	(Achen et al., 1998)
	Feces	60–100% (n = 7 [10 flocks])	(Whyte et al., 2001)
Plant	Pre-scald	77%, > 6 log CFU/g of feather or skin (n = 40)	(Kotula and Pandya, 1995)
	Defeathering	3.9 log CFU/ml of carcasses rinse (n = 24)	(Berrang et al., 2000)
	Evisceration	96–100%, 2.7 log CFU/carcass (n = 48)	(Northcutt et al., 2003)
	Pre-chill	98%, 4.75 log CFU/ml of carcasses rinse (n = 450)	(Stern and Robach, 2003)
	Post-chill	84.7%, 3.03 log CFU/ml of carcasses rinse (n = 450)	(Stern and Robach, 2003)
	Pre-wash	87%, 4.78 log CFU/ml of carcasses rinse (n = 30 [4 processing plants])	(Bashor et al., 2004)
	Post-wash	80%, 4.30 log CFU/ml of carcasses rinse (n = 30 [4 processing plants])	(Bashor et al., 2004)
Retail		90%, > 4 log CFU/carcass (n = 552)	(Walker et al., 2019)

TABLE 3 | Advantages and disadvantages of different prevention and control strategies against *Campylobacter* in poultry production.

Stage	Strategies	Advantage	Disadvantage
Farm	Vaccination	Preventive and promising (Annamalai et al., 2013; Neal-McKinney et al., 2014)	Expensive, highly specific, and difficult (Saxena et al., 2013; Kaakoush et al., 2015)
	Bacteriophages	Caused up to 5 log CFU/g reduction of *C. jejuni* in cecal content of commercial broiler flocks (Kittler et al., 2013)	Dilution in the gut over the time and development of resistance (Labrie et al., 2010; Fischer et al., 2013)
	Bacteriocins	Caused >4 log CFU/g reduction of *C. jejuni* under *in-vitro* settings (Svetoch and Stern, 2010)	Development of antimicrobial resistance by the multidrug efflux pump CmeABC (Mavri and Smole Možina, 2013)
	Probiotics	Part of the chicken gut microbiota (Kaakoush et al., 2015)	Limited reduction of *C. jejuni* after 15 d of oral administration (Santini et al., 2010)
	Short chain fatty acids	Ability to invade the gut epithelium cells (Davidson et al., 2005)	Limited reduction of *C. jejuni* under *in-vitro* settings (Davidson et al., 2005)

effective to reduce *C. jejuni* if administrated at a high MOI within 24 to 48 h prior to slaughter. Phage efficacy can also be improved when phages and hosts are isolated from the same environment. However, this may limit the application of phage therapy to specific poultry farm(s). Therefore, more phages and representative hosts need to be tested.

CAMPYLOBACTER IN POULTRY-PROCESSING PLANTS

Poultry meat and eggs are important sources of dietary proteins, vitamins, and minerals. Poultry production is an intensively growing industry and chicken meat is one of the most produced meats around the world (Ritchie and Roser, 2017). The annual global amount of produced poultry meats has been rising by 10-folds within the last 50 years to approximately 102 million tons (Ritchie and Roser, 2017). Chicken is also one of the most sustainable major sources of dietary proteins as the feed conversion ratio (FCR; kg of feed/kg of edible weight) of chicken meat is only about 40% of the FCR of beef (Wilkinson, 2011). As a large, diverse and vertically integrated system involving animal farming and food processing, poultry production can be a common source of foodborne outbreaks. Either live poultry or poultry meat are important sources of *Campylobacter* and other important foodborne pathogens (Kaakoush et al., 2015). Both on-farm and *in vivo Campylobacter* controls are challenging due to the complexity and diversity of both systems (**Tables 2** and **3**). Alternatively, many studies have focused on controlling *Campylobacter* in the processing facilities.

Campylobacter Survival During Poultry Processing

Poultry processing is considered as an intensive procedure that requires highly trained personnel. One breach in either sanitation or hygiene practices might end in several cases of foodborne illnesses. *Campylobacter* enters a processing plant through any potentially contaminated bird(s) at an initial count as many as 10^9 cells/g of cecal content (Beery et al., 1988; Stern et al., 2001). A single processing plant normally receives birds from multiple farms with variations in their ages, sizes, geographical locations, and production and biosecurity systems that increase the chance of *Campylobacter* contamination. Birds go through different processing steps starting from receiving and hanging until packaging. Processing consists of multiple critical points where *Campylobacter* starts to occur or increase in chicken carcasses (**Table 2**). Steps including scalding, defeathering, evisceration, nick removal, inside and outside (or inside-out) washing can all contribute to cross-contamination of *Campylobacter* in one way or another (**Figure 2**).

Scalding is a quick immersing of poultry carcasses into warm water (51–64°C) for a few seconds up to 2 min so as to loosen the skin follicles for defeathering. Berrang and others investigated the level of *Campylobacter*, total aerobic microbes, *E. coli*, and coliform throughout the poultry-processing plant (Berrang and Dickens, 2000). Total aerobic bacteria clearly decreased throughout the processing steps. In contrast, *Campylobacter* showed the highest recovery (increased from log 1.8 to 3.7 CFU/ml of chicken carcass rinse) compared to all the other bacteria after defeathering. Heating of poultry carcasses followed by chilling during the processing steps are essential in assisting practices for effective defeathering. However, this temperature fluctuation creates several challenges to control microorganisms including *Campylobacter*. For example, skin follicles remain open after scalding that allows bacteria to penetrate the skin and accumulate inside the follicles. Moreover, the follicles close again during chilling, making the poultry decontamination to be highly challenging. In addition, a large shift appears in the native skin microbiome of chicken (Thomas and McMeekin, 1980). The predominately Gram-positive skin microbiota (*e.g.*, *Micrococcus*) is usually detached and replaced by a population mixture consisting of a majority of Gram-negative bacteria. However, the alteration of chicken meat microbiome during processing varies based on multiple factors, including geographical location, season, and bird-to-bird. One common factor is that scalding liquidizes some fats on the skin that became part of chicken juice and other surrounding fluids. The liquid fat solidifies again during the chilling step and creates a lipid film on the surface of chicken meat. Both scanning electron microscopy and transmission electron microscopy showed that scalding and defeathering scraped off the epidermis cells of chicken skin that became smoother and less hydrophobic than normal after processing (Thomas and McMeekin, 1980). The bacterial contaminants were identified within a protective fluid film formed both on the surface and inside deep channels of chicken skin after chilling, which makes microorganisms in chicken meat unapproachable by the antimicrobial agents.

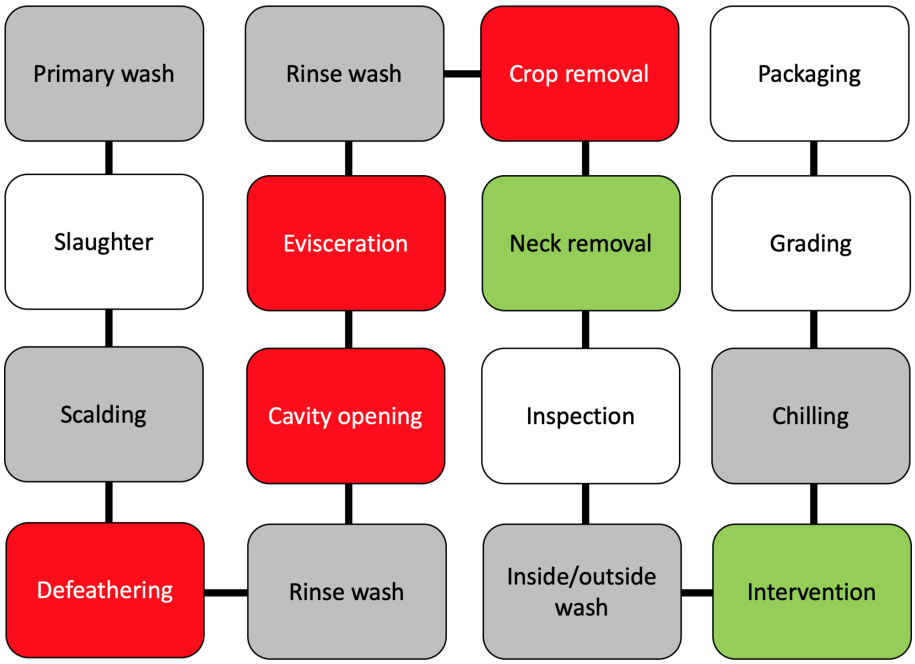

FIGURE 2 | A schematic diagram of raw chicken processing steps. The gray boxes reflect steps that can enhance cross-contamination, the red boxes reflect steps where cross-contamination usually occurs, while green boxes reflect steps that contribute to the mitigation of *Campylobacter*.

Moreover, evisceration is one of the most critical steps of cross-contamination. Colonized gastrointestinal tract of poultry birds carries a large number of *Campylobacter* bacteria that can spread in a wide range, especially in the case of gut leakage. Many in-plant studies confirmed that the number of *Campylobacter*-positive carcasses significantly increased after this process (Berrang et al., 2001; Northcutt et al., 2003; Keener et al., 2004). For example, *Campylobacter*-positive chicken thighs and breasts separately increased from 0% to 90% at a level between 2 and 3 log CFU/g after evisceration (Berrang et al., 2001). Another study identified that *Campylobacter* contamination level was higher on the breast meat than the thigh meat or drumstick (Kotula and Pandya, 1995). Leaking of *Campylobacter* from the gut during evisceration can contaminate the lower half of the carcasses (breast and neck) more than the upper half (thighs and drumstick) as the birds are always hanged upside-down by the feet. The hanging necks of carcasses were also frequently determined to be *Campylobacter*-positive (Kotula and Pandya, 1995).

Poultry carcasses require rapid cooling to prevent the growth of microorganisms after evisceration. Chilling and antimicrobial treatment are usually combined in many processing plants to save energy and rapidly inhibit bacterial growth by washing the carcasses with cold chlorinated water (Keener et al., 2004). Poultry carcasses are usually washed by dipping or spraying using chlorinated water to remove blood, tissue, fragments, and contamination after evisceration. Dipping can cross-contaminate carcasses under commercial processing conditions especially when processing a large number of birds at the time (Bailey et al., 1987; Bilgili et al., 2002; Demirok et al., 2013). In contrast, spray washers tend to reduce the level of cross-contamination on the chicken meat (Keener et al., 2004; Demirok et al., 2013). Several options of spraying systems for poultry carcasses have been used in poultry industry, including brush, cabinet, and inside-out washing systems (Keener et al., 2004). The brush washing system is similar to a car washer where many rubber fingers are used with the aid of water to remove debris and wash the carcasses from the outside. The cabinet washing system contains multiple sprayers in an enclosed system to wash the outside of the carcasses. Inside-out system is a similar enclosed spraying system but used for both external and internal washing at the same time. The machine rotates the carcasses and sprays them from the outside, while probes of single sprayers enter the intestinal cavities of carcasses to wash them from the inside. Many inside-out washing machines spray water at the pressure level between 40 and 180 psi to remove visible fecal contamination and fragments (Keener et al., 2004). Chlorine concentration ranged from 20 to 50 mg/L and water consumption ranged from 100 to 200 L/min.

Antimicrobial Treatments for Poultry Processing

Many laboratory-scale experiments showed that the approved antimicrobials such as acidified sodium, chlorite, cetylpyridinium, chlorine, chlorine dioxide, peroxyacetic acid, and trisodium phosphate could cause up to 5 log reduction of *Campylobacter* in chicken meat (**Table 3**). However, in-plant poultry washers have limited effect on inactivating *Campylobacter* in chicken meat regardless of the efficacy of antimicrobials, water temperature, or

washing system. This could be due to several factors including the presence of large molecules in chicken meat (*e.g.*, proteins and lipids) and *Campylobacter* in chicken skin due to changes induced by processing, sensitivity of chicken skin to heat, oxidation and discoloration, initial microbial load of carcasses, number of processed carcasses per min, interaction or masking of antimicrobials (*e.g.*, chlorine) by organic materials in the processing water, water quality and survival of *Campylobacter* in recycled processing water, poor sanitation, accumulation of lipids, fecal materials, and/or organics at any point through the processing line. It is worth mentioning that there is no effective critical control (*i.e.*, killing) point in processing raw chicken similar to that of the pasteurization step for milk processing (Tresse et al., 2017).

Current Situation of Raw Poultry Product Safety

The prevalence of *Campylobacter* in poultry products is clearly a major food safety challenge for many years. It is important to target chicken as a critical food vehicle of *C. jejuni* due to the high rate of contamination. More on-farm and in-plant control strategies became available in the recent years, but these strategies need improvement to enable effective inactivation of *Campylobacter* at an early stage or in chicken end-products. In 2015, the United State Department of Agriculture, the Food Safety and Inspection Service agency (USDA-FSIS) established a new *Campylobacter* and *Salmonella* performance standard for the contaminated poultry products, raw chicken parts (*e.g.*, breasts, thighs, wings), and not ready-to-eat (NRTE) poultry products (Crim et al., 2015). For example, 8 out of 51 *Campylobacter*-positive broiler carcasses is the maximum acceptable number of randomly tested samples. In 2018, new antimicrobial agents have been approved by the USDA-FSIS to be used in washing poultry carcasses during processing. These include peroxyacetic acid (a mixture of hydrogen peroxide and acetic acid), a mixture of calcium chloride, calcium hypochlorite, sodium chloride, calcium hydroxide, calcium carbonate, sodium triphosphate, and a combination of calcium chloride with sodium bisulfate (Service, 2018). Although *Campylobacter* can be reduced to some extent by antimicrobials, they still might not be reduced to a safe level as only a few hundred cells might cause human illnesses (Black et al., 1988; Hara-Kudo and Takatori, 2011). In addition, in-plant antimicrobial treatment requires intensive amount of water to wash chicken carcasses. For example, a medium size poultry-processing plant spends annually $0.5 to 1 million USD on average on water consumption for washing chicken carcasses and surfaces (Jackson, 1999), but *Campylobacter* reduction is still insufficient.

ALTERNATIVE STRATEGIES TO CONTROL *C. JEJUNI* IN AGRI-FOODS

Plant-Based Antimicrobials

Plant-derived compounds have been used for centuries in medicine, perfumery, cosmetics or being added to foods as oils, herbs or spices (Hyldgaard et al., 2012). For example, herbs and essential oils were initially used in medicine due to their antimicrobial, anti-inflammatory, or antioxidant effects, then their application expanded in agri-foods in the 19th century for their aroma and flavors. These antimicrobials are important secondary metabolites that play major roles in plant defense systems to protect them from microbial infections (Tajkarimi et al., 2010). It was estimated that ~3,000 essential oils have been identified and ~300 are commercially available for flavoring, fragments, or cosmetics (Van de Braak and Leijten, 1999). In addition, essential oils can act as growth promoters in farm animals similar to antibiotics (Brenes and Roura, 2010; Ahmadifar et al., 2011). A histology study showed that feeding different plant extracts to chicken broilers increased the thickness of the mucus layer in the glandular stomach and jejunum (Jamroz et al., 2006). These changes were associated with a large shift in gut microbiota that could hypothetically promote the growth of birds.

Cinnamon is one of the earliest spices used in human history and cinnamon oil is among the most studied essential oils due to its high antimicrobial potency (Ravindran et al., 2003). The genus *Cinnamomum* consists of ~250 different species. *C. verum* and *C. cassia* are the most known and used herbal medicines or spices. These plants are the main natural sources of cinnamon. Cinnamon oil consists of several major antimicrobial compounds, including cinnamaldehyde (70–90%), 1-linalool, p-cymene, and eugenol (Davidson et al., 2005). Aldehyde groups are reactive organic compounds that can crosslink covalently with proteins and nucleic acids through amine groups. Therefore, the mode of action of cinnamaldehyde is inconclusive. Several mechanisms can occur depending on the bioavailability and concentration of the system (Hyldgaard et al., 2012). For example, cytokinesis can be inhibited due to the inhibition of different enzymes by cinnamaldehyde at a low concentration. ATPase inhibition occurs at the sub-lethal concentration, while the alteration of fatty acid composition of cell membrane, cell leakage and cell death occur at the lethal concentration.

In comparison, curcumin is the major active compound of the rhizome of turmeric (*Curcuma longa*). This golden spice is a phenolic pigment responsible for the yellow color of turmeric. Numerous studies have shown that curcumin can effectively inactivate both Gram-negative and Gram-positive bacteria (Rudrappa and Bais, 2008; Kaur et al., 2010; Tyagi et al., 2015). However, limited studies have investigated the antimicrobial mechanism of curcumin. Blocking the assembly of the FtsZ protein essential for forming the FtsZ ring (*i.e.*, Z ring) to initiate cell division in bacteria was identified to be the mode of action against *Bacillus subtilis* and *E. coli* (Kaur et al., 2010). In contrast, curcumin has been found to attenuate several virulence factors, including quorum sensing and biofilm formation in *P. aeruginosa* (Rudrappa and Bais, 2008). A recent study examined the membrane permeability of *S. aureus, Enterococcus faecalis*, *E. coli*, and *P. aeruginosa* after being treated with curcumin (Tyagi et al., 2015). A steady-state fluorescence and flow cytometry analyses showed uptake in the extracellular propidium iodide

(only enters intact bacterial cells by a permeabilizing agent) and leakage of calcein (only leak out of bacterial cells if there is membrane damage due to cell wall membrane damage) in both Gram-positive Gram-negative bacteria. Antimicrobial mechanism of curcumin is different depending on the bacteria studied and the assays used (Han et al., 2006; De et al., 2009; Kaur et al., 2010; Tyagi et al., 2015). More studies are still needed to confirm the antimicrobial mechanism(s) of the action of curcumin.

To the best of our knowledge, the specific antimicrobial mechanism of curcumin against *Campylobacter* has not been investigated. The effect of curcumin against *Helicobacter pylori*, a highly relevant bacterium to *Campylobacter*, has been repeatedly confirmed in several studies (Di Mario et al., 2007; Zaidi et al., 2009; Sarkar et al., 2016; Vetvicka et al., 2016). One study of using a high-throughput screening of 5,000 chemical compounds discovered that the inhibition of *H. pylori* by curcumin was due to the inhibition of shikimate pathway (Han et al., 2006). This pathway is essential for the synthesis of aromatic amino acids (*e.g.*, phenylalanine, tryptophan, and tyrosine) in bacteria, fungi, and higher plants, but not in mammals. Targeting this particular pathway makes curcumin a very safe antimicrobial agent for human consumption. In fact, curcumin showed no toxicity on human health even used at a level as high as 8,000 mg per day (Cheng et al., 2001; Lao et al., 2006). In addition, the antimicrobial activity of curcumin against *H. pylori in vitro* (65 clinical isolates) and *in vivo* during infections in mice were examined. The minimum inhibitory concentration (MIC) of curcumin ranged from 5 to 50 μg/ml regardless of genetic variation of the tested *Helicobacter* strains. Curcumin not only inactivated *H. pylori* during infection but also reduced the gastric damage induced by *H. pylori* infection to almost a normal state. Although limited studies have identified the antimicrobial mechanism of curcumin, available evidence shows its great potential for preventing and treating bacterial contaminations and infections.

Metal Oxide Nanoparticles

Novel applications of nanotechnology and nanomaterials have gained great attention in the recent years. For example, the applications of metal oxide nanoparticles (*e.g.*, Al_2O_3, TiO_2, and ZnO NPs) could inactivate several foodborne pathogens in a variety of agri-food systems (Fernández et al., 2009; Akbar and Anal, 2014; Panea et al., 2014). ZnO was identified to be more effective than other metal oxides (*e.g.*, CuO and Fe_2O_3) against both Gram-negative and Gram-positive bacteria (Azam et al., 2012). In addition, ZnO NPs was more effective against *C. jejuni* than other Gram-negative bacteria including *E. coli* O157: H7 and *S. enterica* (Xie et al., 2011). The direct contact of ZnO NPs (positively charged) with bacterial cell wall (negatively charged) by electrostatic force leads to destabilization and disruption of bacterial outer cell membrane. In addition, semi-conductive property of ZnO allows the generation of reactive oxygen species that can attack different cytoplasmic and extra-cytoplasmic targets after the binding (Sirelkhatim et al., 2015).

Synergism

Antimicrobial combinations have been used since the earliest days of the recorded history to treat illnesses and reduce sufferings (Chou, 2006). Therapeutic use of traditional Chinese herbs is a prime example. Indeed, antibiotic is one of the most important drug discoveries in the modern medicine. However, the emergence of antibiotic resistance to most available antibiotics became a serious public health concern in the recent years and near future (de Kraker et al., 2016). A synergistic combination of antimicrobials can minimize some of the disadvantages associated with the use of antimicrobials, such as the development of bacterial resistance, high dosage, and limited effect (Chou, 2006). Antimicrobial combination has been extensively studied to inactivate some highly challenging bacterial and viral infections including methicillin-resistant *Staphylococcus aureus* infection (An et al., 2011) and human immunodeficiency virus infection (Gaibani et al., 2019).

Synergism is defined as an effect that is greater than the sum of multiple individual effects. Many approaches, hypotheses, methodologies, and models have been used to study the synergism in different fields, including microbiology, pharmacology and enzymology (Chou, 2006). The definition of synergism is a very controversial topic due to the complexity of biological systems and some possible mathematical errors or pitfalls in the combinatorial studies. Some important concepts such as the difference between synergism and enhancement or potentiation are not fully clear. For example, if antimicrobial A has a quantifiable effect (*e.g.*, 10%), while antimicrobial B has no effect (*i.e.*, 0%), and their combination produces an effect greater than antimicrobial A (*e.g.*, 20%), then this is considered as an enhancement or potentiation, but not a synergistic interaction. In contrast, synergism is an effect greater than the sum of multiple quantifiable effects (*e.g.*, 10% + 10% = 30%). In addition, the additive effect has always to be less than 100%. For example, if antimicrobial A and B each affects 20%, the additive effect is not simply 40% because if each antimicrobial produces 70% effect the combined effect cannot be 140%. Chou and Talalay reported the fractional product equation to solve this issue [(1–0.7) (1–0.7) = 0.09] where the additive effect can never exceed 100% (Chou and Talalay, 1984).

Methods for Identifying Antimicrobial Synergism

Three methods are most used in antimicrobial combination studies. These include the disk diffusion method, time killing method and fractional inhibitory concentration index method (FICI) (Odds, 2003; Zhou et al., 2016). Disk diffusion method is a simple visual test that relies on comparing bacterial inhibition zones of diffused (single and combined) antimicrobial agents in the agar plates. Time killing method shows how a bacterial population responds to the antimicrobial treatment at different time intervals in either broth or agar medium. It relies on monitoring the antimicrobial effect of single and combined antimicrobials by calculating the log reduction of lethal and sub-lethal concentrations over time. For example, if antimicrobial A caused 1 log reduction and antimicrobial B caused 1 log reduction, then the additive effect would be 2 log

reduction. In this case, synergism would require effect greater than 2 log reduction (*e.g.*, 1 + 1 = 3). This method is labor-intensive and time-consuming. Thus, a few concentrations of antimicrobials are usually used and combined at a fixed ratio. In contrast, the FICI method (also called microdilution checkerboard) shows a clear visualization of positive/negative inhibitory interactions of multiple ratios of combined antimicrobials. It relies on constructing two antimicrobial combinations in a two-dimension array (*e.g.*, 96-well plate) and comparing the MICs of single and combined treatments. Synergy requires at least a four-fold reduction in the MIC of both antimicrobials combined (*i.e.*, FICI value of ≤ 0.5). The FICI method gained more popularity in the recent years as it is more restricted in identifying synergism, more comprehensive, and easier to construct and interpret than other methods.

Types and Mechanisms of Antimicrobial Synergism

Different types of antimicrobial interactions can occur between antimicrobials depending on their origins and individual mechanisms. It is common to observe synergism between antimicrobials of different mechanisms and different targets (Jia et al., 2009; Oh et al., 2015). For example, combining efflux pump inhibitor(s) with an intracellular antimicrobial(s) can synergize to inactivate microorganisms that use efflux pumps to remove antimicrobials due to antimicrobial accumulation inside the cells (Oh et al., 2015). In addition, antimicrobials may synergize due to the complementary or facilitating collective actions (Jia et al., 2009). Although different antimicrobials may have different targets and mechanisms, they might have overlapping pathways at the molecular level. More importantly, synergism can be used to increase bacterial antibiotic susceptibility.

Applications of Synergism

Bacteria develop resistance to antibiotics *via* different mechanisms (Mavri and Smole Možina, 2013). These include the modification of a receptor or active site of the antibiotic target to prevent or reduce binding, production of enzymes that directly destruct or modify the antibiotics, and/or reducing the accumulation inside the cells by decreasing the outer cell membrane permeability or pumping out the antibiotics using efflux pumps. One of the best applications for antimicrobial synergism is to be against tolerant and/or resistant pathogens that require more than single or additive treatments. For example, Augmetin® is a common commercial antibiotic that consists of a combination of clavulanate acid and amoxicillin to inactivate different pathogens, including β-lactam resistant bacteria (12). The combination of clarithromycin and amoxicillin is part of the standard therapy for *H. pylori* stomach infections (11).

Plant-based antimicrobials are a great source of new alternative antimicrobials. Many recent studies showed that plant-based antimicrobials (*e.g.*, phenolic compounds) synergize with antibiotics (*e.g.*, amikacin, ceftriaxone, cephradine, methicillin, imipenem) (Oh and Jeon, 2015) or metal oxide nanoparticles (Hemaiswarya and Doble, 2010) against various microbes. Oh and Jeon reported synergistic interactions of several phenolic compounds (*e.g.*, gallic acid and taxifolin) in combination with ciprofloxacin or erythromycin against fluoroquinolones- and macrolides-resistant *C. jejuni* isolates (Oh and Jeon, 2015). Phenolic compounds increased membrane permeability as determined by measuring the intracellular uptake of 1-*N*-phenylethylamine. As a result, accumulation of both antibiotics increased substantially inside the bacterial cells. Further testing showed that phenolic compounds increased 1-*N*-phenylethylamine accumulation in an isogenic (knockout) *cmeB* mutant more than that in a wild type *C. jejuni* strain. In addition, the expression level of CmeABC multidrug efflux pump was reduced by several phenolic compounds (*i.e.*, gallic acid and taxifolin). These findings indicated that phenolic compounds increased the influx rate and decreased the efflux rate of antibiotics.

Active Packaging

Food packaging is one of the last steps in food processing to ensure that the food products are contained and delivered in the best condition. Packaging materials and/or the atmospheric condition inside the packaging are used to protect the foods from microbial growth, pathogen contamination, physical damage, chemical degradation, or other effects from the environment. Most of the commercially applied packaging technologies aim to preserve food quality and extend the shelf life of food products. Moreover, data regarding the use of active packaging to control foodborne pathogens in potentially contaminated and high-risk foods is still limited compared to those for spoilage bacteria. For example, modified atmospheric packaging (MAP) was extensively studied for the control of spoilage microorganisms in a variety of food products, such as raw meats, fresh produce, and seafood products (McMillin, 2008). In addition, recent technologies and intervention strategies that are used in food processing allow food packaging to be a suitable component of hurdle technology. Such approach might overcome the challenge of controlling the survival of frequently isolated pathogens from commercially available raw meats, such as *C. jejuni* in chicken, *V. parahaemolyticus* in seafood, and pathogenic *E. coli* in beef.

Fresh poultry, raw meats, and seafoods are considered as high-risk and highly perishable foods. Foods of animal origins including raw milk, raw cheese, and raw meats have a high content of moisture and nutrients. These factors form an ideal environment for rapid growth and/or long survival of many microorganisms, including both spoilage and pathogenic bacteria. Different bacteria have been commonly isolated from fresh chicken meats after processing. These include *Micrococcus*, Gram-positive rods, *Cytophaga-Flavobacterium*, *Pseudomonas*, and Enterobacteriaceae (Thomas and McMeekin, 1980). Thomas and McMeekin identified that poultry carcasses originally carried *Micrococcus* as a part of the skin microbiome, but contamination with the psychrotrophic *Pseudomonas* appeared after processing (Thomas and McMeekin, 1980). A whole-genome sequencing analysis showed that the Firmicutes (mainly Gram-positive) were the most abundant bacterial group based on the phyla level of raw chicken meat after processing (Kim et al., 2017).

Campylobacter and *Salmonella* are the most frequently isolated human pathogens from poultry products at the retail level (Rouger et al., 2017). No correlation was

established between the prevalence of these two poultry-associated pathogens in chicken carcasses collected from 58 slaughterhouses during a 12-month period in France (Hue et al., 2011). Moreover, no correlation was established between the microbial load (*i.e.*, total aerobic count, Enterobacteriaceae, and coliform) and the prevalence of *Campylobacter* in chicken and turkey fresh meat cuts (Fontanot et al., 2014). Thus, the prevalence of *Campylobacter* in poultry end products is random and unpredictable because transmission in farms and cross-contamination during processing can occur at any point, which is not always associated with any other microbial indicators. The large size of poultry industry and production scale makes the detection of *Campylobacter* more challenging in these products. Both *Campylobacter* and *Salmonella* can originally occur at a high level (up to 10^8 CFU/g) in the gastrointestinal tract of birds, but their prevalence in poultry meat varies depending on the cross-contamination incidents. A positive correlation was identified between *Campylobacter* level in chicken caeca and the end products (Hue et al., 2011). *C. jejuni* and *C. coli* were equally prevalent in chicken caeca, but *C. jejuni* was the most frequently isolated one from processed carcasses. These findings highlight the difficulty of preventing the presence of *C. jejuni* in poultry end products from *Campylobacter*-positive carcasses.

Bioactive packaging is an effective method for the control of common foodborne pathogens in foods, including raw meat and fresh produce. It can be used for quality preservation purposes to limit the growth of spoilage microflora, or reduce the prevalence of foodborne pathogens in high-risk foods (Panea et al., 2014; Hakeem et al., 2020). For example, different coliphages and *Listeria* phages were immobilized on active packaging materials to control *E. coli* O104:H4 in alfalfa sprouts during germination and *L. monocytogenes* in cantaloupes during cold storage (Lone et al., 2016). One major issue is that most phages are not stable under dehydration condition, which limit their applications in bioactive packaging (Anany et al., 2011). Thus, many studies have used phages in the absorbing pads that are usually placed under fresh foods to absorb moisture and fluids. This can maintain the quality and freshness of fresh foods and protect phages from desiccation at the same time (Hakeem et al., 2020).

CONCLUDING REMARKS

Campylobacter is one of the leading foodborne pathogens responsible for human gastroenteritis. No effective control method is available to prevent *Campylobacter* contamination either in poultry farms or poultry-processing plants. Both poultry farms and processing systems are complex and require intensive operations. In addition, the use of antibiotics as growth promoters was banned to limit antibiotic resistance in different countries. The efficacy of many approved antimicrobials on the reduction of *Campylobacter* in poultry-processing plants is limited. For all of these aforementioned factors, new generations of antimicrobials including novel synergistic antimicrobial combination are required for *Campylobacter* control and prevention in the agro-ecosystem. Considering the challenges in controlling *C. jejuni* in poultry farms and processing plants, innovative antimicrobial packaging to reduce *C. jejuni* in raw chicken meat at the retail level is needed. Many new effective approaches are available for the control *C. jejuni* to enhance the safety of the end products. These include bioactive packaging of bacteriophages or nanoparticles as well as the use of synergistic combinations of antimicrobials to maximize the advantages and minimize disadvantages associated with their uses.

AUTHOR CONTRIBUTIONS

MH wrote the manuscript. XL supervised, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Achen, M., Morishita, T. Y., and Ley, E. C. (1998). Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Diseases* 42, 732–737. doi: 10.2307/1592708

Acheson, D., and Allos, B. M. (2001). *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Diseases* 32, 1201–1206. doi: 10.1086/319760

Ahmadifar, E., Falahatkar, B., and Akrami, R. (2011). Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, Oncorhynchus mykiss. *J. Appl. Ichthyol.* 27, 1057–1060. doi: 10.1111/j.1439-0426.2011.01763.x

Akbar, A., and Anal, A. K. (2014). Zinc oxide nanoparticles loaded active packaging, a challenge study against *Salmonella* typhimurium and *Staphylococcus aureus in* ready-to-eat poultry meat. *Food Control* 38, 88–95. doi: 10.1016/j.foodcont.2013.09.065

Al-Nehlawi, A., Saldo, J., Vega, L. F., and Guri, S. (2013). Effect of high carbon dioxide atmosphere packaging and soluble gas stabilization pre-treatment on the shelf-life and quality of chicken drumsticks. *Meat Sci.* 94, 1–8. doi: 10.1016/j.meatsci.2012.12.008

An, J., Zuo, G. Y., Hao, X. Y., Wang, G. C., and Li, Z. S. (2011). Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine* 18, 990–993. doi: 10.1016/j.phymed.2011.02.013

Anany, H., Chen, W., Pelton, R., and Griffiths, M. W. (2011). Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157: H7 in meat by using phages immobilized on modified cellulose membranes. *Appl. Environ. Microbiol.* 77, 6379–6387. doi: 10.1128/AEM.05493-11

Annamalai, T., Pina-Mimbela, R., Kumar, A., Binjawadagi, B., Liu, Z., Renukaradhya, G. J., et al. (2013). Evaluation of nanoparticle-encapsulated outer membrane proteins for the control of *Campylobacter jejuni* colonization in chickens. *Poultry Sci.* 92, 2201–2211. doi: 10.3382/ps.2012-03004

Awad, W. A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M., et al. (2016). Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with *Campylobacter jejuni* infection. *Front. Cell. Infect. Microbiol.* 6:3389/fcimb.2016.00154:154. doi: 10.3389/fcimb.2016.00154

Axelsson-Olsson, D., Waldenström, J., Broman, T., Olsen, B., and Holmberg, M. (2005). Protozoan Acanthamoeba polyphaga as a potential reservoir for *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 71, 987–992. doi: 10.1128/AEM.71.2.987-992.2005

Azam, A., Ahmed, A. S., Oves, M., Khan, M. S., Habib, S. S., and Memic, A. (2012). Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: a comparative study. *Int. J. Nanomed.* 7, 6003. doi: 10.2147/IJN.S35347

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

Bailey, J. S., Thomson, J. E., and Cox, N. A. (1987). Contamination of poultry during processing. *Acad. Press Orlando FL.*

Bashor, M. P., Curtis, P. A., Keener, K. M., Sheldon, B. W., Kathariou, S., and Osborne, J. A. (2004). Effects of carcass washers on *Campylobacter* contamination in large broiler processing plants. *Poultry Sci.* 83, 1232–1239. doi: 10.1093/ps/83.7.1232

Batz, M. B., Hoffmann, S., and Morris Jr., J. G. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *J. Food Protect.* 75, 1278–1291. doi: 10.4315/0362-028X.JFP-11-418

BC Center for Disease Control (2017). *British Columbia annual summary of reportable diseases*. British Columbia Center for Disease Control. Available at: http://www.bccdc.ca/resource-gallery/Documents/Statistics and Research/Statistics and Reports/Epid/Annual Reports/2017CDAnnualReportFinal.pdf.

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

Berrang, M. E., and Dickens, J. A. (2000). Presence and level of *Campylobacter* spp. on broiler carcasses throughout the processing plant. *J. Appl. Poultry Res.* 9, 43–47. doi: 10.1093/japr/9.1.43

Berrang, M. E., Dickens, J. A., and Musgrove, M. T. (2000). Effects of hot water application after defeathering on the levels of *Campylobacter*, coliform bacteria, and *Escherichia col*i on broiler carcasses. *Poultry Sci.* 79, 1689–1693. doi: 10.1093/ps/79.11.1689

Berrang, M. E., Ladely, S. R., and Buhr, R. J. (2001). Presence and level of *Campylobacter*, coliforms, *Escherichia coli*, and total aerobic bacteria recovered from broiler parts with and without skin. *J. Food Protect.* 64, 184–188. doi: 10.4315/0362-028x-64.2.184

Bessède, E., Lehours, P., Labadi, L., Bakiri, S., and Mégraud, F. (2014). Comparison of characteristics of patients infected by *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter fetus*. *J. Clin. Microbiol.* 52, 328–330. doi: 10.1128/JCM.03029-13

Bilgili, S. F., Waldroup, A. L., Zelenka, D., and Marion, J. E. (2002). Visible ingesta on prechill carcasses does not affect the microbiological quality of broiler carcasses after immersion chilling. *J. Appl. Poultry Res.* 11, 233–238. doi: 10.1093/japr/11.3.233

Black, R. E., Levine, M. M., Clements, M., Hughes, T. P., and Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Diseases* 157, 472–479. doi: 10.1093/infdis/157.3.472

Blankenship, L. C., and Craven, S. E. (1982). *Campylobacter jejuni* survival in chicken meat as a function of temperature. *Appl. Environ. Microbiol.* 44, 88–92.

Brás, A. M., Chatterjee, S., Wren, B. W., Newell, D. G., and Ketley, J. M. (1999). A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J. Bacteriol.* 181, 3298–3302. doi: 10.1128/JB.181.10.3298-3302.1999

Brenes, A., and Roura, E. (2010). Essential oils in poultry nutrition: Main effects and modes of action. *Anim. Anim. Feed Sci. Technol.* 158, 1–14. doi: 10.1016/j.anifeedsci.2010.03.007

Byrd, J. A., Corrier, D. E., Hume, M. E., Bailey, R. H., Stanker, L. H., and Hargis, B. M. (1998). Incidence of *Campylobacter* in crops of preharvest market-age broiler chickens. *Poultry Sci.* 77, 1303–1305. doi: 10.1093/ps/77.9.1303

Byrd, J., Bailey, R. H., Wills, R., and Nisbet, D. (2007). Recovery of *Campylobacter* from commercial broiler hatchery trayliners. *Poultry Sci.* 86, 26–29. doi: 10.1093/ps/86.1.26

Callicott, K. A., Friðriksdóttir, V., Reiersen, J., Lowman, R., Bisaillon, J.-R., Gunnarsson, E., et al. (2006). Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. *Appl. Environ. Microbiol.* 72, 5794–5798. doi: 10.1128/AEM.02991-05

Carrillo, C. L., Atterbury, R. J., El-Shibiny, A., Connerton, P. L., Dillon, E., Scott, A., et al. (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl. Environ. Microbiol.* 11, 6554–6563. doi: 10.1128/AEM.71.11.6554-6563.2005

Carvalho, C. M., Gannon, B. W., Halfhide, D. E., Santos, S. B., Hayes, C. M., Roe, J. M., et al (2010). The *in-vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiol.* 11, 232. doi: 10.1186/1471-2180-10-232

Casanova, C., Schweiger, A., von Steiger, N., Droz, S., and Marschall, J. (2015). *Campylobacter concisus* pseudo-outbreak caused by improved culture conditions. *J. Clin. Microbiol.* 53, 660–662. doi: 10.1128/JCM.02608-14

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

Chan, Y. C., and Wiedmann, M. (2008). Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. *Crit. Rev. Food Sci. Nutr.* 49, 237–253. doi: 10.1080/10408390701856272

Chandrashekhar, K., Kassem, I.II, and Rajashekara, G. (2017). *Campylobacter jejuni* transducer like proteins: chemotaxis and beyond. *Gut Microbes* 8, 323–334. doi: 10.1080/19490976.2017.1279380

Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., et al. (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* 21, e2900.

Chou, T.-C., and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22, 27–55. doi: 10.1016/0065-2571(84)90007-4

Chou, T.-C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58, 621–681. doi: 10.1124/pr.58.3.10

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

Clark, A. G., and Bueschkens, D. H. (1988). Horizontal spread of human and poultry-derived strains of *Campylobacter jejuni* among broiler chicks held in incubators and shipping boxes. *J. Food Protect.* 51, 438–441. doi: 10.4315/0362-028X-51.6.438

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

Costa, D., and Iraola, G. (2019). Pathogenomics of emerging *Campylobacter* species. *Clin. Microbiol. Rev.* 32, e00072–e00018. doi: 10.1128/CMR.00072-18

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

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

Crim, S. M., Iwamoto, M., Huang, J. Y., Griffin, P. M., Gilliss, D., Cronquist, A. B., et al. (2014). Incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 US site–2013. *Morbid. Mortal. Weekly Rep.* 63, 328. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5779392/pdf/328-332.pdf.

Crim, S. M., Griffin, P. M., Tauxe, R., Marder, E. P., Gilliss, D., Cronquist, A. B., et al. (2015). Preliminary incidence and trends of infection with pathogens

transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 US site–2014. *Morbid. Mortal. Weekly Rep.* 64, 495. https://pubmed.ncbi.nlm.nih.gov/25974634/.

Davidson, P. M., Sofos, J. N., and Branen, A. L. (2005). *Antimicrobials in food* (Boca Raton, Florida, USA: CRC press).

De, R., Kundu, P., Swarnakar, S., Ramamurthy, T., Chowdhury, A., Nair, G. B., et al. (2009). Antimicrobial activity of curcumin against *Helicobacter pylori* isolates from India and during infections in mice. *Antimicrob. Agents Chemother.* 53, 1592–1597. doi: 10.1128/AAC.01242-08

de Kraker, M. E. A., Stewardson, A. J., and Harbarth, S. (2016). Will 10 million people die a year due to antimicrobial resistance by 2050? *PloS Med.* 13, e1002184. doi: 10.1371/journal.pmed.1002184

Demirok, E., Veluz, G., Stuyvenberg, W. V., Castañeda, M. P., Byrd, A., and Alvarado, C. Z. (2013). Quality and safety of broiler meat in various chilling systems. *Poultry Sci.* 92, 1117–1126. doi: 10.3382/ps.2012-02493

Di Mario, F., Cavallaro, L. G., Nouvenne, A., Stefani, N., Cavestro, G. M., Iori, V., et al. (2007). A curcumin-based 1-week triple therapy for eradication of *Helicobacter pylori* infection: something to learn from failure? *Helicobacter* 12, 238–243. doi: 10.1128/AAC.01242-08

Doulgeraki, A.II, Ercolini, D., Villani, F., and Nychas, G.-J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. *Int. J. Food Microbiol.* 157, 130–141. doi: 10.1016/j.ijfoodmicro.2012.05.020

Doyle, M. P., and Roman, D. J. (1982a). Prevalence and survival of *Campylobacter jejuni in* unpasteurized milk. *Appl. Environ. Microbiol.* 44, 1154–1158.

Doyle, M. P., and Roman, D. J. (1982b). Sensitivity of *Campylobacter jejuni* to drying. *J. Food Protect.* 45, 507–510. doi: 10.4315/0362-028X-45.6.507

Doyle, M. P. (1984). Association of *Campylobacter jejuni* with laying hens and eggs. *Appl. Environ. Microbiol.* 47, 533–536.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P., and Connerton, I. (2009). Application of a group II *Campylobacter bacteriophage* to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *J. Food Prot.* 4, 733–740. doi: 10.4315/0362-028X-72.4.733

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

Fernández, A., Soriano, E., López-Carballo, G., Picouet, P., Lloret, E., Gavara, R., et al. (2009). Preservation of aseptic conditions in absorbent pads by using silver nanotechnology. *Food Res. Int.* 42, 1105–1112. doi: 10.1016/j.foodres.2009.05.009

Finch, M. J., and Blake, P. A. (1985). Foodborne outbreaks of campylobacteriosis: the United States experienc–1982. *Am. J. Epidemiologyl.* 122, 262–268. doi: 10.1093/oxfordjournals.aje.a114097

Fischer, S., Kittler, S., Klein, G., and Glünder, G. (2013). Impact of a single phage and a phage cocktail application in broilers on reduction of *Campylobacter jejuni* and development of resistance. *PloS One* 8, e78543. doi: 10.1371/journal.pone.0078543

Fontanot, M., Iacumin, L., Cecchini, F., Comi, G., and Manzano, M. (2014). Rapid detection and differentiation of important *Campylobacter* spp. in poultry samples by dot blot and PCR. *Food Microbiol.* 43, 28–34. doi: 10.1016/j.fm.2014.05.001

Gaibani, P., Ambretti, S., Viale, P., and Re, M. C. (2019). *In-vitro* synergistic activity of meropenem/vaborbactam in combination with ceftazidime/avibactam against KPC-producing Klebsiella pneumoniae. *J. Antimicrob. Chemother.* 74, 1457–1459. doi: 10.1093/jac/dky557

Gast, R. K., and Beard, C. W. (1990). Production of *Salmonella enteritidis*-contaminated eggs by experimentally infected hens. *Avian Diseases* 34, 438–446. doi: 10.2307/1591433

Gaynor, E. C., Wells, D. H., MacKichan, J. K., and Falkow, S. (2005). The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol. Microbiol.* 56, 8–27. doi: 10.1111/j.1365-2958.2005.04525.x

Gormley, F. J., Bailey, R. A., Watson, K. A., McAdam, J., Avendaño, S., Stanley, W. A., et al. (2014). *Campylobacter* colonization and proliferation in the broiler chicken upon natural field challenge is not affected by the bird growth rate or breed. *Appl. Environmental Microbiol.* 80, 6733–6738. doi: 10.1128/AEM.02162-14

Grant, C. C., Konkel, M. E., Cieplak, W., and Tompkins, L. S. (1993). Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni in* nonpolarized and polarized epithelial cell cultures. *Infect. Immunity* 61, 1764–1771.

Guard-Petter, J. (2001). The chicken, the egg and *Salmonella enteritidis*. *Environ. Microbiol.* 3, 421–430. doi: 10.1046/j.1462-2920.2001.00213.x

Hakeem, M. J., Feng, J., Nilghaz, A., Ma, L., Seah, H. C., Konkel, M. E., et al. (2020). Active Packaging of Immobilized Zinc Oxide Nanoparticles Controls *Campylobacter jejuni* in Raw Chicken Meat. *Appl. Environ. Microbiol.* 86, 22. doi: 10.1128/AEM.01195-20

Hald, B., Wedderkopp, A., and Madsen, M. (2000). Thermophilic *Campylobacter* spp. in Danish broiler production: a cross-sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks. *Avian Pathol.* 29, 29. doi: 10.1128/AEM.69.8.4343-4351.2003

Han, C., Wang, L., Yu, K., Chen, L., Hu, L., Chen, K., et al. (2006). Biochemical characterization and inhibitor discovery of shikimate dehydrogenase from *Helicobacter pylori*. *Fed. Eur. Biochem. Societies J.* 273, 4682–4692. doi: 10.1111/j.1742-4658.2006.05469.x

Hara-Kudo, Y., and Takatori, K. (2011). Contamination level and ingestion dose of foodborne pathogens associated with infections. *Epidemiol. Infect.* 139, 1505–1510. doi: 10.1046/j.1462-2920.2001.00213.x

Hazeleger, W. C., Janse, J. D., Koenraad, P. M., Beumer, R. R., Rombouts, F. M., and Abee, T. (1995). Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 61, 2713–2719.

Hazeleger, W. C., Wouters, J. A., Rombouts, F. M., and Abee, T. (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Appl. Environ. Microbiol.* 64, 3917–3922.

Hazeleger, W. C., Bolder, N. M., Beumer, R. R., and Jacobs-Reitsma, W. F. (2008). Darkling beetles (Alphitobius diaperinus) and their larvae as potential vectors for the transfer of *Campylobacter jejuni* and *Salmonella enterica* serovar paratyphi B variant Java between successive broiler flocks. *Appl. Environ. Microbiol.* 74, 6887–6891. doi: 10.1128/AEM.00451-08

Hemaiswarya, S., and Doble, M. (2010). Synergistic interaction of phenylpropanoids with antibiotics against bacteria. *J. Med. Microbiol.* 59, 1469–1476. doi: 10.1099/jmm.0.022426-0

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

Hendrixson, D. R. (2006). A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism. *Mol. Microbiol.* 61, 1646–1659. doi: 10.1111/j.1365-2958.2006.05336.x

Heuer, O. E., Pedersen, K., Andersen, J. S., and Madsen, M. (2001). Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. *Lett. Appl. Microbiol.* 33, 269–274. doi: 10.1046/j.1472-765X.2001.00994.x

Hiett, K. L., Stern, N. J., Fedorka-Cray, P., Cox, N. A., Musgrove, M. T., and Ladely, S. (2002). Molecular subtype analyses of *Campylobacter* spp. from Arkansas and California poultry operations. *Appl. Environ. Microbiol.* 68, 6220–6236. doi: 10.1128/AEM.68.12.6220-6236.2002

Hudson, J. A., Nicol, C., Wright, J., Whyte, R., and Hasell, S. K. (1999). Seasonal variation of *Campylobacter* types from human cases, veterinary cases, raw chicken, milk and water. *J. Appl. Microbiol.* 87, 115–124. doi: 10.1046/j.1365-2672.1999.00806.x

Hue, O., Allain, V., Laisney, M.-J., Le Bouquin, S., Lalande, F., Petetin, I., et al. (2011). *Campylobacter* contamination of broiler caeca and carcasses at the slaughterhouse and correlation with *Salmonella* contamination. *Food Microbiol.* 28, 862–868. doi: 10.1016/j.fm.2010.11.003

Hughes, R.-A., Hallett, K., Cogan, T., Enser, M., and Humphrey, T. (2009). The response of *Campylobacter jejuni* to low temperature differs from that of *Escherichia coli*. *Appl. Environ. Microbiol.* 75, 6292–6298. doi: 10.1128/AEM.00993-09

Humphery, T. J., Henley, A., and Lanning, D. G. (1993). The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. *Epidemiol. Infect.* 110, 601–607. doi: 10.1017/s0950268800051025

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., et al. (2014). *Campylobacter jejuni* is not merely a commensal in commercial

broiler chickens and affects bird welfare. *MBio* 5, e01364–e01314. doi: 10.1128/mBio.01364-14

Hwang, S., Kim, M., Ryu, S., and Jeon, B. (2011). Regulation of oxidative stress response by CosR, an essential response regulator in *Campylobacter jejuni*. *PloS One* 6, e22300. doi: 10.1371/journal.pone.0022300

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* 3:3389/fmicb.2012.00012:12. doi: 10.3389/fmicb.2012.00012

Ijaz, U. Z., Sivaloganathan, L., McKenna, A., Richmond, A., Kelly, C., Linton, M., et al. (2018). Comprehensive longitudinal microbiome analysis of the chicken cecum reveals a shift from competitive to environmental drivers and a window of opportunity for *Campylobacter*. *Front. Microbiol.* 9:3389/fmicb.2018.02452:2452. doi: 10.3389/fmicb.2018.02452

Jackson, W. C. (1999). *Survey shows that poultry processors can save money by conserving water* (North Carolina: Cooperative Extension Service). Available at: https://fbns.ncsu.edu//extension_program/documents/poultry_conserve_water.pdf.

Jacobs-Reitsma, W. F., Van de Giessen, A. W., Bolder, N. M., and Mulder, R. (1995). Epidemiology of *Campylobacter* spp. at two Dutch broiler farms. *Epidemiol. Infect.* 114, 413–421. doi: 10.1017/S0950268800052122

Jacobs-Reitsma, W. F. (1995). *Campylobacter* bacteria in breeder flocks. *Avian Diseases* 39 (2), 355–359.

Jagannathan, A., Constantinidou, C., and Penn, C. W. (2001). Roles of rpoN, fliA, and flgR in Expression of Flagella in *Campylobacter jejuni*. *J. Bacteriol.* 183, 2937–2942. doi: 10.1128/JB.183.9.2937-2942.2001

Jamroz, D., Wertelecki, T., Houszka, M., and Kamel, C. (2006). Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. *J. Anim. Physiol. Anim. Nutr.* 90, 255–268. doi: 10.1111/j.1439-0396.2005.00603.x

Jia, J., Zhu, F., Ma, X., Cao, Z. W., Li, Y. X., and Chen, Y. Z. (2009). Mechanisms of drug combinations: interaction and network perspectives. *Nat. Rev. Drug Discov.* 8, 111. doi: 10.1038/nrd2683

Jorgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D. R., Bolton, F. J., et al. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *Int. J. Food Microbiol.* 76, 151–164. doi: 10.1016/S0168-1605(02)00027-2

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

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

Kärenlampi, R., and Hänninen, M.-L. (2004). Survival of *Campylobacter jejuni on* various fresh produce. *Int. J. Food Microbiol.* 97, 187–195. doi: 10.1016/j.ijfoodmicro.2004.04.019

Karlyshev, A. V., and Wren, B. W. (2001). Detection and Initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using alcian blue dye. *J. Clin. Microbiol.* 39, 279–284. doi: 10.1128/JCM.39.1.279-284.2001

Kaur, S., Modi, N. H., Panda, D., and Roy, N. (2010). Probing the binding site of curcumin in *Escherichia coli* and *Bacillus subtilis* FtsZ–a structural insight to unveil antibacterial activity of curcumin. *Eur. J. Medicinal Chem.* 45, 4209–4214. doi: 10.1016/j.ejmech.2010.06.015

Keener, K. M., Bashor, M. P., Curtis, P. A., Sheldon, B. W., and Kathariou, S. (2004). Comprehensive review of *Campylobacter* and poultry processing. *Compr. Rev. Food Sci. Food Safety* 3, 105–116. doi: 10.1111/j.1541-4337.2004.tb00060.x

Kelly, A. F., Park, S. F., Bovill, R., and Mackey, B. M. (2001). Survival of *Campylobacter jejuni* during stationary phase: evidence for the absence of a phenotypic stationary-phase response. *Appl. Environ. Microbiol.* 67, 2248–2254. doi: 10.1128/AEM.67.5.2248–2254.2001

Kiess, A. S., Kenney, P. B., and Nayak, R. R. (2007). *Campylobacter* detection in commercial turkeys. *Poultry Sci.* 48, 567–572. doi: 10.1080/00071660701573094

Kim, S. A., Park, S. H., Lee, S.II, Owens, C. M., and Ricke, S. C. (2017). Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Sci. Rep.* 7:43354. doi: 10.1038/srep43354

Kittler, S., Fischer, S., Abdulmawjood, A., Glünder, G., and Klein, G. (2013). Effect of bacteriophage application on *Campylobacter jejuni* loads in commercial broiler flocks. *Appl. Environ. Microbiol.* 79, 7525–7533. doi: 10.1128/AEM.02703-13

Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson Donald E, J., and Cieplak Witold, J. (1997). Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejun*i. *Mol. Microbiol.* 24, 953–963. doi: 10.3390/microorganisms8030389

Konkel, M. E., Kim, B. J., Klena, J. D., Young, C. R., and Ziprin, R. (1998). Characterization of the thermal stress response of *Campylobacter jejuni*. *Infect. Immunity* 66, 3666–3672. doi: 10.1128/IAI.66.8.3666-3672.1998

Konkel, M. E., Gray, S. A., Kim, B. J., Garvis, S. G., and Yoon, J. (1999a). Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. *J. Clin. Microbiol.* 37, 510–517. doi: 10.1128/JCM.37.3.510-517.1999

Konkel, M. E., Kim, B. J., Rivera-Amill, V., and Garvis, S. G. (1999b). Bacterial secreted proteins are required for the internalization of *Campylobacter jejun*i into cultured mammalian cells. *Mol. Microbiol.* 32, 691–701. doi: 10.1046/j.1365-2958.1999.01376.x

Kotula, K. L., and Pandya, Y. (1995). Bacterial profile of broiler chickens upon entering the processing plant. *J. Food Protect.* 58, 1326–1329. doi: 10.4315/0362-028X-58.12.1326

Kubota, K., Kasuga, F., Iwasaki, E., Inagaki, S., Sakurai, Y., Komatsu, M., et al. (2011). Estimating the burden of acute gastroenteritis and foodborne illness caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* by using population-based telephone survey data, Miyagi Prefecture, Japato 2006. *J. Food Protect.* 74, 1592–1598. doi: 10.4315/0362-028X.JFP-10-387

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317. doi: 10.1038/nrmicro2315

Lanier, W. A., Hale, K. R., Geissler, A. L., and Dewey-Mattia, D. (2018). Chicken liver–associated outbreaks of campylobacteriosis and salmonellosis, United State–2016: identifying opportunities for prevention. *Foodborne Pathog. Disease* 15, 726–733. doi: 10.1089/fpd.2018.2489

Lao, C. D., Ruffin, M. T., Normolle, D., Heath, D. D., Murray, S.II, Bailey, J. M., et al. (2006). Dose escalation of a curcuminoid formulation. *BMC Complement. Altern. Medicine* 6:10. doi: 10.1186/1472-6882-6-10

Lara-Tejero, M., and Galán, J. E. (2001). CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect. Immunity* 69, 4358–4365. doi: 10.1128/IAI.69.7.4358-4365.2001

Li, M., Havelaar, A. H., Hoffmann, S., Hald, T., Kirk, M. D., Torgerson, P. R., et al. (2019). Global disease burden of pathogens in animal source food. *PloS One* 14, e0216545. doi: 10.1371/journal.pone.0216545

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., et al. (2016). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *Int. J. Food Microbiol.* 217, 49–58. doi: 10.1016/j.ijfoodmicro.2015.10.011

Mace, S., Haddad, N., Zagorec, M., and Tresse, O. (2015). Influence of measurement and control of microaerobic gaseous atmospheres in methods for *Campylobacter* growth studies. *Food Microbiol.* 52, 169–176. doi: 10.1016/j.fm.2015.07.014

Man, S. M. (2011). The clinical importance of emerging *Campylobacter* species. *Nat. Rev. Gastroenterol. Hepatol.* 8, 669. doi: 10.1038/nrgastro.2011.191

Mavri, A., and Smole Možina, S. (2013). Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol.* 160, 304–312. doi: 10.1016/j.ijfoodmicro.2012.11.006

McFadyean, S. J., and Stockman, S. S. (1913). Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire Into Epizootic Abortion. Appendix to Part III, Abortion in Sheep. *HM Stationery Office*.

McMillin, K. W. (2008). Where is MAP going? A review and future potential of modified atmosphere packaging for meat. *Meat Sci.* 80, 43–65. doi: 10.1016/j.meatsci.2008.05.028

Miyamoto, T., Baba, E., Tanaka, T., Sasai, K., Fukata, T., and Arakawa, A. (1997). *Salmonella enteritidis* contamination of eggs from hens inoculated by vaginal,

cloacal, and intravenous routes. *Avian Diseases* 42, 296–303. doi: 10.2307/1592181

Mughini-Gras, L., Smid, J. H., Wagenaar, J. A., De Boer, A., Havelaar, A. H., Friesema, I. H. M., et al. (2014). Campylobacteriosis in returning travellers and potential secondary transmission of exotic strains. *Epidemiol. Infect.* 142, 1277–1288. doi: 10.1017/S0950268813002069

Murphy, C., Carroll, C., and Jordan, K. N. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Neal-McKinney, J. M., Samuelson, D. R., Eucker, T. P., Nissen, M. S., Crespo, R., and Konkel, M. E. (2014). Reducing *Campylobacter jejuni* colonization of poultry via vaccination. *PloS One* 9, e114254. doi: 10.1371/journal.pone.0114254

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

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

Nielsen, H. L., Ejlertsen, T., Engberg, J., and Nielsen, H. (2013). High incidence of *Campylobacter* concisus in gastroenteritis in North Jutland, Denmark: a population-based study. *Clin. Microbiol. Infect.* 19, 445–450. doi: 10.1111/j.1469-0691.2012.03852.x

Northcutt, J. K., Berrang, M. E., Dickens, J. A., Fletcher, D. L., and Cox, N. A. (2003). Effect of broiler age, feed withdrawal, and transportation on levels of coliforms, *Campylobacter*, *Escherichia coli* and *Salmonella* on carcasses before and after immersion chilling. *Poultry Sci.* 82, 169–173. doi: 10.1093/ps/82.1.169

Odds, F. C. (2003). Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* 52:1. doi: 10.1093/ps/82.1.169

Oh, E., and Jeon, B. (2015). Synergistic anti-*Campylobacter jejuni* activity of fluoroquinolone and macrolide antibiotics with phenolic compounds. *Front. Microbiol.* 6:3389/fmicb.2015.01129:1129. doi: 10.3389/fmicb.2015.01129

Oh, E., McMullen, L., and Jeon, B. (2015). High prevalence of hyper-aerotolerant *Campylobacter jejuni* in retail poultry with potential implication in human infection. *Front. Microbiol.* 6:3389/fmicb.2015.01263:1263. doi: 10.3389/fmicb.2015.01263

Panea, B., Ripoll, G., González, J., Fernández-Cuello, Á., and Albertí, P. (2014). Effect of nanocomposite packaging containing different proportions of ZnO and Ag on chicken breast meat quality. *J. Food Engineering* 123, 104–112. doi: 10.1016/j.jfoodeng.2013.09.029

Park, S. (1996). *Microorganisms in foods 5: Characteristics of microbial pathogens* (London, UK: Springer Science & Business Media).

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665. doi: 10.1038/35001088

Pearson, A. D., Greenwood, M., Healing, T. D., Rollins, D., Shahamat, M., Donaldson, J., et al. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 59, 987–996.

Petersen, L., Nielsen, E. M., and On, S. L. W. (2001). Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. *Vet. Microbiol.* 82, 141–154. doi: 10.1016/s0378-1135(01)00382-0

Pothakos, V., Taminiau, B., Huys, G., Nezer, C., Daube, G., and Devlieghere, F. (2014). Psychrotrophic lactic acid bacteria associated with production batch recalls and sporadic cases of early spoilage in Belgium between 2010 and 2014. *Int. J. Food Microbiol.* 191, 157–163. doi: 10.1016/j.ijfoodmicro.2014.09.013

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

Ravel, A., Nesbitt, A., Marshall, B., Sittler, N., and Pollari, F. (2010). Description and burden of travel-related cases caused by enteropathogens reported in a Canadian community. *J. Travel Med. N.* 18, 8–19. doi: 10.1111/j.1708-8305.2010.00471.x

Ravindran, P. N., Nirmal-Babu, K., and Shylaja, M. (2003). Cinnamon and cassia: the genus *Cinnamomum*. *CRC Press*. doi: 10.1201/9780203590874

Ringoir, D. D., and Korolik, V. (2003). Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage *in-vivo*. *Vet. Microbiol.* 92, 225–235. doi: 10.1016/S0378-1135(02)00378-4

Ritchie, H., and Roser, M. (2017). Meat and Dairy Production. *Our World Data*.

Rivera-Amill, V., Kim, B. J., Seshu, J., and Konkel, M. E. (2001). Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J. Infect. Diseases* 183, 1607–1616. doi: 10.1086/320704

Rouger, A., Tresse, O., and Zagorec, M. (2017). Bacterial contaminants of poultry meat: sources, species, and dynamics. *Microorganisms* 5, 50. doi: 10.3390/microorganisms5030050

Rudrappa, T., and Bais, H. P. (2008). Curcumin, a known phenolic from Curcuma longa, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models. *J. Agric. Food Chem.* 56, 1955–1962. doi: 10.1021/jf072591j

Rusin, P. A., Rose, J. B., Haas, C. N., and Gerba, C. P. (1997). "Risk assessment of opportunistic bacterial pathogens in drinking water," in *Reviews of environmental contamination and toxicology* (New York, NY: Springer), 57–83. doi: 10.1007/978-1-4612-1964-4_2

Sahin, O., Morishita, T. Y., and Zhang, Q. (2002). *Campylobacter* colonization in poultry: sources of infection and modes of transmission. *Anim. Health Res. Rev.* 3, 95–105. doi: 10.1079/AHRR200244

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Sarkar, A., De, R., and Mukhopadhyay, A. K. (2016). Curcumin as a potential therapeutic candidate for *Helicobacter pylori* associated diseases. *World J. Gastroenterol.* 22:2736. doi: 10.3748/wjg.v22.i9.2736

Saxena, M., John, B., Mu, M., Van, T. T. H., Taki, A., Coloe, P. J., et al. (2013). Strategies to reduce *Campylobacter* colonisation in chickens. *Proc. Vaccinol.* 7, 40–43. doi: 10.1016/j.provac.2013.06.008

Sears, A., Baker, M. G., Wilson, N., Marshall, J., Muellner, P., Campbell, D. M., et al. (2011). Marked campylobacteriosis decline after interventions aimed at poultry, New Zealand. *Emerg. Infect. Diseases* 17, 1007. doi: 10.3201/eid1706.101272

Service, F. safety and inspection (2018). *Safe and suitable ingredients used in the production of meat, poultry, and egg products*. Food Safety and Inspection Service (FSIS). Available at: https://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES.

Shanker, S., Lee, A., and Sorrell, T. C. (1986). *Campylobacter jejuni* in broilers: the role of vertical transmission. *Epidemiol. Infect.* 96, 153–159. doi: 10.1017/S002217240006592X

Shanker, S., Lee, A., and Sorrell, T. C. (1990). Horizontal transmission of *Campylobacter jejuni* amongst broiler chicks: experimental studies. *Epidemiol. Infect.* 104, 101–110.

Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N. H. M., Ann, L. C., Bakhori, S. K. M., et al. (2015). Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. *Nano Microbiol. Lett.* 7, 219–242. doi: 10.1017/s0950268800054571

Skirrow, M. B. (1977). *Campylobacter enteritis*: a" new" disease. *Br. Med. J.* 2, 9–11. doi: 10.1136/bmj.2.6078.9

Skirrow, M. B. (2006). John McFadyean and the centenary of the first isolation of *Campylobacter* species. *Clin. Infect. Diseases* 43, 1213–1217. doi: 10.1086/508201

Slader, J., Domingue, G., Jørgensen, F., McAlpine, K., Owen, R. J., Bolton, F. J., et al. (2002). Impact of transport crate reuse and of catching and processing on *Campylobacter* and *Salmonella* contamination of broiler chickens. *Appl. Environmntal Microbiol.* 68, 713–719. doi: 10.1128/AEM.68.2.713-719.2002

Smith, K., Reimers, N., Barnes, H. J., Lee, B. C., Siletzky, R., and Kathariou, S. (2004). *Campylobacter* colonization of sibling turkey flocks reared under different management conditions. *J. Food Protect.* 67, 1463–1468. doi: 10.4315/0362-028X-67.7.1463

Snelling, W. J., McKenna, J. P., Hack, C. J., Moore, J. E., and Dooley, J. S. G. (2006). An examination of the diversity of a novel *Campylobacter* reservoir. *Arch. Microbiol.* 186, 31–40. doi: 10.1007/s00203-006-0119-3

Stern, N. J., and Kotula, A. W. (1982). Survival of *Campylobacter jejuni* inoculated into ground beef. *Appl. Environmtal Microbiol.* 44, 1150–1153.

Stern, N. J., and Robach, M. C. (2003). Enumeration of *Campylobacter* spp. in broiler feces and in corresponding processed carcasses. *J. Food Protect.* 66, 1557–1563. doi: 10.4315/0362-028X-66.9.1557

Stern, N. J., Fedorka-Cray, P., Bailey, J. S., Cox, N. A., Craven, S. E., Hiett, K. L., et al. (2001). Distribution of *Campylobacter* spp. in selected US poultry production and processing operations. *J. Food Protect.* 64, 1705–1710. doi: 10.4315/0362-028X-64.11.1705

Svetoch, E. A., and Stern, N. J. (2010). Bacteriocins to control *Campylobacter* spp. in poultry—a review. *Poultry Sci.* 89, 1763–1768. doi: 10.3382/ps.2010-00659

Tajkarimi, M. M., Ibrahim, S. A., and Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control* 21, 1199–1218. doi: 10.1016/j.foodcont.2010.02.003

Thies, F. L., Weishaupt, A., Karch, H., Hartung, H.-P., and Giegerich, G. (1999). Cloning, sequencing and molecular analysis of the *Campylobacter jejuni* groESL bicistronic operon. *Microbiology* 145, 89–98. doi: 10.1099/13500872-145-1-89

Thomas, C. J., and McMeekin, T. A. (1980). Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. *Appl. Environ. Microbiol.* 40, 133–144.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., et al. (2013). Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathog. Disease* 10, 639–648. doi: 10.1089/fpd.2012.1389

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

Turonova, H., Haddad, N., Hernould, M., Chevret, D., Pazlarova, J., and Tresse, O. (2017). Profiling of *Campylobacter jejuni* proteome in exponential and stationary phase of growth. *Front. Microbiol.* 8:3389/fmicb.2017.00913:913. doi: 10.3389/fmicb.2017.00913

Tyagi, P., Singh, M., Kumari, H., Kumari, A., and Mukhopadhyay, K. (2015). Bactericidal activity of curcumin I is associated with damaging of bacterial membrane. *PloS One* 10, e0121313. doi: 10.1371/journal.pone.0121313

Ushanov, L., Lasareishvili, B., Janashia, I., and Zautner, A. E. (2020). Application of Campylobacter jejuni phages: challenges and perspectives. *Animals* 2, 279. doi: 10.3390/ani10020279

Uyttendaele, M., Schukkink, R., Van Gemen, B., and Debevere, J. (1996). Comparison of the nucleic acid amplification system NASBA® and agar isolation for detection of pathogenic *Campylobacters* in naturally contaminated poultry. *J. Food Protect.* 59, 683–687. doi: 10.4315/0362-028X-59.7.683

Van de Braak, S., and Leijten, G. (1999). Essential oils and oleoresins: a survey in the Netherlands and other major markets in the European Union. *Centre Promotion Imports Develop. Countries.* 116.

Van de Giessen, A., Mazurier, S.II, Jacobs-Reitsma, W., Jansen, W., Berkers, P., Ritmeester, W., et al. (1992). Study on the epidemiology and control of *Campylobacter jejuni* in poultry broiler flocks. *Appl. Environ. Microbiol.* 58, 1913–1917.

Van der Stel, A.-X. M., van Mourik, A., Lanniewski, P., van der Putten, J. P. M., Jagusztyn-Krynicka, E. K., and Wosten, M. M. S. M. (2015). The *Campylobacter jejuni* RacRS two-component system activates the glutamate synthesis by directly upregulating γ-glutamyltranspeptidase (GGT). *Front. Microbiol.* 6:3389/fmicb.2015.00567:567. doi: 10.3389/fmicb.2015.00567

Vegge, C. S., Jansen van Rensburg, M. J., Rasmussen, J. J., Maiden, M. C. J., Johnsen, L. G., Danielsen, M., et al. (20161877). Glucose metabolism via the Entner-Doudoroff pathway in Campylobacter: a rare trait that enhances survival and promotes biofilm formation in some isolates. *Front. Microbiol.* 7, 1877. doi: 10.3389/fmicb.2016.01877

Velayudhan, J., Jones, M. A., Barrow, P. A., and Kelly, D. J. (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect. Immunity* 72, 260–268. doi: 10.1128/IAI.72.1.260-268.2004

Véron, M., and Chatelain, R. (1973). Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. *Int. J. System. Evol. Microbiol.* 23, 122–134. doi: 10.1099/00207713-23-2-122

Vetvicka, V., Vetvickova, J., and Fernandez-Botran, R. (2016). Effects of curcumin on *Helicobacter pylori* infection. *Ann. Trans. Medicine's* 4, 479–485. doi: 10.21037/atm.2016.12.52

Vidal, A. B., Colles, F. M., Rodgers, J. D., McCarthy, N. D., Davies, R. H., Maiden, M. C. J., et al. (2016). Genetic diversity of *Campylobacter jejuni* and *Campylobacter coli* isolates from conventional broiler flocks and the impacts of sampling strategy and laboratory method. *Appl. Environ. Microbiol.* 82, 2347–2355. doi: 10.1128/AEM.03693-15

Wagenaar, J. A., Van Bergen, M. A., Mueller, M. A., Wassenaar, T. M., and Carlton, R. M. (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Vet. Microbiol.* 3-4, 275–283. doi: 10.1016/j.vetmic.2005.06.002

Walker, L. J., Wallace, R. L., Smith, J. J., Graham, T., Saputra, T., Symes, S., et al. (2019). Prevalence of *Campylobacter coli* and *Campylobacter jejuni* in Retail Chicken, Beef, Lamb, and Pork Products in Three Australian States. *J. Food Protect.* 82, 2126–2134. doi: 10.4315/0362-028X.JFP-19-146

Wallace, J. S., Stanley, K. N., Currie, J. E., Diggle, P. J., and Jones, K. (1997). Seasonality of thermophilic *Campylobacter* populations in chickens. *J. Appl. Microbiol.* 82, 219–224. doi: 10.1111/j.1365-2672.1997.tb03576.x

Wedderkopp, A., Gradel, K. O., Jørgensen, J. C., and Madsen, M. (2001). Pre-harvest surveillance of *Campylobacter* and *Salmonella* in Danish broiler flocks: a 2-year study. *Int. J. Food Microbiol.* 68, 53–59. doi: 10.1016/S0168-1605(01)00463-9

Whyte, P., Collins, J. D., McGill, K., Monahan, C., and O'mahony, H. (2001). The effect of transportation stress on excretion rates of campylobacters in market-age broilers. *Poultry Sci.* 80, 817–820.

Wilkinson, J. M. (2011). Re-defining efficiency of feed use by livestock. *Animal* 5, 1014–1022. doi: 10.1093/ps/80.6.817

Willis, W. L., and Murray, C. (1997). *Campylobacter jejuni* seasonal recovery observations of retail market broilers. *Poultry Sci.* 76, 314–317. doi: 10.1093/ps/76.2.314

Xie, Y., He, Y., Irwin, P. L., Jin, T., and Shi, X. (2011). Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 77, 2325–2331. doi: 10.1128/AEM.02149-10

Yang, H., Li, Y., and Johnson, M. G. (2001). Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and on chicken skin during poultry scalding and chilling. *J. Food Protect.* 64, 770–776. doi: 10.4315/0362-028X-64.6.770

Yao, R., Burr, D. H., and Guerry, P. (1997). CheY-mediated modulation of *Campylobacter jejun*i virulence. *Mol. Microbiol.* 23, 1021–1031. doi: 10.1046/j.1365-2958.1997.2861650.x

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

Zaidi, S. F. H., Yamada, K., Kadowaki, M., Usmanghani, K., and Sugiyama, T. (2009). Bactericidal activity of medicinal plants, employed for the treatment of gastrointestinal ailments, against *Helicobacter pylori*. *J. Ethnopharmacologyl* 121, 286–291. doi: 10.1016/j.jep.2008.11.001

Zhang, Q. Q., Han, Y. Q., Cao, J. X., Xu, X. L., Zhou, G. H., and Zhang, W. Y. (2012). The spoilage of air-packaged broiler meat during storage at normal and fluctuating storage temperatures. *Poultry Sci.* 91, 208–214. doi: 10.3382/ps.2011-01519

Zhou, X., Seto, S. W., Chang, D., Kiat, H., Razmovski-Naumovski, V., Chan, K., et al. (2016). Synergistic effects of Chinese herbal medicine: a comprehensive review of methodology and current research. *Front. Pharmacol.* 7:3389/fphar.2016.00201:201. doi: 10.3389/fphar.2016.00201

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Metagenomic Analysis of the Gut Microbiota of Wild Mice, a Newly Identified Reservoir of *Campylobacter*

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Campylobacter, the most common etiologic agent of zoonotic gastroenteritis in humans, is present in many reservoirs including livestock animals, wildlife, soil, and water. Previously, we reported a novel *Campylobacter jejuni* strain SCJK02 (MLST ST-8388) from the gut of wild mice (*Micromys minutus*) using culture-dependent methods. However, due to fastidious growth conditions and the presence of viable but non-culturable *Campylobacter* spp., it is unclear whether *M. minutus* is a *Campylobacter* reservoir. This study aimed to: 1) determine the distribution and proportion of *Campylobacter* spp. in the gut microbiota of wild mice using culture-independent methods and 2) investigate the gut microbiota of wild mice and the relationship of *Campylobacter* spp. with other gut microbes. The gut microbiota of 38 wild mice captured from perilla fields in Korea and without any clinical symptoms (18 *M. minutus* and 20 *Mus musculus*) were analyzed. Metagenomic analysis showed that 77.8% (14 of 18) of the captured *M. minutus* harbored *Campylobacter* spp. (0.24–32.92%) in the gut metagenome, whereas none of the captured *M. musculus* carried *Campylobacter* spp. in their guts. Notably, 75% (6 of 8) of *M. minutus* determined to be *Campylobacter*-negative using culture-dependent methods showed a high proportion of *Campylobacter* through metagenome analysis. The results of metagenome analysis and the absence of clinical symptoms suggest that *Campylobacter* may be a component of the normal gut flora of wild *M. minutus*. Furthermore, linear discriminant analysis (LDA) showed that *Campylobacter* was the most enriched genus in the gut microbiota of *M. minutus* (LDA score, 5.37), whereas *Lactobacillus* was the most enriched genus in *M. musculus* (LDA score, –5.96). The differences in the presence of *Campylobacter* between the two species of wild mice may be attributed to the differential abundance of *Campylobacter* and *Lactobacillus* in their respective gut microbiota. In conclusion, the results indicate that wild

M. minutus may serve as a potential *Campylobacter* reservoir. This study presents the first metagenomics analysis of the *M. minutus* gut microbiota to explore its possible role as an environmental *Campylobacter* reservoir and provides a basis for future studies using culture-independent methods to determine the role of environmental reservoirs in *Campylobacter* transmission.

Keywords: *Campylobacter*, wild mouse, *Micromys minutus*, environmental reservoir, gut microbiota, metagenomics, *Lactobacillus*, transmission cycle

INTRODUCTION

Campylobacter is one of the most common etiologic agents of zoonotic gastroenteritis in humans (Kaakoush et al., 2015). Although the most common cause of *Campylobacter* infection is the intake or handling of contaminated poultry, environmental sources such as wildlife, soil, and water are also important infection routes (Whiley et al., 2013; Hofreuter, 2014; Skarp et al., 2016). As an environmental reservoir, wildlife is an emerging source of *Campylobacter* infection *via* the direct transmission of *Campylobacter* to humans or indirectly *via* the wildlife-livestock-human cycle (Kim et al., 2020). While the majority of studies on *Campylobacter* reservoirs in wildlife have been conducted on wild birds, several studies on other hosts, such as deer, boars, and reptiles, have also been conducted (French et al., 2009; Díaz-Sánchez et al., 2013; Patrick et al., 2013; Carbonero et al., 2014). Wild mice are distributed in a wide range of habitats globally and often transmit diverse zoonotic pathogens to humans and livestock, serving as a link between wildlife and the urban community (Razzauti et al., 2015); however, *Campylobacter* in wild mice is not well understood. One study reported *Campylobacter* strains isolated from wild rodents, suggesting wild rodents as a risk factor of *Campylobacter* infection in livestock (Meerburg et al., 2006).

Most studies on *Campylobacter* in wildlife have been conducted using culture-dependent methods, such as the isolation and characterization of bacterial strain (French et al., 2009; Díaz-Sánchez et al., 2013; Patrick et al., 2013; Carbonero et al., 2014). Previously, we reported a novel *C. jejuni* strain SCJK02 (MLST ST-8388) isolated from fecal samples of wild mice (*Micromys minutus*) (Kim et al., 2020). In the previous study, *Campylobacter* was isolated from 63% of *M. minutus*, whereas none was isolated from *Mus musculus*. Considering the limitations of culture-dependent methods, such as fastidious growth conditions and the presence of viable but non-culturable *Campylobacter* spp. (Mihaljevic et al., 2007; Jackson et al., 2009), it is likely that *Campylobacter* was not detected, even if it was present. Therefore, it is essential to apply culture-independent methods together with traditional culture-dependent methods to precisely determine the presence of *Campylobacter* in a host.

The role of the gut microbiota in *Campylobacter*-mediated infection has been reported in several studies (Li et al., 2018; Sun et al., 2018). In humans, the microbiota of poultry workers infected with *Campylobacter* and those resistant to colonization of *Campylobacter* show significant differences in the abundance of certain genera (Dicksved et al., 2014). In laboratory mice, elevated levels of intestinal *Escherichia coli* reduce colonization resistance to *Campylobacter* (Haag et al., 2012), and the gut microbiota composition affects the extraintestinal dissemination of *Campylobacter* (O'Loughlin et al., 2015). In poultry, neonatal chickens transplanted with mature microbiota show a reduced transmission potential of *Campylobacter* (Gilroy et al., 2018). Thus, the infection risk of *Campylobacter* is affected by the gut microbiota of the host through diverse microbe-microbe interactions. Since the gut microbiota of *M. minutus* has not yet been investigated, studies are needed to improve the prediction and prevention of the transmission of *Campylobacter* from wildlife to humans.

This study was conducted to: 1) determine the distribution and proportion of *Campylobacter* spp. in the gut microbiota of wild mice using culture-independent methods and 2) investigate the core microbiota of wild mice and the relationship of *Campylobacter* spp. with other gut microbes. The gut microbiota of 38 wild mice without clinical symptoms (18 *M. minutus* and 20 *M. musculus*) and captured for 2 years from perilla fields in Korea at the end of winter torpor were analyzed. This study is the first to investigate the gut microbiota of *M. minutus* using metagenomics to explore its possible role as an environmental *Campylobacter* reservoir.

MATERIALS AND METHODS

Study Design and Sample Collection

The Institutional Animal Care and Use Committee of Hallym University (approval number Hallym2017-5, Hallym 2018-6) approved this study. Two species of wild mice *(M. minutus* and *M. musculus*) were captured for 2 years from the perilla fields of Chuncheon in Korea at the end of their winter torpor. Information on the wild mice used in this study is included in the supplementary material (**Supplementary Table 1**). All captured mice were transferred to the lab facility immediately. Fresh fecal samples from the mice were collected in single cages and stored at −80°C.

In our previous study, *Campylobacter* was isolated from mice fecal samples using two different culture methods (Kim et al., 2020). Briefly, homogenized fecal samples (in phosphate-buffered saline—PBS) were directly spread onto modified cefoperazone–deoxycholate agar plates (mCCDA; Oxoid Ltd., Hampshire, United Kingdom) containing the CCDA-selective supplement (Oxoid, Ltd.) and plates were incubated at 42°C for 2

days under microaerobic conditions. Next, *Campylobacter*-like colonies were inoculated into Müller–Hinton agar plates (Oxoid Ltd.) and then tested by *Campylobacter* genus-specific polymerase chain reaction (PCR) (Wang et al., 2002). All *Campylobacter*-positive colonies were identified as *C. jejuni* by species-specific PCR (Wang et al., 2002). Additionally, fecal samples that were *Campylobacter*-negative subjected to enrichment in Bolton broth (Oxoid, Ltd.) containing the Bolton broth selective supplement (Oxoid, Ltd., Hampshire, United Kingdom) for 2 days at 42°C under microaerobic conditions. Thereafter, the presence of *C. jejuni* was investigated as above. Of note, results showed that *Campylobacter* was culture-positive in 63.6% of *M. minutus*, and culture-negative in all *M. musculus*.

Here, to investigate the differences in the gut microbiota of *Campylobacter* culture-positive and culture-negative *M. minutus*, 10 fecal samples from culture-positive *M. minutus*, and 8 fecal samples from culture-negative *M. minutus* were used for microbial community analysis. Additionally, to investigate the difference between the gut microbiota of the two wild mice species, 20 fecal samples from *M. musculus* (all *Campylobacter* culture-negative) were used for microbial community analysis.

DNA Extraction and 16S rRNA Sequencing

Metagenomic DNA extraction from fecal samples was performed using the Fast DNA Soil Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The V3–V4 regions of the 16S rRNA gene were amplified using the following primers: 341F; 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 805R; 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. PicoGreen was used to pool and normalize the amplified products. All sequencing processes were performed using an Illumina MiSeq (San Diego, CA, USA) platform at Macrogen, Inc. (Seoul, Korea).

Bioinformatics and Statistical Analyses

The bioinformatics analysis of the sequence data was performed using QIIME 2 (version 2019.10) software package (Bolyen et al., 2019) and *MicrobiomeAnalystR* in R package (Dhariwal et al., 2017). An amplicon sequence variant (ASV) table was generated by filtering, dereplicating, and denoising the raw sequence data using DADA2 (Callahan et al., 2016). A phylogenetic tree of representative sequences was generated using MAFFT (Katoh and Standley, 2013). Taxonomy assignment of the ASV table was conducted at the phylum and genus levels using a naïve Bayes classifier implemented in the q2-feature-classifier (Bokulich et al., 2018) against the SILVA database, version 132 (Quast et al., 2012). ASVs that were classified into the genus *Campylobacter* were further identified at the species-level. For downstream analysis, the sequencing data were normalized *via* rarefication to the minimum library size.

The alpha diversity of the microbial community was measured using the phyloseq package with two metrics, including the number of observed ASVs, which accounts for richness, and the Simpson's and Shannon's indexes, which account for richness and evenness (McMurdie and Holmes, 2013). Differences in alpha diversity between wild mice groups were evaluated using the Mann-Whitney U test. Beta diversity was measured based on Bray-Curtis dissimilarity, and the differences in beta diversity between wild mice groups were evaluated using the analysis of group similarities (ANOSIM) test. Sample core microbiota were defined as those with a minimum abundance of 0.01% and a prevalence of 50% as the cut-off values. Differential abundance analysis of microbiota was performed using linear discriminant analysis effect size (LEFSe), implemented in *MicrobiomeAnalystR* in the R package (Segata et al., 2011). We considered a p value lower than 0.05 to indicate significance. Statistical analyses were performed using SPSS 25 (SPSS, Inc., Chicago, IL, USA) and R version 3.6.3.

RESULTS

Taxonomic Composition of the Gut Microbiota of Wild Mice

To determine the distribution and proportion of *Campylobacter* in the gut microbiota of wild mice, fecal microbiota from 18 *M. minutus* (10 culture-positive, 8 culture-negative) and 20 *M. musculus* (all culture-negative) were compared. No ASV was classified into the genus *Campylobacter* in the gut microbiota of *M. musculus*. The taxonomic composition of the gut microbiota of individual *M. minutus* at the phylum and genus levels are shown in **Figures 1A, B**. *Campylobacter* was present (0.24–32.92%) in the gut microbiota of 14 of 18 *M. minutus* (77.8%) but not in any of the *M. musculus*. The relative abundance of *Campylobacter* in the culture-positive and -negative groups of *M. minutus* showed no significant difference according to the Mann-Whitney U test ($p > 0.05$) (**Figure 1C**). Of note, all ASVs classified into the genus *Campylobacter* were identified as *C. jejuni* at the species-level.

The microbiota of all *M. minutus* samples comprised nine main bacterial phyla including Firmicutes, Bacteroidetes, Epsilonbacteraeota, Proteobacteria, Actinobacteria, Patescibacteria, Deferribacteres, Spirochaetes, and Tenericutes. Firmicutes (45.47%) was the most dominant phylum, followed by Bacteroidetes (38.61%) and Epsilonbacteraeota (7.34%). At the genus level, *Bacteroides* (23.79%) was the most dominant genus, followed by *Lactobacillus* (18.92%), uncultured *Muribaculaceae* (5.96%), *Lachnospiraceae NK4A136 group* (4.67%), uncultured *Lachnospiraceae* (4.65%), *Campylobacter* (4.03%), and *Helicobacter* (3.30%). The microbiota of *M. musculus* comprised seven main bacterial phyla, including Firmicutes, Bacteroidetes, Epsilonbacteraeota, Actinobacteria, Proteobacteria, Patescibacteria, and Deferribacteres. Firmicutes (62.02%) was the most dominant phyla, followed by Bacteroidetes (32.70%) and Epsilonbacteraeota (2.00%). At the genus level, *Lactobacillus* (36.44%) was the most dominant genus, followed by *Bacteroides* (12.99%), uncultured *Muribaculaceae* (5.39%), and *Alistipes* (4.17%) (**Figure 1D**). The taxonomic composition of the gut microbiota of individual *M. musculus* is shown in **Supplementary Figure 1**.

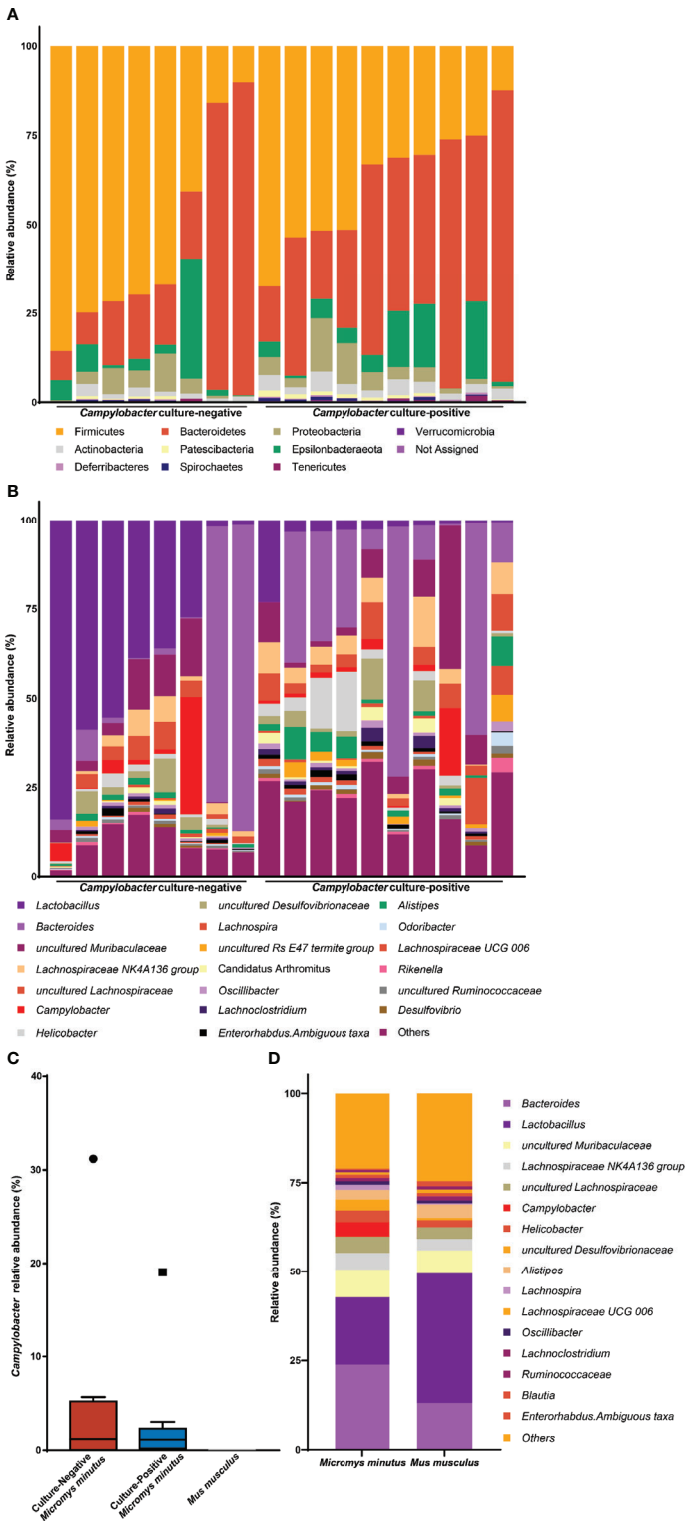

FIGURE 1 | Taxonomic composition of the gut microbiota of wild mice. Taxonomy bar plot of the gut microbiota of *Micromys minutus* at the **(A)** phylum and **(B)** genus levels. **(C)** The relative abundance of *Campylobacter* in the gut microbiota of *Micromys minutus* and *Mus musculus*. The blue and orange boxes represent the relative abundance of *Campylobacter* in the *Campylobacter* culture-positive and culture-negative *M. minutus* groups. Circle (●) and square (■) represent the maximum point of relative abundance of *Campylobacter*, respectively. **(D)** Taxonomic composition of gut microbiota of two species of wild mice (*Micromys minutus* and *Mus musculus*) at the genus level.

Members of the core microbiota of *M. minutus* at the phylum level were identified as Firmicutes, Bacteroidetes, Epsilonbacteraeota, Proteobacteria, and Actinobacteria (**Figures 2A, C**). Members of the core microbiota of *M. minutus* at the genus level were identified as *Bacteroides*, *Lactobacillus*, uncultured *Muribaculaceae*, *Lachnospiraceae NK4A136 group*, uncultured *Lachnospiraceae*, *Helicobacter*, *Campylobacter*, uncultured *Desulfovibrionaceae*, and *Alistipes* (**Figures 2B, D**).

Differences in the Gut Microbiota of *Micromys minutus* According to the Culture Results of *Campylobacter*

When the two culture groups of *M. minutus* were compared using the Mann-Whitney test, no significant differences ($p > 0.05$) were observed in the number of observed ASVs, the Simpson's index and the Shannon's index (**Figure 3A**).

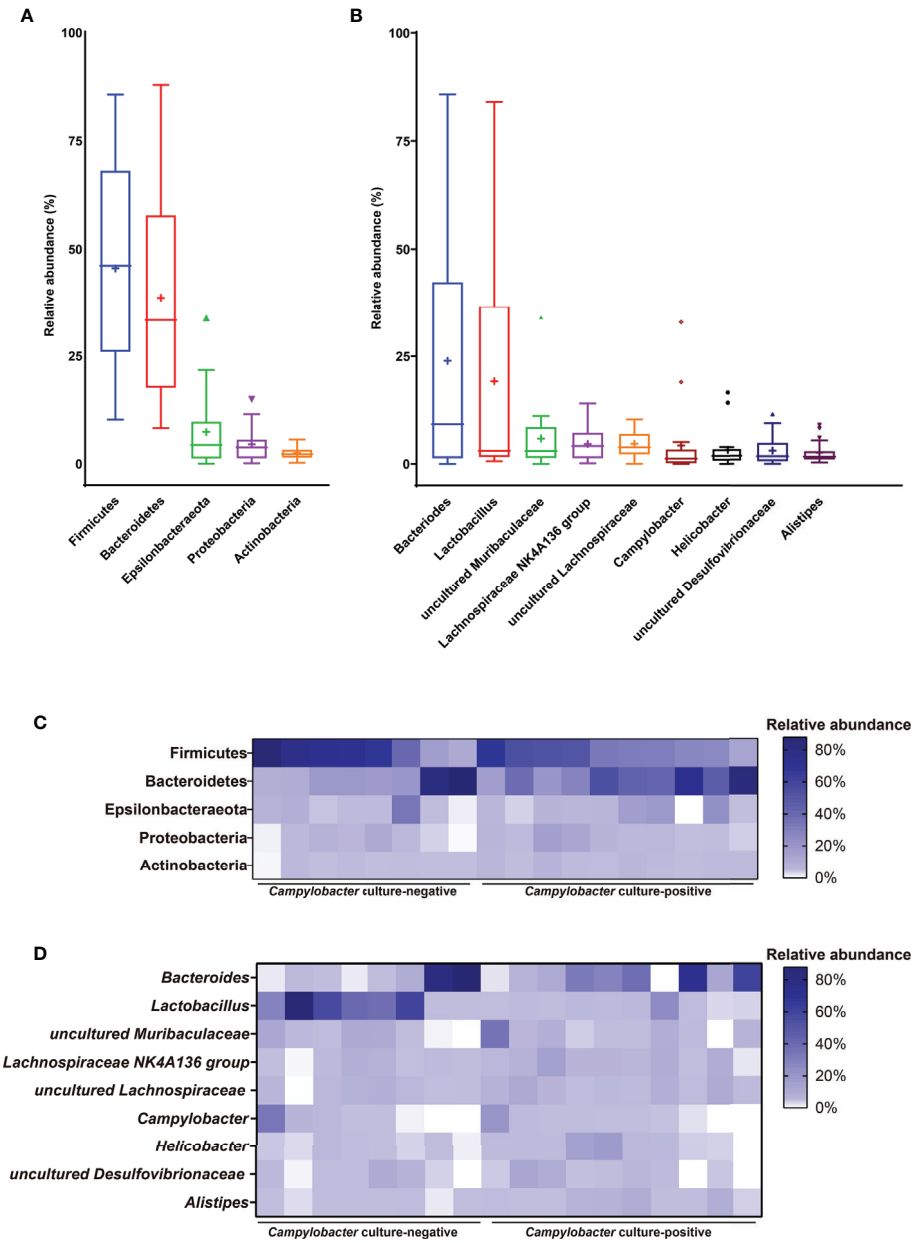

FIGURE 2 | Core gut microbiota of *Micromys minutus*. Box plots showing the relative abundance of the members of the core microbiota at the **(A)** phylum and **(B)** genus levels. Plus sign (+) represents the mean value. Heatmaps showing the relative abundance of core microbiota **(C)** at the phylum and **(D)** genus levels in individual *M. minutus* samples. The X-axis represents the individual samples of *M. minutus*. The Y-axis represents the core taxa. The color scale represents the relative abundance of core taxa in individual samples.

The beta diversity as per the principle coordinate analysis based on Bray-Curtis dissimilarity showed distinct clustering of the gut microbiota of *M. minutus* according to the *Campylobacter* culture results (**Figure 3B**). An ANOSIM test revealed a significant difference in the gut microbiota between the *Campylobacter* culture-positive and -negative groups of *M. minutus* (R: 0.23253, $p < 0.05$). Of note, no significant differences in the beta diversity of the *M. minutus* groups were detected for other factors, such as gender and habitat ($p > 0.05$).

To identify the bacterial taxa with significantly different abundances between wild mice groups, LEFSe was performed. When the *Campylobacter* culture-positive and negative groups of *M. minutus* were compared at the phylum level, Actinobacteria (LDA score −4.89, $p < 0.05$) was the most enriched phylum in the microbiota of *Campylobacter* culture-positive *M. minutus*, followed by Patescibacteria (LDA score −4.4, $p < 0.05$). At the genus level, *Lactobacillus* (LDA score 6.23, $p < 0.05$) was the most enriched genus in the microbiota of *Campylobacter* culture-negative *M. minutus*, whereas *Desulfovibrio* (LDA score −4.5, $p < 0.05$), *Candidatus Saccharimonas* (LDA score −4.4, $p < 0.05$), and *Streptococcus* (LDA score −3.73, $p < 0.05$) were enriched in *Campylobacter* culture-positive *M. minutus* (**Figure 3C**).

Difference in the Gut Microbiota Between Two Species of Wild Mice

When the alpha diversity of two species of wild mice (*M. minutus* and *M. musculus*) was compared using the Mann-Whitney test, no significant differences ($p > 0.05$) were observed in the alpha diversity metrics, including the number of observed ASVs, the Simpson's index, and the Shannon's index (**Figure 4A**).

The beta diversity as per the principle coordinate analysis based on Bray-Curtis dissimilarity showed distinct clustering of the gut microbiota of wild mice according to species (**Figure 4B**). An ANOSIM test revealed a significant difference in the gut microbiota between *M. minutus* and *M. musculus* (R: 0.57627, $p < 0.001$).

When the two species of wild mice (*M. minutus* and *M. musculus*) were compared, the abundance of eight phyla, including Firmicutes, Verrucomicrobia, Deferribacteres, Spirochaetes, Patescibacteria, Actinobacteria, Proteobacteria, and Epsilonbacteraeota were found to be significantly different ($p < 0.05$) based on LEFSe. Firmicutes (LDA score −5.92) was the most enriched phylum in the gut microbiota of *M. musculus*, whereas Epsilonbacteraeota (LDA score 5.43) was the most enriched phylum in the gut microbiota of *M. minutus*, followed by Proteobacteria (LDA score 5.19), Actinobacteria (LDA score 4.69), Patescibacteria (LDA score 4.3), Spirochaetes (LDA score 4.2), Deferribacteres (LDA score 3.96), and Verrucomicrobia (LDA score 3.35). At the genus level, the abundance of all 35 genera was significantly different ($p < 0.05$). *Campylobacter* (LDA score 5.3) was the most enriched genus in *M. minutus*, whereas *Lactobacillus* (LDA score −5.94) was the most enriched genus in *M. musculus* (**Figure 4C**, **Supplementary Table 2**).

DISCUSSION

Previously, we reported a novel *C. jejuni* strain isolated from wild *M. minutus* using a culture-dependent method (Kim et al., 2020). However, the incrimination of *M. minutus* as a reservoir based on culture-dependent methods alone remained unclear because of difficulties in the isolation of *Campylobacter* owing to the fastidious growth conditions required (i.e., microaerophilic) and the presence of viable but non-culturable *Campylobacter* (Mihaljevic et al., 2007; Jackson et al., 2009). Moreover, numerous studies have highlighted the role of a reservoir's microbiota composition in the transmission of a wide range of zoonotic pathogens (Jones et al., 2008; Stecher et al., 2013; Razzauti et al., 2015). However, most studies on the microbiota of wild mice have focused on that of wild *M. musculus*, belonging to the same species as the laboratory mouse, and no study has investigated the microbiota of *M. minutus* (Weldon et al., 2015; Rosshart et al., 2017; Rosshart et al., 2019). Therefore, it is essential to investigate the gut microbiota of *M. minutus* using a culture-independent method to predict the role of *M. minutus* in *Campylobacter* transmission.

The current study revealed that Firmicutes and Bacteroidetes are the most dominant phyla in the gut microbiota of *M. minutus*; in fact, these are the dominant phyla in a wide range of wild rodents (Debebe et al., 2017; Lavrinienko et al., 2018) and are involved in nutrition metabolism and the immune response of the host (Tremaroli and Bäckhed, 2012). Members of Firmicutes play key roles in the degradation of polysaccharides (Flint et al., 2012); thus, the high abundance of Firmicutes in the gut may be related to the food sources and habitats of *M. minutus* (Hata, 2011). At the genus level, *Bacteroides* and *Lactobacillus* were the predominant genera, accounting for nearly half of the microbiota composition. The high abundance of *Bacteroides* and *Lactobacillus* is consistent with the results of another study on omnivorous mammals, including wild mice (*Apodemus sylvaticus*), bears, squirrels, and lemurs (Maurice et al., 2015). The next dominant genera were uncultured *Muribaculaceae*, which is a major component of the mouse gut microbiota and a member of the family *Muribaculaceae*, which was previously known as the S24-7 group (Lagkouvardos et al., 2019), and *Lachnospiraceae NK4A136 group*, a short-chain fatty acid-producing bacteria in the gut (Hu et al., 2019). Therefore, the components of the gut microbiota of *M. minutus* appear to be comparable to those of the gut microbiota of wild rodents reported in previous studies.

Notably, *Campylobacter* was the sixth most abundant genus in the microbiota of all *M. minutus* and varied among samples; this high abundance is inconsistent with previous studies on the microbiota of wild mice (Maurice et al., 2015; Weldon et al., 2015; Rosshart et al., 2017; Rosshart et al., 2019). Moreover, most *M. minutus* harbored *Campylobacter* in their gut metagenome. Of note, this high prevalence of *Campylobacter* in the gut microbiota is similar to that in poultry, which is known to harbor *Campylobacter* as part of the normal gut flora (O'Sullivan et al., 2000; Sahin et al., 2002; Humphrey, 2006). Moreover, the concept of core microbiota considers not only the abundance but also the prevalence to identify microbial communities that exist persistently (Shade et al., 2012; Astudillo-García et al., 2017); thus, *Campylobacter* appears to be a member of the core microbiota of the gut of *M. minutus*. Furthermore, when laboratory mice are infected with

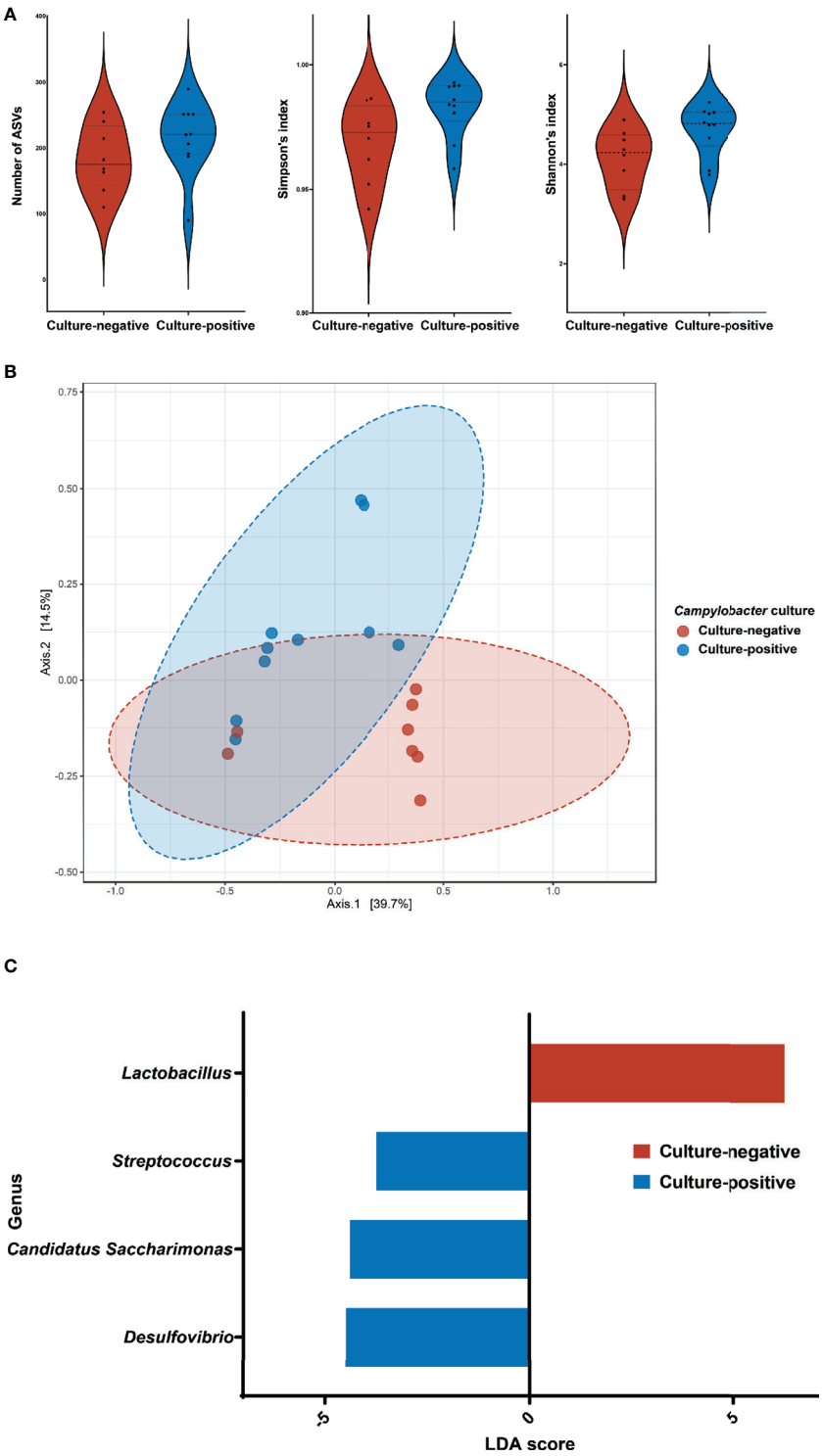

FIGURE 3 | Differences in the gut microbiota of *Micromys minutus* according to *Campylobacter* culture status. **(A)** Alpha diversity of the gut microbiota of two groups of *Micromys minutus*. The distribution of the number of observed amplicon sequence variants, the Simpson's index and the Shannon's index of each group is shown in the box plot. The blue box denotes the *Campylobacter* culture-positive group, and the red box denotes the *Campylobacter* culture-negative group. **(B)** Principle coordinate analysis plot of Bray-Curtis dissimilarity between the gut microbiota of the *Campylobacter* culture-negative and -positive groups of *M. minutus*. Ellipses indicate 95% confidence intervals. **(C)** Histograms of the linear discriminant analysis scores for genera with differential abundance identified using linear discriminant analysis effect size in a culture-positive (blue) and culture-negative (red) group of *Micromys minutus*.

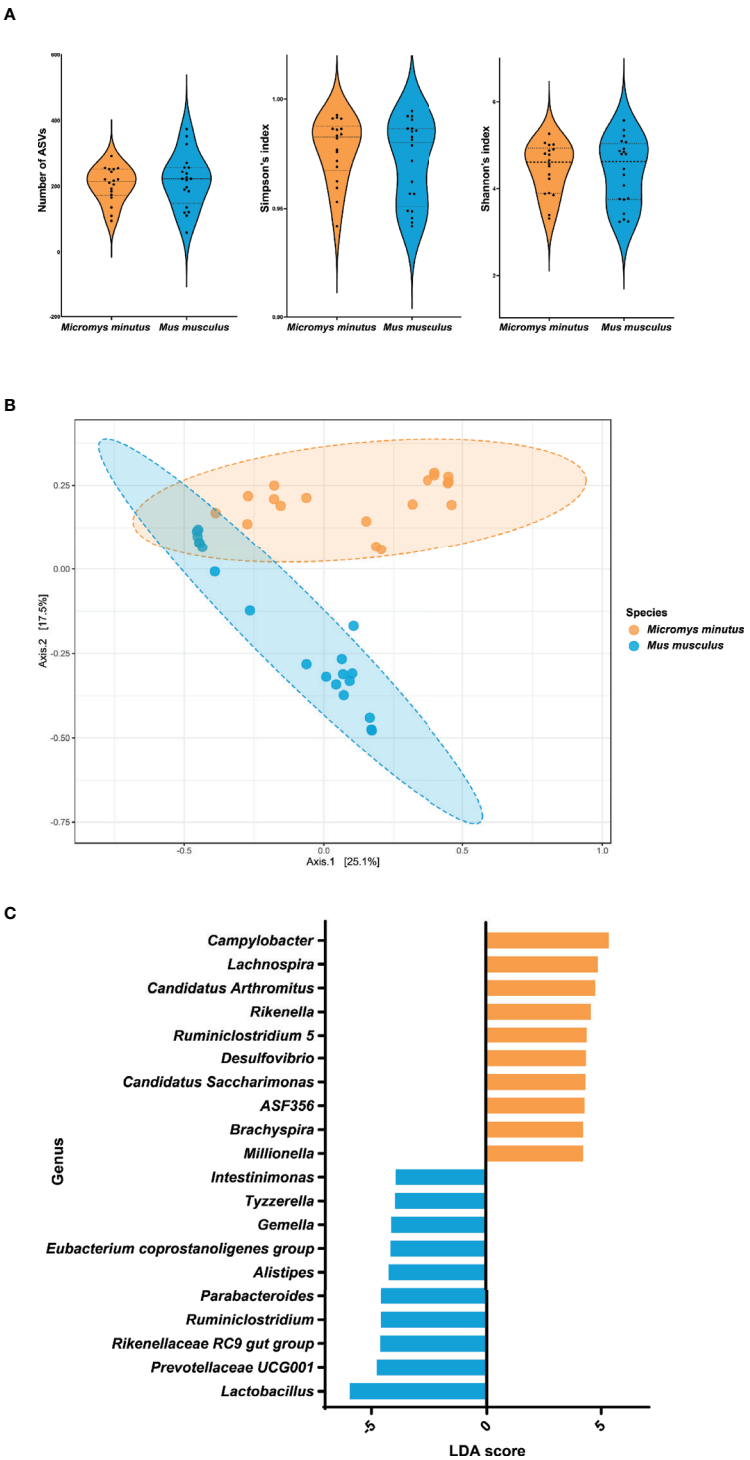

FIGURE 4 | Differences in the gut microbiota of two species of wild mice. **(A)** Alpha diversity of the gut microbiota of two species of wild mice. The distribution of the number of observed amplicon sequence variants, the Simpson's index and the Shannon's index of each group is shown in the box plot. **(B)** Principle coordinate analysis plot of Bray-Curtis dissimilarity between the gut microbiota of *Micromys minutus* (orange) and *Mus musculus* (blue). Ellipses indicate 95% confidence intervals. **(C)** Histograms of the linear discriminant analysis scores for genera with differential abundance identified using linear discriminant analysis effect size in *M. minutus* (orange) and *M. musculus* (blue).

Campylobacter, clinical signs of campylobacteriosis, such as a ruffled coat, hunched posture, lethargy, and diarrhea are observed (Stanfield et al., 1987; Mansfield et al., 2008; Liu et al., 2018). Therefore, if the high abundance and prevalence of *Campylobacter* in the gut microbiota of *M. minutus* were due to an external infection, there would have been clinical signs of campylobacteriosis in *M. minutus*; however, no clinical signs were observed in any captured mice. Considering the results of metagenome analysis and the absence of clinical signs, *Campylobacter* may exist as a normal component of the gut microbiota of *M. minutus*.

The core microbiota of *M. minutus* contained taxa that, in previous studies, were shown to be members of the microbiota of wild mice (*A. sylvaticus*) and laboratory mice, such as *Alistipes* (Maurice et al., 2015) and uncultured *Desulfovibrionaceae* (Zhang et al., 2010). Notably, *Helicobacter*, which can infect humans and other hosts (Bagheri et al., 2015; Tohidpour, 2016) is also a member of the core microbiota of *M. minutus*. Previous studies suggested wild mice (*M. musculus molossinus* and *A. sylvaticus*) as a reservoir of diverse *Helicobacter* strains according to culture-dependent (Won et al., 2002) and culture-independent methods (Maurice et al., 2015); however, the possibility of *M. minutus* as a potential reservoir of other zoonotic pathogens has not been studied. Future studies using culture-dependent methods for further analyses, such as the isolation and characterization of pathogens, are needed to explore the potential of wild mice as a reservoir of other zoonotic pathogens.

Metagenomic analysis results showed that most of the captured *M. minutus* harbored *Campylobacter* in the gut metagenome, regardless of their culture status. Notably, most *M. minutus* that were determined to be *Campylobacter*-negative by culture-dependent methods harbored high proportions of *Campylobacter* in the gut metagenome, indicating that culture-dependent methods alone cannot reliably indicate whether *Campylobacter* is present in the gut. This may be attributed to difficulties in the isolation of *Campylobacter* (as mentioned above) or the cultivation of *Campylobacter* may have been affected by components of the gut microbiota, such as competing flora that inhibit the growth of *Campylobacter* (Jasson et al., 2009; Hazeleger et al., 2016). Moreover, the difference in the microbiota composition between the culture-positive and -negative groups may have affected the isolation of *Campylobacter*. Beta diversity analysis, which showed that the microbiota of *M. minutus* was clustered by the *Campylobacter* culture results rather than by other factors such as gender or habitat, supported this possibility. Differential abundance analysis showed that *Lactobacillus* was the only significantly enriched genus in the culture-negative group compared to that in the culture-positive group. Previous studies revealed that the growth of *Campylobacter* in co-cultures of *Campylobacter* and *Lactobacillus* was significantly lower than that in a single culture of *Campylobacter*, indicating that *Lactobacillus* acts as an antagonist to reduce the level of *Campylobacter* in culture (Wang et al., 2014; Taha-Abdelaziz et al., 2019). These results support the possibility that the relatively high abundance of *Lactobacillus* in the culture-negative group affected the isolation of *Campylobacter* during the culture procedures. As studies on the characteristics of *Lactobacillus* strains isolated from wild mice are lacking, further studies are needed to better understand the antagonistic activities of wild mice-derived *Lactobacillus* strains on *Campylobacter*.

The presence of *Campylobacter* in the gut of the two species of wild mice was also very distinctly different by species. Most *M. minutus* harbored *Campylobacter* in their gut, whereas none of the *M. musculus* harbored *Campylobacter* in their gut. Notably, the presence of *Campylobacter* differed remarkably, despite the fact that the two species of mice were captured in adjacent areas. These results suggest that the different microbiota composition of the two species of wild mice may affect the colonization of *Campylobacter* in the gut. Recent studies showed that components of the gut microbiota provide colonization resistance to *Campylobacter* by competing for nutrition, by modulating the host immune response, and through direct antagonism (Neish, 2009; O'Loughlin et al., 2015; Kampmann et al., 2016); thus, the components of the microbiota in wild *M. musculus* may have prevented the colonization of *Campylobacter* in their gut. Differential abundance analysis to identify significantly enriched taxa in *M. musculus* showed that *Lactobacillus* was the most enriched genus in *M. musculus*. Diverse *Lactobacillus* strains are known to reduce the colonization of *Campylobacter* in the gut (Alemka et al., 2012; Sicard et al., 2017); thus, highly abundant *Lactobacillus* may have played a role as a prophylactic agent against *Campylobacter* in the gut of *M. musculus*. Further studies are needed to demonstrate the interaction of the gut microbiota and colonization of *Campylobacter* in wild mice.

CONCLUSION

This study is the first to investigate the gut microbiota of *M. minutus* using metagenomics to explore its possible role as an environmental *Campylobacter* reservoir. This culture-independent approach indicated that wild *M. minutus* may serve as a reservoir of *Campylobacter*. Metagenomic analysis results revealed that most *M. minutus* harbored high proportions of *Campylobacter* in the gut microbiota regardless of culture status, indicating the necessity of using a culture-independent method together with traditional culture-dependent methods to precisely determine the presence of *Campylobacter*. Considering the high abundance and prevalence of *Campylobacter* in the gut microbiota, and the absence of clinical symptoms, *Campylobacter* may be a component of the normal gut flora of wild *M. minutus*. These findings provide a basis for future studies on the role of environmental reservoirs in the transmission cycle of *Campylobacter* using culture-independent methods.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA656071.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Hallym University.

AUTHOR CONTRIBUTIONS

SC conceived and designed the study. HS, JK, and J-HG performed the sampling and experiments. HS, WK, and HN analyzed the data. JGS and JKS prepared and reviewed the manuscript. HS made a great contribution to the experiments, data analysis, and preparing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.596149/full#supplementary-material

REFERENCES

Alemka, A., Corcionivoschi, N., and Bourke, B. (2012). Defense and adaptation: the complex inter-relationship between *Campylobacter jejuni* and mucus. *Front. Cell. Infect. Microbiol.* 2, 15. doi: 10.3389/fcimb.2012.00015

Astudillo-García, C., Bell, J. J., Webster, N. S., Glasl, B., Jompa, J., Montoya, J. M., et al. (2017). Evaluating the core microbiota in complex communities: A systematic investigation. *Environ. Microbiol.* 19, 1450–1462. doi: 10.1111/1462-2920.13647

Bagheri, N., Azadegan-Dehkordi, F., Shirzad, H., Rafieian-Kopaei, M., Rahimian, G., and Razavi, A. (2015). The biological functions of IL-17 in different clinical expressions of *Helicobacter pylori*-infection. *Microb. Pathog.* 81, 33–38. doi: 10.1016/j.micpath.2015.03.010

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6, 90. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Carbonero, A., Paniagua, J., Torralbo, A., Arenas-Montes, A., Borge, C., and García-Bocanegra, I. (2014). *Campylobacter* infection in wild artiodactyl species from southern Spain: Occurrence, risk factors and antimicrobial susceptibility. *Comp. Immunol. Microbiol. Infect. Dis.* 37, 115–121. doi: 10.1016/j.cimid.2014.01.001

Debebe, T., Biagi, E., Soverini, M., Holtze, S., Hildebrandt, T. B., Birkemeyer, C., et al. (2017). Unraveling the gut microbiome of the long-lived naked mole-rat. *Sci. Rep.* 7, 1–9. doi: 10.1038/s41598-017-10287-0

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* 45, W180–W188. doi: 10.1093/nar/gkx295

Díaz-Sánchez, S., Sánchez, S., Herrera-León, S., Porrero, C., Blanco, J. E., Dahbi, G., et al. (2013). Prevalence of Shiga toxin-producing *Escherichia coli*, *Salmonella spp.* and *Campylobacter spp.* in large game animals intended for consumption: Relationship with management practices and livestock influence. *Vet. Microbiol.* 163, 274–281. doi: 10.1016/j.vetmic.2012.12.026

Dicksved, J., Ellström, P., Engstrand, L., and Rautelin, H. (2014). Susceptibility to *Campylobacter* infection is associated with the species composition of the human fecal microbiota. *MBio* 5, e01212-14. doi: 10.1128/mBio.01212-14

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3, 289–306. doi: 10.4161/gmic.19897

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

Gilroy, R., Chaloner, G., Wedley, A., Lacharme-Lora, L., Jopson, S., and Wigley, P. (2018). *Campylobacter jejuni* transmission and colonisation in broiler chickens is inhibited by Faecal Microbiota Transplantation. *bioRxiv* 476119. doi: 10.1101/476119

Haag, L.-M., Fischer, A., Otto, B., Plickert, R., Kühl, A. A., Göbel, U. B., et al. (2012). Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. *PLoS One* 7, e35988. doi: 10.1371/journal.pone.0035988

Hata, S. (2011). Nesting Characteristics of Harvest Mice (*Micromys minutus*) in Three Types of Japanese Grasslands with Different Inundation Frequencies. *Mammal Study* 36, 49–53. doi: 10.3106/041.036.0106

Hazeleger, W. C., Jacobs-Reitsma, W. F., and de, H. M. W. (2016). Quantification of growth of *Campylobacter* and extended spectrum β-lactamase producing bacteria sheds light on black box of enrichment procedures. *Front. Microbiol.* 7, 1430. doi: 10.3389/fmicb.2016.01430

Hofreuter, D. (2014). Defining the metabolic requirements for the growth and colonization capacity of *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.* 4, 137. doi: 10.3389/fcimb.2014.00137

Hu, S., Wang, J., Xu, Y., Yang, H., Wang, J., Xue, C., et al. (2019). Anti-inflammation effects of fucosylated chondroitin sulphate from: Acaudina molpadioides by altering gut microbiota in obese mice. *Food Funct.* 10, 1736–1746. doi: 10.1039/c8fo02364f

Humphrey, T. (2006). Are happy chickens safer chickens? Poultry welfare and disease susceptibility. *Br. Poult. Sci.* 47, 379–391. doi: 10.1080/00071660600829084

Jackson, D. N., Davis, B., Tirado, S. M., Duggal, M., Van Frankenhuyzen, J. K., Deaville, D., et al. (2009). Survival mechanisms and culturability of *Campylobacter jejuni* under stress conditions. *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* 96, 377–394. doi: 10.1007/s10482-009-9378-8

Jasson, V., Sampers, I., Botteldoorn, N., López-Gálvez, F., Baert, L., Denayer, S., et al. (2009). Characterization of *Escherichia coli* from raw poultry in Belgium and impact on the detection of *Campylobacter jejuni* using Bolton broth. *Int. J. Food Microbiol.* 135, 248–253. doi: 10.1016/j.ijfoodmicro.2009.09.007

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., et al. (2008). Global trends in emerging infectious diseases. *Nature* 451, 990–993. doi: 10.1038/nature06536

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

Kampmann, C., Dicksved, J., Engstrand, L., and Rautelin, H. (2016). Composition of human faecal microbiota in resistance to *Campylobacter* infection. *Clin. Microbiol. Infect.* 22, 61.e1–61.e8. doi: 10.1016/j.cmi.2015.09.004

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kim, J., Guk, J. H., Mun, S. H., An, J. U., Kim, W., Lee, S., et al. (2020). The Wild Mouse (*Micromys minutus*): Reservoir of a Novel *Campylobacter jejuni* Strain. *Front. Microbiol.* 10, 3066. doi: 10.3389/fmicb.2019.03066

Lagkouvardos, I., Lesker, T. R., Hitch, T. C. A., Gálvez, E. J. C., Smit, N., Neuhaus, K., et al. (2019). Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. *Microbiome* 7, 1–15. doi: 10.1186/s40168-019-0637-2

Lavrinienko, A., Mappes, T., Tukalenko, E., Mousseau, T. A., Møller, A. P., Knight, R., et al. (2018). Environmental radiation alters the gut microbiome of the bank vole Myodes glareolus. *ISME J.* 12, 2801–2806. doi: 10.1038/s41396-018-0214-x

Li, Z., Quan, G., Jiang, X., Yang, Y., Ding, X., Zhang, D., et al. (2018). Effects of metabolites derived from gut microbiota and hosts on pathogens. *Front. Cell. Infect. Microbiol.* 8, 314. doi: 10.3389/fcimb.2018.00314

Liu, F., Ma, R., Wang, Y., and Zhang, L. (2018). The Clinical Importance of *Campylobacter concisus* and Other Human Hosted *Campylobacter* Species. *Front. Cell. Infect. Microbiol.* 8, 243. doi: 10.3389/fcimb.2018.00243

Mansfield, L. S., Patterson, J. S., Fierro, B. R., Murphy, A. J., Rathinam, V. A., Kopper, J. J., et al. (2008). Genetic background of IL-10–/– mice alters host–pathogen interactions with *Campylobacter jejuni* and influences disease phenotype. *Microb. Pathog.* 45, 241–257. doi: 10.1016/j.micpath.2008.05.010

Maurice, C. F., Cl Knowles, S., Ladau, J., Pollard, K. S., Fenton, A., Pedersen, A. B., et al. (2015). Marked seasonal variation in the wild mouse gut microbiota. *ISME J.* 9, 2423–2434. doi: 10.1038/ismej.2015.53

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* 8, e61217. doi: 10.1371/journal.pone.0061217

Meerburg, B. G., Jacobs-Reitsma, W. F., Wagenaar, J. A., and Kijlstra, A. (2006). Presence of *Salmonella* and *Campylobacter spp.* in wild small mammals on organic farms. *Appl. Environ. Microbiol.* 72, 960–962. doi: 10.1128/AEM.72.1.960-962.2006

Mihaljevic, R. R., Sikic, M., Klancnik, A., Brumini, G., Mozina, S. S., and Abram, M. (2007). Environmental stress factors affecting survival and virulence of *Campylobacter jejuni*. *Microb. Pathog.* 43, 120–125. doi: 10.1016/j.micpath.2007.03.004

Neish, A. S. (2009). Microbes in Gastrointestinal Health and Disease. *Gastroenterology* 136, 65–80. doi: 10.1053/j.gastro.2008.10.080

O'Loughlin, J. L., Samuelson, D. R., Braundmeier-Fleming, A. G., White, B. A., Haldorson, G. J., Stone, J. B., et al. (2015). The intestinal microbiota influences *Campylobacter jejuni* colonization and extraintestinal dissemination in mice. *Appl. Environ. Microbiol.* 81, 4642–4650. doi: 10.1128/AEM.00281-15

O'Sullivan, N. A., Fallon, R., Carroll, C., Smith, T., and Maher, M. (2000). Detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in broiler chicken samples using a PCR/DNA probe membrane based colorimetric detection assay. *Mol. Cell. Probes* 14, 7–16. doi: 10.1006/mcpr.1999.0274

Patrick, M. E., Gilbert, M. J., Blaser, M. J., Tauxe, R. V., Wagenaar, J. A., and Fitzgerald, C. (2013). Human infections with new subspecies of *Campylobacter fetus*. *Emerg. Infect. Dis.* 19, 1678–1680. doi: 10.3201/eid1910.130883

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Razzauti, M., Galan, M., Bernard, M., Maman, S., Klopp, C., Charbonnel, N., et al. (2015). A comparison between transcriptome sequencing and 16S metagenomics for detection of bacterial pathogens in wildlife. *PLoS Negl. Trop. Dis.* 9, 1–21. doi: 10.1371/journal.pntd.0003929

Rosshart, S. P., Vassallo, B. G., Angeletti, D., Hutchinson, D. S., Morgan, A. P., Takeda, K., et al. (2017). Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. *Cell* 171, 1015–1028.e13. doi: 10.1016/j.cell.2017.09.016

Rosshart, S. P., Herz, J., Vassallo, B. G., Hunter, A., Wall, M. K., Badger, J. H., et al. (2019). Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science (80-.)* 365, eaaw4361. doi: 10.1126/science.aaw4361

Sahin, O., Morishita, T. Y., and Zhang, Q. (2002). *Campylobacter* colonization in poultry: sources of infection and modes of transmission. *Anim. Heal. Res. Rev.* 3, 95–105. doi: 10.1079/ahrr200244

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Shade, A., Hogan, C. S., Klimowicz, A. K., Linske, M., McManus, P. S., and Handelsman, J. (2012). Culturing captures members of the soil rare biosphere. *Environ. Microbiol.* 14, 2247–2252. doi: 10.1111/j.1462-2920.2012.02817.x

Sicard, J. F., Bihan, G., Vogeleer, P., Jacques, M., and Harel, J. (2017). Interactions of intestinal bacteria with components of the intestinal mucus. *Front. Cell. Infect. Microbiol.* 7, 387. doi: 10.3389/fcimb.2017.00387

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

Stanfield, J. T., McCardell, B. A., and Madden, J. M. (1987). *Campylobacter* diarrhea in an adult mouse model. *Microb. Pathog.* 3, 155–165. doi: 10.1016/0882-4010(87)90092-1

Stecher, B., Berry, D., and Loy, A. (2013). Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiol. Rev.* 37, 793–829. doi: 10.1111/1574-6976.12024

Sun, X., Winglee, K., Gharaibeh, R. Z., Gauthier, J., He, Z., Tripathi, P., et al. (2018). Microbiota-Derived Metabolic Factors Reduce Campylobacteriosis in Mice. *Gastroenterology* 154, 1751–1763.e2. doi: 10.1053/j.gastro.2018.01.042

Taha-Abdelaziz, K., Astill, J., Kulkarni, R. R., Read, L. R., Najarian, A., Farber, J. M., et al. (2019). In vitro assessment of immunomodulatory and anti-*Campylobacter* activities of probiotic lactobacilli. *Sci. Rep.* 9, 1–15. doi: 10.1038/s41598-019-54494-3

Tohidpour, A. (2016). CagA-mediated pathogenesis of *Helicobacter pylori*. *Microb. Pathog.* 93, 44–55. doi: 10.1016/j.micpath.2016.01.005

Tremaroli, V., and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–249. doi: 10.1038/nature11552

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., et al. (2002). Colony Multiplex PCR Assay for Identification and Differentiation of *Campylobacter* jejuni, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp *fetus*. *J. Clin. Microbiol.* 40, 4744 LP – 4747. doi: 10.1128/JCM.40.12.4744-4747.2002

Wang, G., Zhao, Y., Tian, F., Jin, X., Chen, H., Liu, X., et al. (2014). Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*. *Food Control* 44, 49–57. doi: 10.1016/j.foodcont.2014.03.042

Weldon, L., Abolins, S., Lenzi, L., Bourne, C., Riley, E. M., and Viney, M. (2015). The gut microbiota of wild mice. *PLoS One* 10, 1–15. doi: 10.1371/journal.pone.0134643

Whiley, H., van den Akker, B., Giglio, S., and Bentham, R. (2013). The role of environmental reservoirs in human campylobacteriosis. *Int. J. Environ. Res. Public Health* 10, 5886–5907. doi: 10.3390/ijerph10115886

Won, Y. S., Yoon, J. H., Lee, C. H., Kim, B. H., Hyun, B. H., and Choi, Y. K. (2002). *Helicobacter muricola* sp. nov., a novel *Helicobacter* species isolated from the ceca and feces of Korean wild mouse (*Mus musculus molossinus*). *FEMS Microbiol. Lett.* 209, 43–49. doi: 10.1111/j.1574-6968.2002.tb11107.x

Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., et al. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *ISME J.* 4, 232–241. doi: 10.1038/ismej.2009.112

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REVIEW
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Revisiting *Campylobacter jejuni* Virulence and Fitness Factors: Role in Sensing, Adapting, and Competing

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Campylobacter jejuni is the leading cause of bacterial foodborne gastroenteritis world wide and represents a major public health concern. Over the past two decades, significant progress in functional genomics, proteomics, enzymatic-based virulence profiling (EBVP), and the cellular biology of *C. jejuni* have improved our basic understanding of this important pathogen. We review key advances in our understanding of the multitude of emerging virulence factors that influence the outcome of *C. jejuni*–mediated infections. We highlight, the spatial and temporal dynamics of factors that promote *C. jejuni* to sense, adapt and survive in multiple hosts. Finally, we propose cohesive research directions to obtain a comprehensive understanding of *C. jejuni* virulence mechanisms.

Keywords: *Campylobacter jejuni*, virulence, host-pathogen, sensing, adaptation, stress and survival

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INTRODUCTION

Campylobacters are the leading cause of bacterial foodborne gastroenteritis in the world. There are 31 different species[1] and 10 sub-species within the genus *Campylobacter* (Garcia-Sanchez et al., 2018; Wilkinson et al., 2018). The *Campylobacter* genus encompasses several clinically relevant species, such as *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter lari*, *and Campylobacter upsaliensis* (Kaakoush et al., 2015; Garcia-Sanchez et al., 2018). This review focuses on *C. jejuni* subsp. *jejuni* which is the most relevant clinically (Skirrow, 1977; Skirrow, 2006). *C. jejuni* is responsible for 80%–90% of the diagnosed cases of *Campylobacter* infections (Facciola et al., 2017). *C. jejuni* colonizes the gastrointestinal (GI) tract of a wide variety of food-producing animals such as poultry, cattle, sheep and swine (**Figure 1**). However, poultry, particularly chickens are the major source of human infection (Humphrey et al., 2014; Ijaz et al., 2018; McKenna et al., 2020). Outbreaks of *C. jejuni* infections are also associated with exposure to contaminated soil, unpasteurized milk and untreated water sources (Korlath et al., 1985; Hudson et al., 1999; Bronowski et al., 2014; Artursson et al., 2018). Clinical symptoms of *C. jejuni* infection can be watery or bloody diarrhea accompanied by abdominal cramps, nausea, fever and sometimes vomiting (Blaser, 1997; Hansson et al., 2018; Igwaran and Okoh, 2019). Although *C. jejuni* infection is acute and self-limiting, in a small number of patients (1:1000) post infection sequalae can lead to severe neurological disorders such as Guillain-Barré syndrome (Yuki et al., 1993; Nachamkin et al., 1998; Sheikh et al., 1998a; Sheikh et al., 1998b; Houliston et al., 2011). According to a recent report by the World Health Organization (WHO), *C. jejuni* is responsible for 96 million cases of enteric

[1] http://www.bacterio.net/campylobacter.html

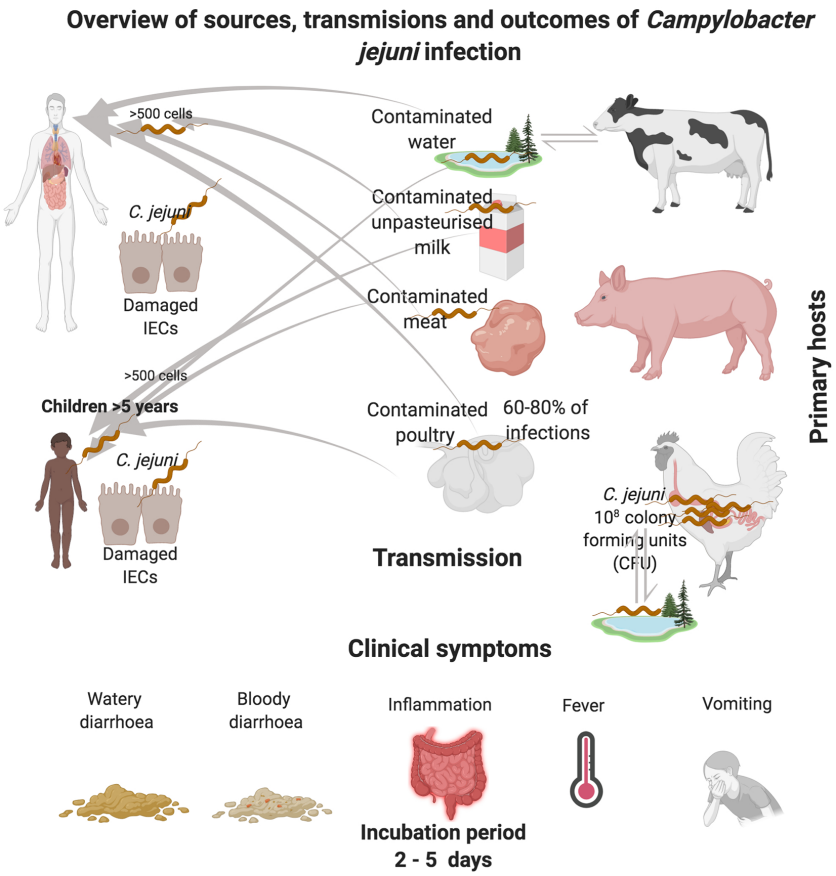

FIGURE 1 | Overview of *C. jejuni* reservoirs and transmission routes of infection. *C. jejuni* reside in the GI tract of chickens, where the bacteria can be spread through consumption of contaminated poultry products. *C. jejuni* transmission can also occur *via* the consumption of contaminated raw cows drinking milk (RDM) which can occur during the milking process, most commonly *via* fecal contamination of udders. Pigs are also recognized as reservoirs of *C. jejuni*. Contamination of the environment can also occur *via* host fecal contamination. *C. jejuni* can persist for long periods in feces, milk and water, especially at temperatures close to 4°C. In adverse conditions, *C jejuni* converts to a viable nonculturable form that can be reactivated when ingested.

infection globally each year (Havelaar et al., 2015; Bailey et al., 2018). In the United Kingdom, *C. jejuni* is responsible for more than 700,000 cases, of which 22,000 hospitalisations and more than 100 deaths occur each year (Bronowski et al., 2014; John et al., 2017). The economic burden associated with *C. jejuni* infection in the United Kingdom is estimated to be £1 billion per year (Bronowski et al., 2014). Moreover, in the European Union (EU), *C. jejuni* is responsible for estimated cases of 9 million with an economic burden of around €2.4 billion each year (https://www.efsa.europa.eu/en/topics/topic/campylobacter). According to the United States Centers for Disease Control, *C. jejuni* is responsible for an estimated 1.5 million human infections each year[2] with a staggering economic burden of between $1.3 to 6.8 billion dollars per year.

C. jejuni does not possess classical virulence factors observed in bacterial enteropathogens such as enterotoxigenic *Escherichia coli* and *Salmonella* spp. (Gaytan et al., 2016; Park et al., 2018). However, *C. jejuni* has a complex array of fitness and virulence factors (Cróinín and Backert, 2012; Backert and Hofreuter, 2013; Backert et al., 2013) which aid the bacterium to respond to the defense mounted by the host; *C. jejuni* can adhere, invade and temporarily survive inside human intestinal epithelial cells (IECs) *in vitro*. We review recent progress made in understanding *C. jejuni* pathogenesis. We highlight findings from several approaches that pioneered the integration of selective mutagenesis, phenotypic assays, high-resolution proteomics and 'omics. Finally, we describe challenges ahead for successful research in understanding how *C. jejuni* causes disease in humans.

C. JEJUNI VIRULENCE FACTORS, A BREAKTHROUGH IN UNDERSTANDING THE MISSING LINK

In early 2000, the availability of the full genome sequence of *C. jejuni* NCTC 11168, isolated from the feces of a diarrheic patient in

[2]https://www.cdc.gov/campylobacter/technical.html

1977 by Martin Skirrow, marked a new era in the study of the pathogenesis of this major enteric pathogen (Skirrow, 1977; Parkhill et al., 2000). The annotation of the full genome sequence revealed the absence of genes encoding for a non-flagellar type 3 protein secretion system (NF-T3SS). This finding has raised an intriguing question: Does *C. jejuni* sense, inject and secrete putative virulence factors into host cells? In contrast to the absence of NF-T3SS, the genome sequence shed light on the presence of a genomic locus encoding a novel bacterial protein *N*-glycosylation (*pgl*) system, absent in other enteropathogens (Szymanski et al., 1999; Linton et al., 2005). This 11 gene locus encodes for all the necessary enzymes for *N*-linked *pgl* system to produce a conserved heptasaccharide consisting of GalNAc–α1,4-GalNAc–α1,4 (Glcβ1,3)-GalNAc–α1,4-GalNAc–α1,4-GalNAc–α1,3-Bac (Bac is bacillosamine or 2,4,-diacetamido-2,4,6-trideoxyglucose (Young et al., 2002; Jervis et al., 2012). *C. jejuni* conserved heptasaccharide has been found to modify up to 100 periplasmic and membrane-bound proteins while it also appears to be responsible for multiple cell functions (Cain et al., 2019; Abouelhadid et al., 2019; Abouelhadid et al., 2020). A feature of the availability of *C. jejuni* genome sequence was the identification and characterization of different glycostructures. In addition to the *N*-linked *pgl* system, other studies have facilitated systematic analysis of genes encoding for flagellar biosynthesis and modification (Jagannathan et al., 2001; Hendrixson and DiRita, 2003; Konkel et al., 2004), lipooligosaccharide (LOS) (Parker et al., 2005; Parker et al., 2008; Kanipes et al., 2008; Hameed et al., 2020) and capsule polysaccharide (CPS) (Karlyshev et al., 2001; Karlyshev et al., 2005). In parallel, the genome sequence of *C. jejuni* identified a large repertoire of phase-variable genes (Guerry et al., 2002; Aidley et al., 2018). The genome sequence of *C. jejuni* further accelerated characterization of repertoire of virulence and fitness factors such as putative adhesins (Konkel et al., 2005), proteases (Brondsted et al., 2005), autotransporters (Ashgar et al., 2007), chemotaxis regulatory genes (Marchant et al., 2002) and the cytolethal distending toxin (CDT) (Purdy et al., 2000). Sequencing the genomes of various *C. jejuni* isolates have also elucidated strain-specific genetic diversity, noticeably the finding of the putative pVir plasmid in *C. jejuni* strain 81–176 (Bacon et al., 2000). Because of the high genome plasticity of *C. jejuni*, genome sequencing also facilitated genome-wide association studies (GWAS) which provided insight into the prevalence of *C. jejuni* virulence genes, antimicrobial resistance markers as well as relatedness of human clinical isolates (Sheppard et al., 2013; Buchanan et al., 2017). Understanding the genetic variability of *C. jejuni* isolates is important for defining key factors that contribute to its ability to host adaptation and evolution. Some *C. jejuni* strains are restricted to specific host while there are *C. jejuni* strains with multi-host lineages. Defining how *C. jejuni* adapts to hosts is an enduring challenge. However, study has demonstrated that one factor that is driving rapid *C. jejuni* host adaptation is gain and loss of *panBCD* genes encoding for vitamin B_5 biosynthesis pathway (Sheppard et al., 2013). Recently, the advent of large scale genome sequencing has also identified *C. jejuni* isolates possessing Type VI Secretion System (T6SS) (Corcionivoschi et al., 2015; Ugarte-Ruiz et al., 2015), offering the potential to better understand the role of T6SS in *C. jejuni* pathogenesis (Liaw et al., 2019).

C. JEJUNI IN THE HOST-PATHOGEN CROSSTALK: VIRULENCE AND FITNESS FACTORS

In its natural environment *C. jejuni* adapts, survives and proliferates in the nutrient-rich mucous layer of the avian GI tract. *C. jejuni* growth in chicken ceca exceeds 10^8 colony-forming units per g of cecal contents (CFU)/g (Dhillon et al., 2006; Hermans et al., 2011; Gormley et al., 2014). The transition of *C. jejuni* from nutrient-rich chicken ceca to the environment exposes *C. jejuni* to perturbations. These perturbations unveil *C. jejuni* to atmospheric oxygen (ca. 21% O_2) and temperature fluctuations which thus alter *C. jejuni* nutrient acquisition and metabolism. In the context of human infection, *C. jejuni* faces additional stresses such as peristalsis and expulsion in the GI tract. *C. jejuni* also faces endogenous stresses ranging from oxidative, nitrosative, pH fluctuations and cationic stresses. The ability to persist in spite of various stresses indicate *C. jejuni* harbors complex virulence and fitness factors (Hermans et al., 2012). These virulence and fitness factors do not only confer protection but also play a role in the ability of *C. jejuni* to sense, adapt and compete the constantly changing host microenvironments, working for example as sensors and/signal molecules, adhesins for host receptors, and/or effectors for invasion and intracellular survival.

C. jejuni interaction and invasion of human IECs induce numerous downstream host signaling pathways. *C. jejuni* activates mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK) and p38, leading to the induction of a potent pro-inflammatory cytokine interleukin-8 (IL-8) (MacCallum et al., 2005). IL-8 is an important pro-inflammatory cytokine of IECs and acts as a chemotactic factor of immune cells. However, it is hypothesized that induction IL-8 from human IECs which is found to correlate with an increase in circulating neutrophils to the site of infection can inadvertently exacerbate the classical acute inflammatory symptoms. *C. jejuni* induction of Erk and p38 signaling pathways is dependent on bacterial *de novo* protein synthesis and a functional flagellum (Jin et al., 2003; Watson and Galan, 2005).

C. JEJUNI FLAGELLA: FUNCTION AND VIRULENCE

C. jejuni produces two polar flagella at each pole of the cell, termed as amphitrichous flagellation. *C. jejuni* flagella is a multifunctional organelle which enables the bacterium to avoid hostile environments including forceful peristalsis and expulsion from the GI tract. *C. jejuni* flagella also enable the bacterium to penetrate the viscous mucosa lining of the human IECs, and to reach the distal ileum, jejunum and colon. Thus, *C. jejuni* flagella

promotes bacteria motility, chemotaxis and avian colonization. Besides mediating these virulence attributes, *C. jejuni* flagellar also promotes adhesion and invasion into human IECs *in vitro* (Black et al., 1988; Grant et al., 1993; Szymanski et al., 1995; Konkel et al., 1999), biofilm formation (Svensson et al., 2014) and non-flagella protein export. The latter enables *C. jejuni* to secrete ∼18 putative virulence-associated proteins termed *Campylobacter* invasion antigens (Cia) (Konkel et al., 2004; Christensen et al., 2009). Some of *C. jejuni* Cia proteins are required for invading human IECs *in vitro*, for instance CiaC plays a role in invasion whereas CiaI is required for intracellular survival in human IECs (Buelow et al., 2011; Neal-McKinney and Konkel, 2012). Interestingly study showed CiaD involves in maximal activation of the MAP kinase signaling pathways Erk 1/2 and p38 resulting in the secretion of IL-8 (Samuelson et al., 2013).

C. jejuni flagella synthesis and glycan modification involves over 50 flagellum-related genes. The flagellum is composed of three major parts, the basal body, which crosses the bacterial cell membrane, as well as a flagellar-associated cytoplasmic ring, the hook complex and the flagellar filament. Debates had focused on finding relationships between *C. jejuni* flagellum, motility, colonization and secretion. *C. jejuni* flagellar filament contributes to bacterial motility (Wassenaar et al., 1991; Guerry et al., 1991), adherence and colonization. The flagellar filament is composed of subunits of FlaA and FlaB proteins. *C. jejuni* flagellin proteins are *O*-linked glycosylated and the *O*-linked glycosylation is specific to the serine and threonine residues on a flagellin subunit which is modified by pseudaminic acid (Pse) and derivatives containing acetyl and acetamindino groups (PseAcOAc or PseAm, respectively (Thibault et al., 2001; Schirm et al., 2005). Sometimes *C. jejuni* flagellin subunits are modified with legionaminic acid (Leg), moieties (Logan et al., 2009; Schoenhofen et al., 2009; Howard et al., 2009). *C. jejuni* flagellar subunit FlaA rather than FlaB is essential for *C. jejuni* motility. This is supported by evidence that showed a mutation of the *flaA* gene led to the generation of non-flagellated and non-motile cells (Nuijten et al., 1990; Wassenaar et al., 1991). By contrast, the mutation of *flaB*, has no impact on *C. jejuni* flagella synthesis and motility. These findings suggest that FlaA protein, rather than motility, is essential for *C. jejuni* optimal colonization in chickens (Wassenaar et al., 1993). However, subsequent studies have identified *C. jejuni* mutant with normal but paralyzed flagella that is also non-motile and had a reduced ability to colonize chickens (Yao et al., 1994). The role *of C. jejuni* flagella in chicken colonization is further confirmed through mutation of the flagellar motor genes *MotA* and *MotB* which are essential for the rotation of the flagella. A *motAB* mutant produced non-motile cells with a full-length flagellum that is unable to rotate, thus unable to colonize chickens (Hendrixson and DiRita, 2004). Other *C. jejuni* flagella genes that have been studied include the flagellar sigma factor σ^{28} (*fliA*) and the alternative sigma factor σ^{54} (*rpoN*). These two sigma factors regulate a large number of genes that are responsible for the expression and function of *C. jejuni* flagella. For example, sigma σ^{28} is known to regulate the major flagellin gene *flaA* and some other late flagellar genes which control synthesis of proteins forming motor and chemotaxis proteins. On the other hand, *C. jejuni* σ^{54} involves the transcription of genes encoding for the hook, basal body, and minor flagellin *flaB*. In the context of host colonization and infection, mutation of σ^{54} (*rpoN)* gene results a non-motile cells that are unable to colonize chickens (Fernando et al., 2007), adhere to and invade into human IECs *in vitro* (Wassenaar et al., 1991). Also, *C. jejuni* flagellar functions as an organelle to secrete flagellar co-expressed determinants (Feds) which are required for efficient invasion of human IECs *in vitro* (Song et al., 2004; Barrero-Tobon and Hendrixson, 2012). A unique feature of *C. jejuni* flagellar filament is its mechanism to escape immune interaction with Toll-like receptor 5 (TLR5). TLR5s are found at the basolateral side of the human IECs and recognize a highly conserved epitope in bacterial flagellin. However, *C. jejuni* flagellar filament evades TLR5 activation because it fails to make complementary contacts with the TLR5 LRR9 loop (Song et al., 2017). This is attributed to sequence divergence of *C. jejuni* flagellin particularly the highly conserved epitope found in most γ-proteobacteria and Firmicutes bacterial flagellin. Recently, specific amino acids found in *C. jejuni* flagellar filament have been shown to mediate weakened binding to human TLR5 (Kreutzberger et al., 2020).

C. JEJUNI CAPSULAR POLYSACCHARIDE (CPS)

The first evidence of a CPS at the surface of *C. jejuni* was reported in 2001 (Karlyshev et al., 2001). *C. jejuni* CPS is found on the outermost layer of the cell surface of the bacterium and it is composed of a rare structure of diverse repeating units of sugars (Karlyshev et al., 2005; McNally et al., 2005; Gilbert et al., 2007). *C. jejuni* CPS possess a heptoses sugar with an unusual configuration (e.g., ido, gulo, and altro) and nonstoichiometric modifications on the sugars, including ethanolamine, aminoglycerol, and O-methyl phosphoramidate (MeOPN). Unsurprisingly, *C. jejuni* CPS is the major sero-determinant of the Penner serotyping scheme of *C. jejuni* strains (Karlyshev et al., 2000). Currently, there are more than 47 different *C. jejuni* Penner serotypes of the bacterial CPS with some forming related serotype complexes (Poly et al., 2015). The structural variations of *C. jejuni* CPS reflects differences in the genetic content of the genomic locus that drives CPS biosynthesis (Karlyshev et al., 2005). *C. jejuni* CPS contains homopolymeric tracts which are prone to phase variation. As expected, homopolymeric tracts allow a rapid on/off switching of the *C. jejuni* CPS genes resulting in variations in CPS arrangements even in *C. jejuni* isolates that have identical gene contents. In addition to the phase variation observed in CPS sugar composition, *C. jejuni* CPS is also modified with ethanolamine, glycerol, and nonstoichiometric MeOPN modifications in approximately 75% of *C. jejuni* strains (Thota et al., 2018).

C. jejuni CPS plays a role in bacteria pathogenicity (Guerry et al., 2012; Bolton, 2015). *C. jejuni* CPS is required to resist

complement-mediated killing (Bacon et al., 2001; Keo et al., 2011), invade into human IECs *in vitro* (Bachtiar et al., 2007; Corcionivoschi et al., 2009), colonization of chickens (Jones et al., 2004), and diarrheal disease in ferrets (Bacon et al., 2001). Consistently, the nonstoichiometric modification of CPS with MeOPN has also been demonstrated to be essential for complement resistance. The role of CPS in *C. jejuni* resistance to complement-mediated killing is supported by evidence showing *C. jejuni* expressing full CPS structure but lacking MeOPN, displayed the same pattern of serum killing as a nonencapsulated *kpsM* mutant, which lacked CPS. Also, study, using *Galeria mellonella* larvae infection model demonstrated *C. jejuni* expressing full CPS but lacking specific MeOPN modification to be significantly attenuated in virulence (Champion et al., 2010). This same study suggested the structure of the MeOPN moiety has a remarkable similarities to the active structures of organophosphorous pesticides (McNally et al., 2007), therefore, the virulence attenuation of *C. jejuni* expressing full CPS but lacking specific MeOPN may be due to a consequence of toxicity provided by the MeOPN. However, from virulence perspective, the role of *C. jejuni* CPS in serum resistance is still unclear as *C. jejuni* induces human β-defensins 2 and 3 (hBD2 and hBD3) from human IECs *in vitro* (Zilbauer et al., 2005).

C. JEJUNI PUTATIVE ADHESINS

Adhesins play an important role in the pathogenesis of bacteria to adhere, colonize, and invade into hosts. *C. jejuni* adherence to human IECs *in vitro* involves putative adhesins decorated on its outer membrane (OM) surface. *C. jejuni* adhesins seem to have alternate primary functions, yet some can target the same host receptor such as fibronectin. Once *C. jejuni* adheres to fibronectin on the basolateral side of human IECs, it is preceded by secondary steps that orchestrate cellular invasion (Konkel et al., 2020). The most highly investigated adhesins in *C. jejuni* that exist almost in mutually exclusive fashion are *Campylobacter* adhesion to fibronectin (CadF) and fibronectin-like protein A (FlpA). *C. jejuni* adhesins (CadF and FlpA) are highly conserved among *C. jejuni* strains. CadF and FlpA proteins are important for *C. jejuni* adherence to human IECs and colonization of chickens (Konkel et al., 2020). A *C. jejuni cadF* mutant displays reduced ability to adhere to human IECs and chicken hepatoma cell line, LMH cells. *C. jejuni cadF* mutant is also unable to adhere to immobilized fibronectin (Talukdar et al., 2020). *C. jejuni* FlpA also promotes *C. jejuni* adherence to human IECs *in vitro* and plays a role in *C. jejuni* colonization of chickens (Flanagan et al., 2009; Konkel et al., 2010; Larson et al., 2013). There are additional *C. jejuni* surface-exposed adhesins, such as *Campylobacter* adhesion protein A (CapA), PEB1 (Kervella et al., 1993; Pei et al., 1998) and PEB4 (Asakura et al., 2007). These adhesins which also play a role in *C. jejuni* adherence to human and chicken IECs *in vitro* represent the multifactorial ability of *C. jejuni* virulence mechanisms. However, study suggested that PEB1 is not required for adhering to chicken LMH cells but rather as a transporter of amino acids aspartate and glutamate (Leon-Kempis Mdel et al., 2006). Unfortunately, an important gap in our current knowledge is the lack of mechanistic insight as to how *C. jejuni* orchestrates adherence steps to IECs. This is due in part to the fact that some of the adhesins identified to date display an overlap in binding mechanisms, a factor that confounds straightforward analysis of *C. jejuni* adhesion mechanisms. It is hypothesized that these *C. jejuni* different adhesins are required in the multiple steps of infection. First, to adhere to the mucosal layer at the luminal side of human IECs and then to adhere to the fibronectin receptor at the basolateral side of IECs.

OTHER *C. JEJUNI* OUTER MEMBRANE CHANNELS

C. jejuni produces numerous virulence and/or fitness proteins that function as major outer membrane proteins (MOMPs). Two of the most well characterized MOMPs in *C. jejuni* are MOMP and OMP50. *C. jejuni* MOMP is also referred to as PorA. In contrast to *E. coli*, *C. jejuni* possesses only one MOMP that is present in all isolates and is highly (but not absolutely) conserved in other *Campylobacters* (Ferrara et al., 2016). *C. jejuni*, MOMP, is a 44-kDa protein, with sequence signature typical of β-barrel porin seen in other enteropathogens (Amako et al., 1996; Ferrara et al., 2016). *C. jejuni*, MOMP is relatively well characterized compared to OMP50. As might be expected, considering its association with the outer surface of the bacterial cell, *C. jejuni* MOMP exhibits substrate selectivity and functions as a control channel for the entry/exit of nutrients and other specific molecules (Dhanasekar et al., 2017). Mutation of *porA* have been thought to be lethal due to critical structural and transport functions. However, inactivation of *porA* enhances sensitivity to certain hydrophilic antibiotics (Iovine, 2013). Unlike MOMP, which is present in most *Campylobacter*s, Omp50 is only found in *C. jejuni* and *C. lari* strains, but not in *C. coli* (Dedieu et al., 2008). The synthesis of Omp50 is tightly regulated by the host microenvironment. For example, *C. jejuni Omp50* is down-regulated in chicken cecum and up-regulated in rabbit ileal loop (Stintzi et al., 2005; Woodall et al., 2005). Mutation of *Omp50* substantially reduced *C. jejuni* motility and invasion, while it also involves bacterium decreased Nox1-dependent ROS generation (Corcionivoschi et al., 2012).

C. JEJUNI PUTATIVE PROTEASES: NEW PERSPECTIVE IN VIRULENCE INVOLVEMENT

Recent characterization of *C. jejuni* putative proteases represent an important step forward in the efforts to dissect *C. jejuni* pathogenesis. As opposed to traditional candidate-mutant experimental approaches, a proteomics analysis coupled with

enzymatic-based virulence profiling (EBVP) have shed light on the specific role of *C. jejuni* putative proteases in adhesion to and invasion into human IECs *in vitro*. *C. jejuni* secretes outer membrane vesicles (OMVs) that contain three active serine proteases (HtrA, Cj0511, and Cj1365c) (Elmi et al., 2012). The mechanism responsible for the abundance of these serine proteases in OMVs remains elusive. However, *C. jejuni* proteases have been demonstrated to contribute targeted damage to human IECs *in vitro* (Elmi et al., 2016). Treatment of human IECs with active protease result in cleavage of IECs tight and adherens junction proteins, namely occludin and E-cadherin. The targeted proteolytic activity of *C. jejuni* OMVs also enhance *C. jejuni* adhesion to and invasion into IECs *in vitro* (Elmi et al., 2016). Moreover, follow-up study has shown that bile salt sodium taurocholate (ST) upregulates *C. jejuni* expression of *htrA*, *Cj0511*, *Cj1365*, and the *cdtABC* operon, highlighting the importance of bacterium adaptation to host metabolites (Elmi et al., 2018). Furthermore, recent study has demonstrated that physiological concentrations of ST regulates *C. jejuni* OMVs production through changes in expression of the maintenance of lipid asymmetry (MLA) pathway (Davies et al., 2019). Although most of the examples discussed above had focused on the role of serine proteases in virulence, it should be remembered that *C. jejuni* OMVs also contain a cocktail of virulence and fitness factors, including stress response enzymes, adhesins, CDT, lipoproteins and other metalloproteases, which also play an important role in bacterial virulence. Thus, suggestions have been raised that *C. jejuni* OMVs might also function as fitness and survival factors, allowing the bacterium to adapt new niches, adhere to surfaces, translocate rapidly across IECs, and resist antibiotics and other deleterious circumstances.

C. JEJUNI FITNESS AND VIRULENCE FACTORS: ROLE IN STRESS ADAPTATION, TEMPERATURE, NUTRIENT SENSING, AND METABOLIC REWIRING

As *C. jejuni* transitions from nutritionally rich ceca in the GI tract of chickens to accidentally infect humans, the bacterium faces formidable stresses. Here, the term "stress" refers to environmental and human host stresses that reduce *C. jejuni* fitness or negatively impact on its virulence. Unlike other enteropathogens, *C. jejuni* does not have homologs of the classical stress response regulators such as SoxRS and OxyR found in *E. coli* and *Salmonella* spp. respectively. SoxRS regulates response to redox-active compounds while OxyR responds to hydrogen peroxide (Nunoshiba et al., 1992; Zheng et al., 1998). In addition, *C. jejuni* lacks transcription regulators such as cold shock protein A (CspA) and leucine-responsive regulatory protein (Lrp) (Calvo and Matthews, 1994; Murphy et al., 2006; Keto-Timonen et al., 2016). Besides, *C. jejuni* does not possess the classical alternative sigma factors such as RpoS (σ^{38}) although it has limited sigma factors including RpoD (σ^{70}), RpoN (σ^{54}), and RpoF/FliA (σ^{28}). Interestingly, *C. jejuni* possesses unique and yet unresolved mechanisms to survive under various stress conditions. *C. jejuni* utilizes OmpR-type response regulators such as *Campylobacter* oxidative stress regulator (CosR) (Hwang et al., 2011), peroxide-sensing regulator (PerR) (Palyada et al., 2009) and Multiple Antibiotic Resistance Regulator, MarR-type regulators designated for response to peroxide stress (Gundogdu et al., 2016). *C. jejuni* CosR is a pleiotropic regulator that controls the expression of genes involved in various cellular processes, especially genes that involve in macromolecule biosynthesis, metabolism, and oxidative stress response (Kim et al., 2015b). The genes that CosR regulates mostly encode for stress response-related proteins such as the DNA binding protein from starved cells (Dps), rubredoxin oxidoreductase/rubrerythrin (Rrc), alkyl hydroperoxide reductase (AhpC), and superoxide dismutase (SodB). On the other hand PerR, non-OxyR-dependent regulator, controls transcription of peroxide as well as the superoxide defense genes particularly under oxidative stress conditions. For instance, *perR* mutation abrogates the transcriptional response of *ahpC*, *katA*, and *sodB* to oxidants (Kim et al., 2015a).

C. jejuni also possesses global transcriptional regulators such as carbon starvation regulator (CsrA), ortholog of the *E. coli* global posttranscriptional regulator CsrA. In addition, *C. jejuni* has two-component regulatory systems (TCRS) such as *Campylobacter* planktonic growth regulator (CprRS) (Svensson et al., 2015; El Abbar et al., 2019). Mutation of *csrA* results in *C. jejuni* cells with altered motility, biofilm formation, adherence to and invasion of human IECs cells and resistance to oxidative stress (Fields and Thompson, 2008). CprRS is two-component systems regulator typically consisting of a sensor kinase and a response regulator. The CprR response regulator is essential and mutation to the *cprR*, is lethal to *C. jejuni*, but a *cprS* mutation, results in decreased expression of *SodB*, *Rrc* and *LuxS*. *C. jejuni* also possesses a ferric uptake regulator (Fur) to control the expression of a range of oxidative stress genes, to prevent the build-up of toxic levels of iron within the cell (Butcher et al., 2012). In addition to the stress-responsive regulators, *C. jejuni* KatA and SodB proteins play critical roles in detoxification, SodB detoxifies free radicals O_2^- while KatA contributes for the detoxification of H_2O_2 (Atack and Kelly, 2009). SodB also contributes to *C. jejuni* chicken colonization and intracellular survival in human IECs *in vitro* (Palyada et al., 2009; Novik et al., 2010). *C. jejuni* cell surface structures such as flagella, CPS, LOS and OM also can act at the interface between the bacterium and the extracellular environment. These cellular surface structures assist *C. jejuni* to sense environmental and host stresses, in principle, inducing a collective response to protect the bacterium from damage caused by stresses.

Environmental Stress Survival and Adaptation

In light of its relatively small genome (1.6–1.7 Mb), it remains enigmatic how *C. jejuni* senses, adapts and persists in diverse environmental stresses. *C. jejuni* requires optimal oxygen concentrations of approximately 5%–10% for growth; however, the bacterium can survive in the environment, which is rich in

oxygen (ca. 21% O_2). This variation in oxygen concentration constraints *C. jejuni* to rewire its physiology to adapt flexible metabolic pathways. The requirement of 5%–10% O_2 for growth is governed by single class I-type Ribonucleotide Reductase (RNR) (Burnham and Hendrixson, 2018). This is an oxygen-dependent enzyme that catalyses the *de novo* conversion of ribonucleotides diphosphates (NDPs) to deoxyribonucleotides diphosphates (dNDPs), and therefore plays a pivotal role in maintaining *C. jejuni* synthesis of deoxyribonucleotide (dNTP). Besides, *C. jejuni* also possesses a highly-branched respiratory chain feature that facilitates the use of oxygen as an electron acceptor for one of two respiratory oxidases, cytochrome c oxidase (CcoNOQP), a cbb3-type cytochrome c oxidoreductase and a bd-type (CioAB or CydAB) quinol oxidase (Guccione et al., 2017; van der Stel and Wosten, 2019). The sensitivity of *C. jejuni* pyruvate: acceptor oxidoreductase (POR) and the TCA cycle 2-oxoglutarate: acceptor oxidoreductase (OOR) to oxygen has been suggested as one of the explanations of the so-called '*C. jejuni*-oxygen paradox' - that is, why *C. jejuni* is unable to proliferate in aerobic environment. Also, atmospheric levels of oxygen inactivate *C. jejuni* L-serine dehydratase (SdaA), which catalyses the deamination of serine and converts serine into pyruvate which is further converted to acetyl CoA, which is oxidized *via* the TCA cycle to carbon dioxide and free energy. SdaA is essential for colonization of the avian gut (Velayudhan et al., 2004). The ability of *C. jejuni* to tolerate oxygen in the environment can also vary between strains. For instance, study has found a higher prevalence of some strain genotypes in environmental samples attributing these variations in oxygen tolerance (Champion et al., 2005; Bronowski et al., 2014). Besides, another study has reported atypical *C. jejuni* Bf strain that is oxygen tolerant (Rodrigues et al., 2015; Bronnec et al., 2016a). This strain has been demonstrated to have protective mechanisms against oxidative stress which is thought to be mediated by regulation of genes involved in oxidative stress response and biofilm formation (Bronnec et al., 2016b). Interestingly, recent assessment of *C. jejuni* phospholipidome profile has indicated that *C. jejuni* phospholipidome have an unusually high percentage of lysophospholipid. Lysophospholipids are small bioactive lipid molecules characterized by a single carbon chain and a polar head group. It is hypothesized lysophospholipid allows *C. jejuni* to be motile under low O_2 conditions (Cao et al., 2020a). This is a significant observation considering the requirement of *C. jejuni* to adapt to the low oxygen deep in the mucus layer of the human GI tract. This could give *C. jejuni* competitive advantage when competing with other microbiota that colonize the mucosal layer as it transitions into the IECs. In addition, the ability of *C. jejuni* to sense environmental oxygen have been thought to correlate altering its membrane lipid composition which could be crucial for biofilm formation.

C. jejuni Biofilm: Environmental Adaptation and Persister Phenomena

C. jejuni adaptation to an oxygen-rich environment such as contaminated freshwater, poultry meat or raw milk can be attributed to the ability of the bacterium to form biofilms on different substrates. *C. jejuni* can attach and persist on a variety of abiotic and biotic surfaces, and several studies have reported on the viable but non-culturable (VBNC) state (Teh et al., 2014; Magajna and Schraft, 2015). *C. jejuni* cells switch to VBNC state to survive better under adverse environmental conditions. In the environments, *C. jejuni* is exposed to high oxygen tension, limited nutrient availability, heat, acidic pH, temperatures fluctuations and antimicrobials. These environmental constraints are known to stimulate increased *C. jejuni* biofilm formation to a relatively high level, supporting the proposal that *C. jejuni* forms biofilm as a survival strategy outside of the avian host. *C. jejuni* forms increased biofilm in oxygen-rich conditions compared to microaerobic conditions (Reuter et al., 2010). It is commonly agreed that all *C. jejuni* strains form biofilm, however, the ability of *C. jejuni* to form biofilm appears to be strain-dependent (Melo et al., 2017). Interestingly, *C. jejuni* mutant strains deficient in genes encoding for key oxidative stress resistance enzymes such as alkyl hydroperoxide reductase (AhpC) or *C. jejuni*'s sole catalase enzyme (KatA) have been shown to have an increased ability to form biofilm (Oh and Jeon, 2014). This is attributed to the accumulation of reactive oxygen species (ROS) which may serve as a trigger to increase the level of biofilm formed in response to increased oxidative stress. Overexpression of *ahpC* is correlated with decreased biofilm formation, and treatment of the *ahpC* mutant with antioxidants reduces biofilm formation (Oh and Jeon, 2014). *C. jejuni* lacks the classical two-component regulatory systems involved in biofilm formation found in other bacteria, such as GacSA in *Pseudomonas aeruginosa*, however, *C. jejuni* biofilm formation is thought to be under the control of a complex array of regulatory factors that respond to a variety of environmental signals. These complex regulatory factors include global regulator CsrA, *Campylobacter* oxidative stress regulator (CosR), stringent response regulator (SpotT) and CprRS, which have been shown to play an important role in biofilm formation in *C. jejuni* under aerobic conditions (Gaynor et al., 2005; Fields and Thompson, 2008; Svensson et al., 2015; El Abbar et al., 2019). Mutations of *cosR*, *cprRS*, and, *spotT* increase biofilm formation under aerobic conditions, while mutation of the gene encoding for global regulator (CsrA) decreases the ability of *C. jejuni* to form biofilms when grown in static culture as well as increased sensitivity to oxidative stresses (Fields and Thompson, 2008). Interestingly, in other enteric bacteria *spoT* mutation decreases biofilm formation (He et al., 2012). In *C. jejuni*, the mutation of *spoT* alters the expression of genes related to redox balance, metabolism, energy production, and conversion pathways while CosR, a key orphan regulator in the maturation of biofilm, has also been shown to affect the expression of the antimicrobial efflux pump CmeABC (Turonova et al., 2015). CprRS is two-component systems regulator typically consisting of a sensor kinase and a response regulator. The CprR response regulator is essential and deletion of the cprS sensor kinase enhances biofilms. Current evidence suggests that CprRS likely regulates genes related to aspects of the *C. jejuni* surface structures (Svensson et al., 2015). The molecular mechanism of *C. jejuni*

biofilm formation also appears to indirectly correlate with factors required for fitness and virulence. For instance, mutation of the flagella genes *flaA*, *flaB* and the cell surface modification genes *pgp1* and *waaF* have been shown to increase biofilm formation (Reeser et al., 2007). This indicates that *C. jejuni* increases biofilm formation as a survival strategy during stress. Interestingly, a recent study suggests *C. jejuni* does not form biofilms under conditions encountered in the environment but attaches to surfaces or biofilms of other species (Teh et al., 2014; Teh et al., 2019). This is an attractive proposal supporting the notion that *C. jejuni* is a poor biofilm initiator, and is likely to form enhanced biofilms in a "mixed-species biofilm" with other bacteria such as *P. aeruginosa*, *Enterococcus faecalis* and *Staphylococcus simulans*.

C. jejuni Temperature Stress Adaptation

Temperature is a prominent signal used by many enteric pathogens. The strategies enteric pathogens use to sense temperature variation across space, hosts and time broadly acts as a mechanism to adjust bacterial survival and virulence. For *C. jejuni*, the transition from its primary chicken host (42°C) to the environment, the bacterium experiences temperature variation. This temperature variation confines proliferation and shifts *C. jejuni* physiology forcing the bacterium to coordinate fitness and virulence regulatory systems. It is puzzling that *C. jejuni* lacks classical RpoS homolog (Parkhill et al., 2000) and cold shock proteins (Oh et al., 2019), yet *C. jejuni* has the ability to survive in low and/or high nonpermissive temperature growth conditions before reaching human host. *C. jejuni* doesn't also grow temperatures below ~ 30°C, however the bacterium survives temperature growth range between 4°C to 33°C (Hazeleger et al., 1998). *C. jejuni* survives better at 4°C in various biological milieu than at 25°C (Murphy et al., 2006). *C. jejuni* also survives in water, at low temperatures, for up to 4 months (Oberheim et al., 2020). The ability of *C. jejuni* to survive in cold temperatures is different among strains, with *C. jejuni* strains isolated from human infection being significantly more capable of prolonged survival at 4°C than poultry-derived strains (Chan et al., 2001). Intriguingly *C. jejuni* also survives extreme freezing temperatures (−20°C) for several weeks (Bhaduri and Cottrell, 2004).

C. jejuni genes associated with oxygen tolerance, starvation and osmotic stress are essential for the bacterium to survive in the low temperature. This perplexing physiology of *C. jejuni* seems to be the bottleneck to the efforts aimed to eradicate the risk of *C. jejuni* to human health. The ability of *C. jejuni* to rapidly sense and adapt to cold temperature is largely driven at the transcriptional level (Bronowski et al., 2017). Studies focusing on human infections, use *in vitro* human IECs grown at 37°C to mimic the temperature that the bacteria encounters inside human host. *C. jejuni* ability to sense 37°C is crucial to optimize its fitness and adjust expression of its virulence genes. *C. jejuni* is more invasive into human IECs cultured at 37°C than IECs cultures at 42°C (Aroori et al., 2013). Although the exact mechanism of *C. jejuni* response to temperature stress is not yet explicitly known, changes in temperature are known to affect expression of bacterial heat shock proteins (HSP). *C. jejuni* possesses two-component regulatory systems (TCSs) such as reduced ability to colonize response regulator (RacRS). RacRS function to assist the bacteria to overcome stresses associated with heat shock response. In addition, *C. jejuni* RacR is required for avian colonization and growth while mutation of *racR* alters the expression of selected proteins in both temperature-dependent and independent manners (Hazeleger et al., 1998; Wouters et al., 2000).

C. jejuni Acid Stress Adaptation

C. jejuni grows at optimal pH range of 6.5–7.5, while it is also able to survive pH range as low as 5.5 and as high as 8.5. However, *C. jejuni* encounters acidic conditions either in the environment or within the gut of the various hosts that it colonizes. In the context of human infection, *C. jejuni* survives passage through the stomach, where the concentration of acid is high and the pH ranges 1.5–3.5. The molecular strategies that *C. jejuni* uses to sense, adapt and survive the luminal acid concentration in the stomach upon ingestion and within the phagosomes and phagolysosomes of human IECs is not currently known. However, *C. jejuni* tolerance to human GI tract luminal acid is important for disease development. So far, it is hypothesized *C. jejuni* lacks proteins required for acid tolerance such as urease protein found in *Helicobacter pylori*. However, it is intriguing that with low infectious dose of (500–800 bacteria), *C. jejuni* cells survive the gastric acid of the human stomach and continue down to reach the small intestine. Study has demonstrated some *C. jejuni* strains can survive acid exposure at pH 3.5 and above for up to 30 min (Le et al., 2012). Another study has suggested adaptation of *C. jejuni* to the luminal acid concentration in humans requires genes mediating various cellular processes, including those involved in motility, metabolism, stress response, DNA repair and surface polysaccharide biosynthesis (Reid et al., 2008). For instance, *C. jejuni Rpo*N, a classical flagellar transcriptional regulator, which is historically known to play an important role in motility has been demonstrated to be important for the resistance of *C. jejuni* to various stresses including acid stress. This suggested flagella mediated motility is critical for both initial navigation through the acid environment in the GI tract lumen and mucus layer to IECs attachment. *C. jejuni* adaptation to low pH stress also involved the differential expression of genes involve in respiratory pathways, the upregulation of genes for phosphate transport, and the repression of energy generation and intermediary metabolism genes (Reid et al., 2008). Recent study that investigated acid-stressed adaptation of *C. jejuni* under iron-enriched conditions has shown the capacity of *C. jejuni* to survive acid stress is greatly enhanced in presence of iron (Askoura et al., 2020). However, limited information is available about the role which human host microbiota plays in the pathophysiology of *C. jejuni* adaptation in acidity along the gut, although it is evident that many species of the microbiota are able to generate metabolites that have bearing on the composition of GI tract luminal acidity. For example, lactate which is an organic acid that is found in the upper GI tract of human and avian species

can act as a chemoattractant signal of *C. jejuni* (Bernalier-Donadille, 2010; Hofreuter, 2014).

C. jejuni Metabolic Sensing and Adaptation

While, as discussed above, *C. jejuni* has complex stress response mechanisms, its ability to resist stresses overlaps its ability to adapt to different metabolic requirements. *C. jejuni* sequenced strain NCTC11168 lacks the glycolytic enzymes glucokinase (Glk) and phosphofructokinase (PfkA) of the classical Embden-Meyerhof-Parnas (EMP) pathway (Parkhill et al., 2000; Guccione et al., 2008; Hofreuter, 2014). *C. jejuni* was once considered to be non-saccharolytic since *C. jejuni* sequenced strain NCTC11168 lacks genes encoding for the complete pentose phosphate (PPP) or Entner-Doudoroff (ED) pathway. Interestingly, few isolates of *C. jejuni* subsp. *doylei* encode a complete ED pathway which suggests the potential to catabolize glucose (Vegge et al., 2016; Garber et al., 2020). The inability to utilize glucose has necessitated *C. jejuni* to utilize amino acids such as serine, aspartate, glutamate, glutamine, proline and asparagine as carbon and energy sources (Stahl et al., 2012; Hofreuter, 2014; Szymanski, 2015). Most *C. jejuni* strains preferentially use serine, aspartate, glutamate, and proline, although certain *C. jejuni* strains can also utilize asparagine and glutamine (Thompson and Gaynor, 2008; van der Hooft et al., 2018). This unique ability to metabolize only a few amino acids allows the bacterium to utilize efficient strategies to include host nutrients into its anabolic processes, to fuel its metabolic pathways and to support its survival and adaptation in hosts with largely commensalism outcome in avian species or pathogenesis in humans. For instance, *C. jejuni* catabolism of serine and aspartate enhances the ability of the bacterium to colonize the avian gut (Hermans et al., 2011), while a *C. jejuni* mutant that is lacking an oxygen-labile serine dehydratase and unable to catabolize serine is demonstrated to be incapable of colonizing chickens (Velayudhan et al., 2004). Furthermore, *C. jejuni* rewires its metabolic requirements during avian colonization and human infection. *C. jejuni* has the ability to adopt an asaccharolytic lifestyle, likely as a strategy to evade microbiome competition. It is known that certain *C. jejuni* strains metabolize sugars such as L-fucose (Stahl et al., 2011). These *C. jejuni* strains possess an operon for L-fucose utilisation which until recently has been known to be limited to some *C. coli* and *C. jejuni* subsp. *doylei* strains. L-fucose acts as a chemoattractant for *C. jejuni* (Dwivedi et al., 2016). Interestingly, *C. jejuni* binds to α1, 2-fucosylated glycans, however the L-fucose catabolism is not essential for *C. jejuni* colonization of avian species (Muraoka and Zhang, 2011; Stahl et al., 2011). Furthermore, *C. jejuni* lacks fucosidase enzyme which is essential for the release of the L-fucose from glycosylated host proteins such as mucin. A study recently demonstrated that *C. jejuni* fucose positive strain utilisation of L-fucose is dependent on the fucosidase activity of the gastrointestinal bacterium *Bacteriodes fragilis* (Garber et al., 2020). This same study also revealed that *C. jejuni* becomes more invasive toward human Caco-2 cells in the presence of an exogenous fucosidases from *B. fragilis*.

Recently, examining the idea of a host nutritional role in *C. jejuni* adaptation and pathogenesis, studies showed that *C. jejuni* senses and utilizes catabolic end products of the intestinal microbiota such as short-chain fatty acids (SCFAs) butyrate and acetate, CO_2-derived hydrogen carbonate, and free amino acids and di-/or oligopeptides, which are released by microbiota from dietary or endogenous proteins (Gao et al., 2017). The ability of *C. jejuni* to sense SCFAs positively regulates many *C. jejuni* amino acids uptake and catabolism systems that are essential for host colonization. SCFAs are found in abundance in the lower regions of the intestinal tracts of avian species and humans where they play a major role in host physiology through nutritional, regulatory, and immunomodulatory functions. However, in the context of *C. jejuni* avian and human colonization, the abundance of butyrate and acetate in the lower GI tract provides the bacterium with a competitive advantage to thrive in this niche (Burnham and Hendrixson, 2018). A prevailing belief is that *C. jejuni* has the ability to spatially differentiate between sections of the GI tract by sensing the presence of acetate and butyrate, and thereby modifying the transcription of its colonization factors (Goodman et al., 2020). This enables *C. jejuni* to obtain sufficient nutrients and resources to allow for optimal survival and persistence in both avian and human intestinal tracts. *C. jejuni* specifically senses butyrate *via* a noncanonical TCS termed BumSR (Goodman et al., 2020). BumS functions as a phosphatase, *via* a noncanonical mechanism for signal transduction in place of a sensor kinase, to control the activity of the cognate BumR response regulator. BumS phosphorylates BumR in response to the presence of butyrate. *C. jejuni* genes known to be induced after sensing butyrate and acetate include genes encoding for nutrient acquisition systems, energy generation pathways, and colonization factors (Goodman et al., 2020). In addition, acetate which is more abundant in the gut is preferred metabolite for *C. jejuni* once the rate-limiting step of carbohydrate metabolism is surpassed in stationary phase. *C. jejuni* also catabolizes organic acids such as lactate which is abundant in the upper gut of avian hosts (Luethy et al., 2017).

CONCLUSIONS AND FUTURE DIRECTIONS

Recent developments in the understanding of *C. jejuni* pathogenesis have combined several experimental approaches that link the functional characterization of various putative genes. Although this is important, characterizing *C. jejuni* virulence and fitness factors requires an integrative approach. In the future, an ideal experiment should involve the use of single-gene inactivations and phenotypic assays, incorporated with integrative multi-omics approach including, transcriptomics, proteomics and metabolomics. This should reveal comprehensive findings that would contribute to the characterization of *C. jejuni* pathogenesis. This approach will also guide us to re-focus on re-characterization of many *C. jejuni* virulence-associated genes that have not yet been fully characterized. From our perspective, the incorporation of integrative multi-omics and phenotypic assays in *C. jejuni* research promises enormous potential. However, there are many challenges

and thus, opportunities for further development of experiments involving multi-omics technology. Also, future studies of *C. jejuni* should include refining, optimisation and normalization of experimental design and protocols that represent ideal settings for *C. jejuni* and host cells, allowing researchers to reproduce data. Unsurprisingly, there are a plethora of *C. jejuni* studies that use experimental approaches that give an insight into the selected role of *C. jejuni* putative virulence associate genes. For instance, in stress survival, adhesion, invasion and intracellular survival, however, few studies provide information about the function of such putative genes. Also, integration of *C. jejuni* virulence characterizations with spatial analysis at the various time point and *C. jejuni* strains variability is needed to improve our understanding of *C. jejuni* pathogenesis.

AUTHOR'S NOTE

For the purpose of this review, we define a virulence factor as a protein (such as a toxin) or macromolecular structure (such as flagellum) that contribute to the ability of the bacteria to cause disease and a fitness factor as a protein or macromolecular structure that, while not required for virulence, offers a competitive advantage during infection.

AUTHOR CONTRIBUTIONS

AE: Conceived and designed the structure of the manuscript; AE Created Figure 1; AE, OG, and FN: Wrote the manuscript; AE, BW, ND, OG, and FN: Read and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abouelhadid, S., North, S. J., Hitchen, P., Vohra, P., Chintoan-Uta, C., Stevens, M., et al. (2019). Quantitative Analyses Reveal Novel Roles for N-Glycosylation in a Major Enteric Bacterial Pathogen. *mBio* 10. doi: 10.1128/mBio.00297-19

Abouelhadid, S., Raynes, J., Bui, T., Cuccui, J., and Wren, B. W. (2020). Characterization of Posttranslationally Modified Multidrug Efflux Pumps Reveals an Unexpected Link between Glycosylation and Antimicrobial Resistance. *mBio* 11. doi: 10.1128/mBio.02604-20

Aidley, J., Wanford, J. J., Green, L. R., Sheppard, S. K., and Bayliss, C. D. (2018). PhasomeIt: an 'omics' approach to cataloguing the potential breadth of phase variation in the genus Campylobacter. *Microb. Genom.* 4. doi: 10.1099/mgen.0.000228

Amako, K., Wai, S. N., Umeda, A., Shigematsu, M., and Takade, A. (1996). Electron microscopy of the major outer membrane protein of Campylobacter jejuni. *Microbiol. Immunol.* 40, 749–754. doi: 10.1111/j.1348-0421.1996.tb01136.x

Aroori, S. V., Cogan, T. A., and Humphrey, T. J. (2013). The effect of growth temperature on the pathogenicity of Campylobacter. *Curr. Microbiol.* 67, 333–340. doi: 10.1007/s00284-013-0370-1

Artursson, K., Schelin, J., Thisted Lambertz, S., Hansson, I., and Olsson Engvall, E. (2018). Foodborne pathogens in unpasteurized milk in Sweden. *Int. J. Food Microbiol.* 284, 120–127. doi: 10.1016/j.ijfoodmicro.2018.05.015

Asakura, H., Yamasaki, M., Yamamoto, S., and Igimi, S. (2007). Deletion of peb4 gene impairs cell adhesion and biofilm formation in Campylobacter jejuni. *FEMS Microbiol. Lett.* 275, 278–285. doi: 10.1111/j.1574-6968.2007.00893.x

Ashgar, S. S., Oldfield, N. J., Wooldridge, K. G., Jones, M. A., Irving, G. J., Turner, D. P., et al. (2007). CapA, an autotransporter protein of Campylobacter jejuni, mediates association with human epithelial cells and colonization of the chicken gut. *J. Bacteriol.* 189, 1856–1865. doi: 10.1128/JB.01427-06

Askoura, M., Youns, M., and Halim Hegazy, W. A. (2020). Investigating the influence of iron on Campylobacter jejuni transcriptome in response to acid stress. *Microb. Pathog.* 138, 103777. doi: 10.1016/j.micpath.2019.103777

Atack, J. M., and Kelly, D. J. (2009). Oxidative stress in Campylobacter jejuni: responses, resistance and regulation. *Future Microbiol.* 4, 677–690. doi: 10.2217/fmb.09.44

Bachtiar, B. M., Coloe, P. J., and Fry, B. N. (2007). Knockout mutagenesis of the kpsE gene of Campylobacter jejuni 81116 and its involvement in bacterium-host interactions. *FEMS Immunol. Med. Microbiol.* 49, 149–154. doi: 10.1111/j.1574-695X.2006.00182.x

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

Backert, S., Boehm, M., Wessler, S., and Tegtmeyer, N. (2013). Transmigration route of Campylobacter jejuni across polarized intestinal epithelial cells: paracellular, transcellular or both? *Cell Commun. Signal* 11, 72. doi: 10.1186/1478-811X-11-72

Bacon, D. J., Alm, R. A., Burr, D. H., Hu, L., Kopecko, D. J., Ewing, C. P., et al. (2000). Involvement of a plasmid in virulence of Campylobacter jejuni 81-176. *Infect. Immun.* 68, 4384–4390. doi: 10.1128/IAI.68.8.4384-4390.2000

Bacon, D. J., Szymanski, C. M., Burr, D. H., Silver, R. P., Alm, R. A., and Guerry, P. (2001). A phase-variable capsule is involved in virulence of Campylobacter jejuni 81-176. *Mol. Microbiol.* 40, 769–777. doi: 10.1046/j.1365-2958.2001.02431.x

Bailey, R. A., Kranis, A., Psifidi, A., Watson, K. A., Rothwell, L., Hocking, P. M., et al. (2018). Colonization of a commercial broiler line by Campylobacter is under limited genetic control and does not significantly impair performance or intestinal health. *Poult. Sci.* 97, 4167–4176. doi: 10.3382/ps/pey295

Barrero-Tobon, A. M., and Hendrixson, D. R. (2012). Identification and analysis of flagellar coexpressed determinants (Feds) of Campylobacter jejuni involved in colonization. *Mol. Microbiol.* 84, 352–369. doi: 10.1111/j.1365-2958.2012.08027.x

Bernalier-Donadille, A. (2010). [Fermentative metabolism by the human gut microbiota]. *Gastroenterol. Clin. Biol.* 34 (Suppl 1), S16–S22. doi: 10.1016/S0399-8320(10)70016-6

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

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., and Blaser, M. J. (1988). Experimental Campylobacter jejuni infection in humans. *J. Infect. Dis.* 157, 472–479. doi: 10.1093/infdis/157.3.472

Blaser, M. J. (1997). Epidemiologic and clinical features of Campylobacter jejuni infections. *J. Infect. Dis.* 176 (Suppl 2), S103–S105. doi: 10.1086/513780

Bolton, D. J. (2015). Campylobacter virulence and survival factors. *Food Microbiol.* 48, 99–108. doi: 10.1016/j.fm.2014.11.017

Brondsted, L., Andersen, M. T., Parker, M., Jorgensen, K., and Ingmer, H. (2005). The HtrA protease of Campylobacter jejuni is required for heat and oxygen tolerance and for optimal interaction with human epithelial cells. *Appl. Environ. Microbiol.* 71, 3205–3212. doi: 10.1128/AEM.71.6.3205-3212.2005

Bronnec, V., Haddad, N., Cruveiller, S., Hernould, M., Tresse, O., and Zagorec, M. (2016a). Draft Genome Sequence of Campylobacter jejuni Bf, an Atypical

Strain Able To Grow under Aerobiosis. *Genome Announc.* 4. doi: 10.1128/genomeA.00120-16

Bronnec, V., Turonova, H., Bouju, A., Cruveiller, S., Rodrigues, R., Demnerova, K., et al. (2016b). Adhesion, Biofilm Formation, and Genomic Features of Campylobacter jejuni Bf, an Atypical Strain Able to Grow under Aerobic Conditions. *Front. Microbiol.* 7, 1002. doi: 10.3389/fmicb.2016.01002

Bronowski, C., James, C. E., and Winstanley, C. (2014). Role of environmental survival in transmission of Campylobacter jejuni. *FEMS Microbiol. Lett.* 356, 8–19. doi: 10.1111/1574-6968.12488

Bronowski, C., Mustafa, K., Goodhead, I., James, C. E., Nelson, C., Lucaci, A., et al. (2017). Campylobacter jejuni transcriptome changes during loss of culturability in water. *PloS One* 12, e0188936. doi: 10.1371/journal.pone.0188936

Buchanan, C. J., Webb, A. L., Mutschall, S. K., Kruczkiewicz, P., Barker, D. O. R., Hetman, B. M., et al. (2017). A Genome-Wide Association Study to Identify Diagnostic Markers for Human Pathogenic Campylobacter jejuni Strains. *Front. Microbiol.* 8, 1224. doi: 10.3389/fmicb.2017.01224

Buelow, D. R., Christensen, J. E., Neal-Mckinney, J. M., and Konkel, M. E. (2011). Campylobacter jejuni survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol. Microbiol.* 80, 1296–1312. doi: 10.1111/j.1365-2958.2011.07645.x

Burnham, P. M., and Hendrixson, D. R. (2018). Campylobacter jejuni: collective components promoting a successful enteric lifestyle. *Nat. Rev. Microbiol.* 16, 551–565. doi: 10.1038/s41579-018-0037-9

Butcher, J., Sarvan, S., Brunzelle, J. S., Couture, J. F., and Stintzi, A. (2012). Structure and regulon of Campylobacter jejuni ferric uptake regulator Fur define apo-Fur regulation. *Proc. Natl. Acad. Sci. U. S. A.* 109, 10047–10052. doi: 10.1073/pnas.1118321109

Cain, J. A., Dale, A. L., Niewold, P., Klare, W. P., Man, L., White, M. Y., et al. (2019). Proteomics Reveals Multiple Phenotypes Associated with N-linked Glycosylation in Campylobacter jejuni. *Mol. Cell Proteomics* 18, 715–734. doi: 10.1074/mcp.RA118.001199

Calvo, J. M., and Matthews, R. G. (1994). The leucine-responsive regulatory protein, a global regulator of metabolism in Escherichia coli. *Microbiol. Rev.* 58, 466–490. doi: 10.1128/MR.58.3.466-490.1994

Cao, X., Brouwers, J., Van Dijk, L., Van De Lest, C. H. A., Parker, C. T., Huynh, S., et al. (2020a). Dataset of the phospholipidome and transcriptome of Campylobacter jejuni under different growth conditions. *Data Brief* 33, 106349. doi: 10.1016/j.dib.2020.106349

Cao, X., Brouwers, J. F. H. M., Van Dijk, L., van de Lest, C. H. A., Parker, C. T., Huynh, S., et al. (2020b). Pathogen Campylobacter jejuni: Lysophosholipids Are Required for Motility atLow Oxygen Availability. *J. Mol. Biol.* 432 (19), 5244-5258. doi: 10.1016/j.jmb.2020.07.012

Champion, O. L., Gaunt, M. W., Gundogdu, O., Elmi, A., Witney, A. A., Hinds, J., et al. (2005). Comparative phylogenomics of the food-borne pathogen Campylobacter jejuni reveals genetic markers predictive of infection source. *Proc. Natl. Acad. Sci. U. S. A.* 102, 16043–16048. doi: 10.1073/pnas.0503252102

Champion, O. L., Karlyshev, A. V., Senior, N. J., Woodward, M., La Ragione, R., Howard, S. L., et al. (2010). Insect infection model for Campylobacter jejuni reveals that O-methyl phosphoramidate has insecticidal activity. *J. Infect. Dis.* 201, 776–782. doi: 10.1086/650494

Chan, K. F., Le Tran, H., Kanenaka, R. Y., and Kathariou, S. (2001). Survival of clinical and poultry-derived isolates of Campylobacter jejuni at a low temperature (4 degrees C). *Appl. Environ. Microbiol.* 67, 4186–4191. doi: 10.1128/AEM.67.9.4186-4191.2001

Christensen, J. E., Pacheco, S. A., and Konkel, M. E. (2009). Identification of a Campylobacter jejuni-secreted protein required for maximal invasion of host cells. *Mol. Microbiol.* 73, 650–662. doi: 10.1111/j.1365-2958.2009.06797.x

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

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

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

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

Davies, C., Taylor, A. J., Elmi, A., Winter, J., Liaw, J., Grabowska, A. D., et al. (2019). Sodium Taurocholate Stimulates Campylobacter jejuni Outer Membrane Vesicle Production via Down-Regulation of the Maintenance of Lipid Asymmetry Pathway. *Front. Cell. Infect. Microbiol.* 9, 177. doi: 10.3389/fcimb.2019.00177

Dedieu, L., Pages, J. M., and Bolla, J. M. (2008). The omp50 gene is transcriptionally controlled by a temperature-dependent mechanism conserved among thermophilic Campylobacter species. *Res. Microbiol.* 159, 270–278. doi: 10.1016/j.resmic.2008.03.002

Dhanasekar, N. N., Aliouane, S., Winterhalter, M., Pages, J. M., and Bolla, J. M. (2017). Peptide translocation across MOMP, the major outer membrane channel from Campylobacter jejuni. *Biochem. Biophys. Rep.* 11, 79–83. doi: 10.1016/j.bbrep.2017.06.007

Dhillon, A. S., Shivaprasad, H. L., Schaberg, D., Wier, F., Weber, S., and Bandli, D. (2006). Campylobacter jejuni infection in broiler chickens. *Avian Dis.* 50, 55–58. doi: 10.1637/7411-071405R.1

Dwivedi, R., Nothaft, H., Garber, J., Xin Kin, L., Stahl, M., Flint, A., et al. (2016). L-fucose influences chemotaxis and biofilm formation in Campylobacter jejuni. *Mol. Microbiol.* 101, 575–589. doi: 10.1111/mmi.13409

El Abbar, F. M., Li, J., Owen, H. C., Daugherty, C. L., Fulmer, C. A., Bogacz, M., et al. (2019). RNA Binding by the Campylobacter jejuni Post-transcriptional Regulator CsrA. *Front. Microbiol.* 10, 1776. doi: 10.3389/fmicb.2019.01776

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D. C., Inglis, N. F., et al. (2012). Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect. Immun.* 80, 4089–4098. doi: 10.1128/IAI.00161-12

Elmi, A., Nasher, F., Jagatia, H., Gundogdu, O., Bajaj-Elliott, M., Wren, B., et al. (2016). Campylobacter jejuni outer membrane vesicle-associated proteolytic activity promotes bacterial invasion by mediating cleavage of intestinal epithelial cell E-cadherin and occludin. *Cell Microbiol.* 18, 561–572. doi: 10.1111/cmi.12534

Elmi, A., Dorey, A., Watson, E., Jagatia, H., Inglis, N. F., Gundogdu, O., et al. (2018). The bile salt sodium taurocholate induces Campylobacter jejuni outer membrane vesicle production and increases OMV-associated proteolytic activity. *Cell Microbiol.* 20. doi: 10.1111/cmi.12814

Facciola, A., Riso, R., Avventuroso, E., Visalli, G., Delia, S. A., and Lagana, P. (2017). Campylobacter: from microbiology to prevention. *J. Prev. Med. Hyg.* 58, E79–E92.

Fernando, U., Biswas, D., Allan, B., Willson, P., and Potter, A. A. (2007). Influence of Campylobacter jejuni fliA, rpoN and flgK genes on colonization of the chicken gut. *Int. J. Food Microbiol.* 118, 194–200. doi: 10.1016/j.ijfoodmicro.2007.07.038

Ferrara, L. G. M., Wallat, G. D., Moynie, L., Dhanasekar, N. N., Aliouane, S., Acosta-Gutierrez, S., et al. (2016). MOMP from Campylobacter jejuni Is a Trimer of 18-Stranded beta-Barrel Monomers with a Ca(2+) Ion Bound at the Constriction Zone. *J. Mol. Biol.* 428, 4528–4543. doi: 10.1016/j.jmb.2016.09.021

Fields, J. A., and Thompson, S. A. (2008). Campylobacter jejuni CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. *J. Bacteriol.* 190, 3411–3416. doi: 10.1128/JB.01928-07

Flanagan, R. C., Neal-Mckinney, J. M., Dhillon, A. S., Miller, W. G., and Konkel, M. E. (2009). Examination of Campylobacter jejuni putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. *Infect. Immun.* 77, 2399–2407. doi: 10.1128/IAI.01266-08

Gao, B., Vorwerk, H., Huber, C., Lara-Tejero, M., Mohr, J., Goodman, A. L., et al. (2017). Metabolic and fitness determinants for in vitro growth and intestinal colonization of the bacterial pathogen Campylobacter jejuni. *PloS Biol.* 15, e2001390. doi: 10.1371/journal.pbio.2001390

Garber, J. M., Nothaft, H., Pluvinage, B., Stahl, M., Bian, X., Porfirio, S., et al. (2020). The gastrointestinal pathogen Campylobacter jejuni metabolizes sugars with potential help from commensal Bacteroides vulgatus. *Commun. Biol.* 3, 2. doi: 10.1038/s42003-019-0727-5

Garcia-Sanchez, L., Melero, B., and Rovira, J. (2018). Campylobacter in the Food Chain. *Adv. Food Nutr. Res.* 86, 215–252. doi: 10.1016/bs.afnr.2018.04.005

Gaynor, E. C., Wells, D. H., Mackichan, J. K., and Falkow, S. (2005). The Campylobacter jejuni stringent response controls specific stress survival and virulence-associated phenotypes. *Mol. Microbiol.* 56, 8–27. doi: 10.1111/j.1365-2958.2005.04525.x

Gaytan, M. O., Martinez-Santos, V.II, Soto, E., and Gonzalez-Pedrajo, B. (2016). Type Three Secretion System in Attaching and Effacing Pathogens. *Front. Cell Infect. Microbiol.* 6, 129. doi: 10.3389/fcimb.2016.00129

Gilbert, M., Mandrell, R. E., Parker, C. T., Li, J., and Vinogradov, E. (2007). Structural analysis of the capsular polysaccharide from Campylobacter jejuni RM1221. *Chembiochem* 8, 625–631. doi: 10.1002/cbic.200600508

Goodman, K. N., Powers, M. J., Crofts, A. A., Trent, M. S., and Hendrixson, D. R. (2020). Campylobacter jejuni BumSR directs a response to butyrate via sensor phosphatase activity to impact transcription and colonization. *Proc. Natl. Acad. Sci. U. S. A.* 117, 11715–11726. doi: 10.1073/pnas.1922719117

Gormley, F. J., Bailey, R. A., Watson, K. A., Mcadam, J., Avendano, S., Stanley, W. A., et al. (2014). Campylobacter colonization and proliferation in the broiler chicken upon natural field challenge is not affected by the bird growth rate or breed. *Appl. Environ. Microbiol.* 80, 6733–6738. doi: 10.1128/AEM.02162-14

Grant, C. C., Konkel, M. E., Cieplak, W. Jr., and Tompkins, L. S. (1993). Role of flagella in adherence, internalization, and translocation of Campylobacter jejuni in nonpolarized and polarized epithelial cell cultures. *Infect. Immun.* 61, 1764–1771. doi: 10.1128/IAI.61.5.1764-1771.1993

Guccione, E., Leon-Kempis Mdel, R., Pearson, B. M., Hitchin, E., Mulholland, F., Van Diemen, P. M., et al. (2008). Amino acid-dependent growth of Campylobacter jejuni: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol. Microbiol.* 69, 77–93. doi: 10.1111/j.1365-2958.2008.06263.x

Guccione, E. J., Kendall, J. J., Hitchcock, A., Garg, N., White, M. A., Mulholland, F., et al. (2017). Transcriptome and proteome dynamics in chemostat culture reveal how Campylobacter jejuni modulates metabolism, stress responses and virulence factors upon changes in oxygen availability. *Environ. Microbiol.* 19, 4326–4348. doi: 10.1111/1462-2920.13930

Guerry, P., Alm, R. A., Power, M. E., Logan, S. M., and Trust, T. J. (1991). Role of two flagellin genes in Campylobacter motility. *J. Bacteriol.* 173, 4757–4764. doi: 10.1128/JB.173.15.4757-4764.1991

Guerry, P., Szymanski, C. M., Prendergast, M. M., Hickey, T. E., Ewing, C. P., Pattarini, D. L., et al. (2002). Phase variation of Campylobacter jejuni 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infect. Immun.* 70, 787–793. doi: 10.1128/IAI.70.2.787-793.2002

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

Gundogdu, O., Da Silva, D. T., Mohammad, B., Elmi, A., Wren, B. W., Van Vliet, A. H., et al. (2016). The Campylobacter jejuni Oxidative Stress Regulator RrpB Is Associated with a Genomic Hypervariable Region and Altered Oxidative Stress Resistance. *Front. Microbiol.* 7, 2117. doi: 10.3389/fmicb.2016.02117

Hameed, A., Woodacre, A., Machado, L. R., and Marsden, G. L. (2020). An Updated Classification System and Review of the Lipooligosaccharide Biosynthesis Gene Locus in Campylobacter jejuni. *Front. Microbiol.* 11, 677. doi: 10.3389/fmicb.2020.00677

Hansson, I., Sandberg, M., Habib, I., Lowman, R., and Engvall, E. O. (2018). Knowledge gaps in control of Campylobacter for prevention of campylobacteriosis. *Transbound. Emerg. Dis.* 65 (Suppl 1), 30–48. doi: 10.1111/tbed.12870

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. *PloS Med.* 12, e1001923. doi: 10.1371/journal.pmed.1001923

Hazeleger, W. C., Wouters, J. A., Rombouts, F. M., and Abee, T. (1998). Physiological activity of Campylobacter jejuni far below the minimal growth temperature. *Appl. Environ. Microbiol.* 64, 3917–3922. doi: 10.1128/AEM.64.10.3917-3922.1998

He, H., Cooper, J. N., Mishra, A., and Raskin, D. M. (2012). Stringent response regulation of biofilm formation in Vibrio cholerae. *J. Bacteriol.* 194, 2962–2972. doi: 10.1128/JB.00014-12

Hendrixson, D. R., and Dirita, V. J. (2003). Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in Campylobacter jejuni is associated with formation of the flagellar secretory apparatus. *Mol. Microbiol.* 50, 687–702. doi: 10.1046/j.1365-2958.2003.03731.x

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

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

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

Hofreuter, D. (2014). Defining the metabolic requirements for the growth and colonization capacity of Campylobacter jejuni. *Front. Cell. Infect. Microbiol.* 4, 137. doi: 10.3389/fcimb.2014.00137

Houliston, R. S., Vinogradov, E., Dzieciatkowska, M., Li, J., St Michael, F., Karwaski, M. F., et al. (2011). Lipooligosaccharide of Campylobacter jejuni: similarity with multiple types of mammalian glycans beyond gangliosides. *J. Biol. Chem.* 286, 12361–12370. doi: 10.1074/jbc.M110.181750

Howard, S. L., Jagannathan, A., Soo, E. C., Hui, J. P., Aubry, A. J., Ahmed, I., et al. (2009). Campylobacter Jejuni Glycosylation Island Important In Cell Charge, Legionaminic Acid Biosynthesis, And Colonization Of Chickens. *Infect. Immun.* 77, 2544–2556. doi: 10.1128/IAI.01425-08

Hudson, J. A., Nicol, C., Wright, J., Whyte, R., and Hasell, S. K. (1999). Seasonal Variation Of Campylobacter Types From Human Cases, Veterinary Cases, Raw Chicken, Milk And Water. *J. Appl. Microbiol.* 87, 115–124. doi: 10.1046/j.1365-2672.1999.00806.x

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., et al. (2014). Campylobacter Jejuni Is Not Merely A Commensal In Commercial Broiler Chickens And Affects Bird Welfare. *Mbio* 5, E01364–E01314. doi: 10.1128/mBio.01364-14

Hwang, S., Kim, M., Ryu, S., and Jeon, B. (2011). Regulation of oxidative stress response by CosR, an essential response regulator in Campylobacter jejuni. *PloS One* 6, e22300. doi: 10.1371/journal.pone.0022300

Igwaran, A., and Okoh, A. II. (2019). Human campylobacteriosis: A public health concern of global importance. *Heliyon* 5, e02814. doi: 10.1016/j.heliyon.2019.e02814

Ijaz, U. Z., Sivaloganathan, L., McKenna, A., Richmond, A., Kelly, C., Linton, M., et al. (2018). Comprehensive Longitudinal Microbiome Analysis of the Chicken Cecum Reveals a Shift from Competitive to Environmental Drivers and a Window of Opportunity for Campylobacter. *Front. Microbiol.* 9, 2452. doi: 10.3389/fmicb.2018.02452

Iovine, N. M. (2013). Resistance mechanisms in Campylobacter jejuni. *Virulence* 4, 230–240. doi: 10.4161/viru.23753

Jagannathan, A., Constantinidou, C., and Penn, C. W. (2001). Roles of rpoN, fliA, and flgR in expression of flagella in Campylobacter jejuni. *J. Bacteriol.* 183, 2937–2942. doi: 10.1128/JB.183.9.2937-2942.2001

Jervis, A. J., Butler, J. A., Lawson, A. J., Langdon, R., Wren, B. W., and Linton, D. (2012). Characterization of the structurally diverse N-linked glycans of Campylobacter species. *J. Bacteriol.* 194, 2355–2362. doi: 10.1128/JB.00042-12

Jin, S., Song, Y. C., Emili, A., Sherman, P. M., and Chan, V. L. (2003). JlpA of Campylobacter jejuni interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. *Cell Microbiol.* 5, 165–174. doi: 10.1046/j.1462-5822.2003.00265.x

John, D. A., Williams, L. K., Kanamarlapudi, V., Humphrey, T. J., and Wilkinson, T. S. (2017). The Bacterial Species Campylobacter jejuni Induce Diverse Innate Immune Responses in Human and Avian Intestinal Epithelial Cells. *Front. Microbiol.* 8, 1840. doi: 10.3389/fmicb.2017.01840

Jones, M. A., Marston, K. L., Woodall, C. A., Maskell, D. J., Linton, D., Karlyshev, A. V., et al. (2004). Adaptation of Campylobacter jejuni NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infect. Immun.* 72, 3769–3776. doi: 10.1128/IAI.72.7.3769-3776.2004

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

Kanipes, M.II, Tan, X., Akelaitis, A., Li, J., Rockabrand, D., Guerry, P., et al. (2008). Genetic analysis of lipooligosaccharide core biosynthesis in Campylobacter jejuni 81-176. *J. Bacteriol.* 190, 1568–1574. doi: 10.1128/JB.01696-07

Karlyshev, A. V., Linton, D., Gregson, N. A., Lastovica, A. J., and Wren, B. W. (2000). Genetic and biochemical evidence of a Campylobacter jejuni capsular polysaccharide that accounts for Penner serotype specificity. *Mol. Microbiol.* 35, 529–541. doi: 10.1046/j.1365-2958.2000.01717.x

Karlyshev, A. V., Mccrossan, M. V., and Wren, B. W. (2001). Demonstration of polysaccharide capsule in Campylobacter jejuni using electron microscopy. *Infect. Immun.* 69, 5921–5924. doi: 10.1128/IAI.69.9.5921-5924.2001

Karlyshev, A. V., Champion, O. L., Churcher, C., Brisson, J. R., Jarrell, H. C., Gilbert, M., et al. (2005). Analysis of Campylobacter jejuni capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol. Microbiol.* 55, 90–103. doi: 10.1111/j.1365-2958.2004.04374.x

Keo, T., Collins, J., Kunwar, P., Blaser, M. J., and Iovine, N. M. (2011). Campylobacter capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. *Virulence* 2, 30–40. doi: 10.4161/viru.2.1.14752

Kervella, M., Pages, J. M., Pei, Z., Grollier, G., Blaser, M. J., and Fauchere, J. L. (1993). Isolation and characterization of two Campylobacter glycine-extracted proteins that bind to HeLa cell membranes. *Infect. Immun.* 61, 3440–3448. doi: 10.1128/IAI.61.8.3440-3448.1993

Keto-Timonen, R., Hietala, N., Palonen, E., Hakakorpi, A., Lindstrom, M., and Korkeala, H. (2016). Cold Shock Proteins: A Minireview with Special Emphasis on Csp-family of Enteropathogenic Yersinia. *Front. Microbiol.* 7, 1151. doi: 10.3389/fmicb.2016.01151

Kim, J. C., Oh, E., Hwang, S., Ryu, S., and Jeon, B. (2015a). Non-selective regulation of peroxide and superoxide resistance genes by PerR in Campylobacter jejuni. *Front. Microbiol.* 6, 126. doi: 10.3389/fmicb.2015.00126

Kim, J. C., Oh, E., Kim, J., and Jeon, B. (2015b). Regulation of oxidative stress resistance in Campylobacter jejuni, a microaerophilic foodborne pathogen. *Front. Microbiol.* 6, 751. doi: 10.3389/fmicb.2015.00751

Konkel, M. E., Kim, B. J., Rivera-Amill, V., and Garvis, S. G. (1999). Bacterial secreted proteins are required for the internaliztion of Campylobacter jejuni into cultured mammalian cells. *Mol. Microbiol.* 32, 691–701. doi: 10.1046/j.1365-2958.1999.01376.x

Konkel, M. E., Klena, J. D., Rivera-Amill, V., Monteville, M. R., Biswas, D., Raphael, B., et al. (2004). Secretion of virulence proteins from Campylobacter jejuni is dependent on a functional flagellar export apparatus. *J. Bacteriol.* 186, 3296–3303. doi: 10.1128/JB.186.11.3296-3303.2004

Konkel, M. E., Christensen, J. E., Keech, A. M., Monteville, M. R., Klena, J. D., and Garvis, S. G. (2005). Identification of a fibronectin-binding domain within the Campylobacter jejuni CadF protein. *Mol. Microbiol.* 57, 1022–1035. doi: 10.1111/j.1365-2958.2005.04744.x

Konkel, M. E., Larson, C. L., and Flanagan, R. C. (2010). Campylobacter jejuni FlpA binds fibronectin and is required for maximal host cell adherence. *J. Bacteriol.* 192, 68–76. doi: 10.1128/JB.00969-09

Konkel, M. E., Talukdar, P. K., Negretti, N. M., and Klappenbach, C. M. (2020). Taking Control: Campylobacter jejuni Binding to Fibronectin Sets the Stage for Cellular Adherence and Invasion. *Front. Microbiol.* 11, 564. doi: 10.3389/fmicb.2020.00564

Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C., and Robinson, R. A. (1985). A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J. Infect. Dis.* 152, 592–596. doi: 10.1093/infdis/152.3.592

Kreutzberger, M. A. B., Ewing, C., Poly, F., Wang, F., and Egelman, E. H. (2020). Atomic structure of the Campylobacter jejuni flagellar filament reveals how epsilon Proteobacteria escaped Toll-like receptor 5 surveillance. *Proc. Natl. Acad. Sci. U.S.A.* 117, 16985–16991. doi: 10.1073/pnas.2010996117

Larson, C. L., Samuelson, D. R., Eucker, T. P., O'loughlin, J. L., and Konkel, M. E. (2013). The fibronectin-binding motif within FlpA facilitates Campylobacter jejuni adherence to host cell and activation of host cell signaling. *Emerg. Microbes Infect.* 2, e65. doi: 10.1038/emi.2013.65

Le, M. T., Porcelli, I., Weight, C. M., Gaskin, D. J., Carding, S. R., and Van Vliet, A. H. (2012). Acid-shock of Campylobacter jejuni induces flagellar gene expression and host cell invasion. *Eur. J. Microbiol. Immunol. (Bp)* 2, 12–19. doi: 10.1556/EuJMI.2.2012.1.3

Leon-Kempis Mdel, R., Guccione, E., Mulholland, F., Williamson, M. P., and Kelly, D. J. (2006). The Campylobacter jejuni PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids. *Mol. Microbiol.* 60, 1262–1275. doi: 10.1111/j.1365-2958.2006.05168.x

Liaw, J., Hong, G., Davies, C., Elmi, A., Sima, F., Stratakos, A., et al. (2019). The Campylobacter jejuni Type VI Secretion System Enhances the Oxidative Stress Response and Host Colonization. *Front. Microbiol.* 10, 2864. doi: 10.3389/fmicb.2019.02864

Linton, D., Dorrell, N., Hitchen, P. G., Amber, S., Karlyshev, A. V., Morris, H. R., et al. (2005). Functional analysis of the Campylobacter jejuni N-linked protein glycosylation pathway. *Mol. Microbiol.* 55, 1695–1703. doi: 10.1111/j.1365-2958.2005.04519.x

Logan, S. M., Hui, J. P., Vinogradov, E., Aubry, A. J., Melanson, J. E., Kelly, J. F., et al. (2009). Identification of novel carbohydrate modifications on Campylobacter jejuni 11168 flagellin using metabolomics-based approaches. *FEBS J.* 276, 1014–1023. doi: 10.1111/j.1742-4658.2008.06840.x

Luethy, P. M., Huynh, S., Ribardo, D. A., Winter, S. E., Parker, C. T., and Hendrixson, D. R. (2017). Microbiota-Derived Short-Chain Fatty Acids Modulate Expression of Campylobacter jejuni Determinants Required for Commensalism and Virulence. *mBio* 8. doi: 10.1128/mBio.00407-17

Maccallum, A., Haddock, G., and Everest, P. H. (2005). Campylobacter jejuni activates mitogen-activated protein kinases in Caco-2 cell monolayers and in vitro infected primary human colonic tissue. *Microbiol. (Reading)* 151, 2765–2772. doi: 10.1099/mic.0.27979-0

Magajna, B., and Schraft, H. (2015). Evaluation of Propidium Monoazide and Quantitative PCR To Quantify Viable Campylobacter jejuni Biofilm and Planktonic Cells in Log Phase and in a Viable but Nonculturable State. *J. Food Prot.* 78, 1303–1311. doi: 10.4315/0362-028X.JFP-14-583

Marchant, J., Wren, B., and Ketley, J. (2002). Exploiting genome sequence: predictions for mechanisms of Campylobacter chemotaxis. *Trends Microbiol.* 10, 155–159. doi: 10.1016/S0966-842X(02)02323-5

Mcnally, D. J., Jarrell, H. C., Li, J., Khieu, N. H., Vinogradov, E., Szymanski, C. M., et al. (2005). The HS:1 serostrain of Campylobacter jejuni has a complex teichoic acid-like capsular polysaccharide with nonstoichiometric fructofuranose branches and O-methyl phosphoramidate groups. *FEBS J.* 272, 4407–4422. doi: 10.1111/j.1742-4658.2005.04856.x

McKenna, A., Ijaz, U. Z., Kelly, C., Linton, M., Sloan, W. T., Green, B. D., et al. (2020). Impact of industrial production system parameters on chicken microbiomes: mechanisms to improve performance and reduce Campylobacter. *Microbiome* 8, 128. doi: 10.1186/s40168-020-00908-8

Mcnally, D. J., Lamoureux, M. P., Karlyshev, A. V., Fiori, L. M., Li, J., Thacker, G., et al. (2007). Commonality and biosynthesis of the O-methyl phosphoramidate capsule modification in Campylobacter jejuni. *J. Biol. Chem.* 282, 28566–28576. doi: 10.1074/jbc.M704413200

Melo, R. T., Mendonca, E. P., Monteiro, G. P., Siqueira, M. C., Pereira, C. B., Peres, P., et al. (2017). Intrinsic and Extrinsic Aspects on Campylobacter jejuni Biofilms. *Front. Microbiol.* 8, 1332. doi: 10.3389/fmicb.2017.01332

Muraoka, W. T., and Zhang, Q. (2011). Phenotypic and genotypic evidence for L-fucose utilization by Campylobacter jejuni. *J. Bacteriol.* 193, 1065–1075. doi: 10.1128/JB.01252-10

Murphy, C., Carroll, C., and Jordan, K. N. (2006). Environmental survival mechanisms of the foodborne pathogen Campylobacter jejuni. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Nachamkin, I., Allos, B. M., and Ho, T. (1998). Campylobacter species and Guillain-Barre syndrome. *Clin. Microbiol. Rev.* 11, 555–567. doi: 10.1128/CMR.11.3.555

Neal-Mckinney, J. M., and Konkel, M. E. (2012). The Campylobacter jejuni CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells. *Front. Cell Infect. Microbiol.* 2, 31. doi: 10.3389/fcimb.2012.00031

Novik, V., Hofreuter, D., and Galan, J. E. (2010). Identification of Campylobacter jejuni genes involved in its interaction with epithelial cells. *Infect. Immun.* 78, 3540–3553. doi: 10.1128/IAI.00109-10

Nuijten, P. J., Van Asten, F. J., Gaastra, W., and Van Der Zeijst, B. A. (1990). Structural and functional analysis of two Campylobacter jejuni flagellin genes. *J. Biol. Chem.* 265, 17798–17804. doi: 10.1016/S0021-9258(18)38234-6

Nunoshiba, T., Hidalgo, E., Amabile Cuevas, C. F., and Demple, B. (1992). Two-stage control of an oxidative stress regulon: the Escherichia coli SoxR protein triggers redox-inducible expression of the soxS regulatory gene. *J. Bacteriol.* 174, 6054–6060. doi: 10.1128/JB.174.19.6054-6060.1992

Oberheim, J., Hoser, C., Luchters, G., and Kistemann, T. (2020). Small-scaled association between ambient temperature and campylobacteriosis incidence in Germany. *Sci. Rep.* 10, 17191. doi: 10.1038/s41598-020-73865-9

Oh, E., and Jeon, B. (2014). Role of alkyl hydroperoxide reductase (AhpC) in the biofilm formation of Campylobacter jejuni. *PloS One* 9, e87312. doi: 10.1371/journal.pone.0087312

Oh, E., Andrews, K. J., Mcmullen, L. M., and Jeon, B. (2019). Tolerance to stress conditions associated with food safety in Campylobacter jejuni strains isolated from retail raw chicken. *Sci. Rep.* 9, 11915. doi: 10.1038/s41598-019-48373-0

Palyada, K., Sun, Y. Q., Flint, A., Butcher, J., Naikare, H., and Stintzi, A. (2009). Characterization of the oxidative stress stimulon and PerR regulon of Campylobacter jejuni. *BMC Genomics* 10, 481. doi: 10.1186/1471-2164-10-481

Park, D., Lara-Tejero, M., Waxham, M. N., Li, W., Hu, B., Galan, J. E., et al. (2018). Visualization of the type III secretion mediated Salmonella-host cell interface using cryo-electron tomography. *Elife* 7. doi: 10.7554/eLife.39514.018

Parker, C. T., Horn, S. T., Gilbert, M., Miller, W. G., Woodward, D. L., and Mandrell, R. E. (2005). Comparison of Campylobacter jejuni lipooligosaccharide biosynthesis loci from a variety of sources. *J. Clin. Microbiol.* 43, 2771–2781. doi: 10.1128/JCM.43.6.2771-2781.2005

Parker, C. T., Gilbert, M., Yuki, N., Endtz, H. P., and Mandrell, R. E. (2008). Characterization of lipooligosaccharide-biosynthetic loci of Campylobacter jejuni reveals new lipooligosaccharide classes: evidence of mosaic organizations. *J. Bacteriol.* 190, 5681–5689. doi: 10.1128/JB.00254-08

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X. Z., Kopecko, D. J., et al. (1998). Mutation in the peb1A locus of Campylobacter jejuni reduces interactions with epithelial cells and intestinal colonization of mice. *Infect. Immun.* 66, 938–943. doi: 10.1128/IAI.66.3.938-943.1998

Poly, F., Serichantalergs, O., Kuroiwa, J., Pootong, P., Mason, C., Guerry, P., et al. (2015). Updated Campylobacter jejuni Capsule PCR Multiplex Typing System and Its Application to Clinical Isolates from South and Southeast Asia. *PloS One* 10, e0144349. doi: 10.1371/journal.pone.0144349

Purdy, D., Buswell, C. M., Hodgson, A. E., Mc, A. K., Henderson, I., and Leach, S. A. (2000). Characterisation of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni. *J. Med. Microbiol.* 49, 473–479. doi: 10.1099/0022-1317-49-5-473

Reeser, R. J., Medler, R. T., Billington, S. J., Jost, B. H., and Joens, L. A. (2007). Characterization of Campylobacter jejuni biofilms under defined growth conditions. *Appl. Environ. Microbiol.* 73, 1908–1913. doi: 10.1128/AEM.00740-06

Reid, A. N., Pandey, R., Palyada, K., Whitworth, L., Doukhanine, E., and Stintzi, A. (2008). Identification of Campylobacter jejuni genes contributing to acid adaptation by transcriptional profiling and genome-wide mutagenesis. *Appl. Environ. Microbiol.* 74, 1598–1612. doi: 10.1128/AEM.01508-07

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

Rodrigues, R. C., Pocheron, A. L., Hernould, M., Haddad, N., Tresse, O., and Cappelier, J. M. (2015). Description of Campylobacter jejuni Bf, an atypical aero-tolerant strain. *Gut Pathog.* 7, 30. doi: 10.1186/s13099-015-0077-x

Samuelson, D. R., Eucker, T. P., Bell, J. A., Dybas, L., Mansfield, L. S., and Konkel, M. E. (2013). The Campylobacter jejuni CiaD effector protein activates MAP kinase signaling pathways and is required for the development of disease. *Cell Commun. Signal* 11, 79. doi: 10.1186/1478-811X-11-79

Schirm, M., Schoenhofen, I. C., Logan, S. M., Waldron, K. C., and Thibault, P. (2005). Identification of unusual bacterial glycosylation by tandem mass spectrometry analyses of intact proteins. *Anal. Chem.* 77, 7774–7782. doi: 10.1021/ac051316y

Schoenhofen, I. C., Vinogradov, E., Whitfield, D. M., Brisson, J. R., and Logan, S. M. (2009). The CMP-legionaminic acid pathway in Campylobacter: biosynthesis involving novel GDP-linked precursors. *Glycobiology* 19, 715–725. doi: 10.1093/glycob/cwp039

Sheikh, K. A., Ho, T. W., Nachamkin, I., Li, C. Y., Cornblath, D. R., Asbury, A. K., et al. (1998a). Molecular mimicry in Guillain-Barre syndrome. *Ann. N. Y. Acad. Sci.* 845, 307–321. doi: 10.1111/j.1749-6632.1998.tb09683.x

Sheikh, K. A., Nachamkin, I., Ho, T. W., Willison, H. J., Veitch, J., Ung, H., et al. (1998b). Campylobacter jejuni lipopolysaccharides in Guillain-Barre syndrome: molecular mimicry and host susceptibility. *Neurology* 51, 371–378. doi: 10.1212/WNL.51.2.371

Sheppard, S. K., Didelot, X., Meric, G., Torralbo, A., Jolley, K. A., Kelly, D. J., et al. (2013). Genome-wide association study identifies vitamin B5 biosynthesis as a host specificity factor in Campylobacter. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11923–11927. doi: 10.1073/pnas.1305559110

Skirrow, M. B. (1977). Campylobacter enteritis: a "new" disease. *Br. Med. J.* 2, 9–11. doi: 10.1136/bmj.2.6078.9

Skirrow, M. B. (2006). John McFadyean and the centenary of the first isolation of Campylobacter species. *Clin. Infect. Dis.* 43, 1213–1217. doi: 10.1086/508201

Song, Y. C., Jin, S., Louie, H., Ng, D., Lau, R., Zhang, Y., et al. (2004). FlaC, a protein of Campylobacter jejuni TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. *Mol. Microbiol.* 53, 541–553. doi: 10.1111/j.1365-2958.2004.04175.x

Song, W. S., Jeon, Y. J., Namgung, B., Hong, M., and Yoon, S.II (2017). A conserved TLR5 binding and activation hot spot on flagellin. *Sci. Rep.* 7, 40878. doi: 10.1038/srep40878

Stahl, M., Friis, L. M., Nothaft, H., Liu, X., Li, J., Szymanski, C. M., et al. (2011). L-fucose utilization provides Campylobacter jejuni with a competitive advantage. *Proc. Natl. Acad. Sci. U. S. A.* 108, 7194–7199. doi: 10.1073/pnas.1014125108

Stahl, M., Butcher, J., and Stintzi, A. (2012). Nutrient acquisition and metabolism by Campylobacter jejuni. *Front. Cell. Infect. Microbiol.* 2, 5. doi: 10.3389/fcimb.2012.00005

Stintzi, A., Marlow, D., Palyada, K., Naikare, H., Panciera, R., Whitworth, L., et al. (2005). Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of Campylobacter jejuni. *Infect. Immun.* 73, 1797–1810. doi: 10.1128/IAI.73.3.1797-1810.2005

Svensson, S. L., Pryjma, M., and Gaynor, E. C. (2014). Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of Campylobacter jejuni. *PloS One* 9, e106063. doi: 10.1371/journal.pone.0106063

Svensson, S. L., Huynh, S., Parker, C. T., and Gaynor, E. C. (2015). The Campylobacter jejuni CprRS two-component regulatory system regulates aspects of the cell envelope. *Mol. Microbiol.* 96, 189–209. doi: 10.1111/mmi.12927

Szymanski, C. M., King, M., Haardt, M., and Armstrong, G. D. (1995). Campylobacter jejuni motility and invasion of Caco-2 cells. *Infect. Immun.* 63, 4295–4300. doi: 10.1128/IAI.63.11.4295-4300.1995

Szymanski, C. M., Yao, R., Ewing, C. P., Trust, T. J., and Guerry, P. (1999). Evidence for a system of general protein glycosylation in Campylobacter jejuni. *Mol. Microbiol.* 32, 1022–1030. doi: 10.1046/j.1365-2958.1999.01415.x

Szymanski, C. M. (2015). Are campylobacters now capable of carbo-loading? *Mol. Microbiol.* 98, 805–808. doi: 10.1111/mmi.13162

Talukdar, P. K., Negretti, N. M., Turner, K. L., and Konkel, M. E. (2020). Molecular Dissection of the Campylobacter jejuni CadF and FlpA Virulence Proteins in Binding to Host Cell Fibronectin. *Microorganisms* 8. doi: 10.3390/microorganisms8030389

Teh, A. H., Lee, S. M., and Dykes, G. A. (2014). Does Campylobacter jejuni form biofilms in food-related environments? *Appl. Environ. Microbiol.* 80, 5154–5160. doi: 10.1128/AEM.01493-14

Teh, A. H. T., Lee, S. M., and Dykes, G. A. (2019). Association of some Campylobacter jejuni with Pseudomonas aeruginosa biofilms increases attachment under conditions mimicking those in the environment. *PloS One* 14, e0215275. doi: 10.1371/journal.pone.0215275

Thibault, P., Logan, S. M., Kelly, J. F., Brisson, J. R., Ewing, C. P., Trust, T. J., et al. (2001). Identification of the carbohydrate moieties and glycosylation motifs in Campylobacter jejuni flagellin. *J. Biol. Chem.* 276, 34862–34870. doi: 10.1074/jbc.M104529200

Thompson, S. A., and Gaynor, E. C. (2008). Campylobacter jejuni host tissue tropism: a consequence of its low-carb lifestyle? *Cell Host Microbe* 4, 409–410. doi: 10.1016/j.chom.2008.10.010

Thota, V. N., Ferguson, M. J., Sweeney, R. P., and Lowary, T. L. (2018). Synthesis of the Campylobacter jejuni 81-176 Strain Capsular Polysaccharide Repeating Unit Reveals the Absolute Configuration of its O-Methyl Phosphoramidate Motif. *Angew. Chem. Int. Ed. Engl.* 57, 15592–15596. doi: 10.1002/anie.201810222

Turonova, H., Briandet, R., Rodrigues, R., Hernould, M., Hayek, N., Stintzi, A., et al. (2015). Biofilm spatial organization by the emerging pathogen Campylobacter jejuni: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions. *Front. Microbiol.* 6, 709. doi: 10.3389/fmicb.2015.00709

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

Van Der Hooft, J. J. J., Alghefari, W., Watson, E., Everest, P., Morton, F. R., Burgess, K. E. V., et al. (2018). Unexpected differential metabolic responses of Campylobacter jejuni to the abundant presence of glutamate and fucose. *Metabolomics* 14, 144. doi: 10.1007/s11306-018-1438-5

Van Der Stel, A. X., and Wosten, M. (2019). Regulation of Respiratory Pathways in Campylobacterota: A Review. *Front. Microbiol.* 10, 1719. doi: 10.3389/fmicb.2019.01719

Vegge, C. S., Jansen Van Rensburg, M. J., Rasmussen, J. J., Maiden, M. C., Johnsen, L. G., Danielsen, M., et al. (2016). Glucose Metabolism via the Entner-Doudoroff Pathway in Campylobacter: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates. *Front. Microbiol.* 7, 1877. doi: 10.3389/fmicb.2016.01877

Velayudhan, J., Jones, M. A., Barrow, P. A., and Kelly, D. J. (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by Campylobacter jejuni. *Infect. Immun.* 72, 260–268. doi: 10.1128/IAI.72.1.260-268.2004

Wassenaar, T. M., Bleumink-Pluym, N. M., and Van Der Zeijst, B. A. (1991). Inactivation of Campylobacter jejuni flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. *EMBO J.* 10, 2055–2061. doi: 10.1002/j.1460-2075.1991.tb07736.x

Wassenaar, T. M., Van Der Zeijst, B. A., Ayling, R., and Newell, D. G. (1993). Colonization of chicks by motility mutants of Campylobacter jejuni demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* 139 (Pt 6), 1171–1175. doi: 10.1099/00221287-139-6-1171

Watson, R. O., and Galan, J. E. (2005). Signal transduction in Campylobacter jejuni-induced cytokine production. *Cell Microbiol.* 7, 655–665. doi: 10.1111/j.1462-5822.2004.00498.x

Wilkinson, D. A., O'donnell, A. J., Akhter, R. N., Fayaz, A., Mack, H. J., Rogers, L. E., et al. (2018). Updating the genomic taxonomy and epidemiology of Campylobacter hyointestinalis. *Sci. Rep.* 8, 2393. doi: 10.1038/s41598-018-20889-x

Woodall, C. A., Jones, M. A., Barrow, P. A., Hinds, J., Marsden, G. L., Kelly, D. J., et al. (2005). Campylobacter jejuni gene expression in the chick cecum: evidence for adaptation to a low-oxygen environment. *Infect. Immun.* 73, 5278–5285. doi: 10.1128/IAI.73.8.5278-5285.2005

Wouters, J. A., Rombouts, F. M., Kuipers, O. P., De Vos, W. M., and Abee, T. (2000). The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. *Syst. Appl. Microbiol.* 23, 165–173. doi: 10.1016/S0723-2020(00)80001-6

Yao, R., Burr, D. H., Doig, P., Trust, T. J., Niu, H., and Guerry, P. (1994). Isolation of motile and non-motile insertional mutants of Campylobacter jejuni: the role of motility in adherence and invasion of eukaryotic cells. *Mol. Microbiol.* 14, 883–893. doi: 10.1111/j.1365-2958.1994.tb01324.x

Young, N. M., Brisson, J. R., Kelly, J., Watson, D. C., Tessier, L., Lanthier, P. H., et al. (2002). Structure of the N-linked glycan present on multiple glycoproteins in the Gram-negative bacterium, Campylobacter jejuni. *J. Biol. Chem.* 277, 42530–42539. doi: 10.1074/jbc.M206114200

Yuki, N., Taki, T., Inagaki, F., Kasama, T., Takahashi, M., Saito, K., et al. (1993). A bacterium lipopolysaccharide that elicits Guillain-Barre syndrome has a GM1 ganglioside-like structure. *J. Exp. Med.* 178, 1771–1775. doi: 10.1084/jem.178.5.1771

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279, 1718–1721. doi: 10.1126/science.279.5357.1718

Zilbauer, M., Dorrell, N., Boughan, P. K., Harris, A., Wren, B. W., Klein, N. J., et al. (2005). Intestinal innate immunity to Campylobacter jejuni results in induction of bactericidal human beta-defensins 2 and 3. *Infect. Immun.* 73, 7281–7289. doi: 10.1128/IAI.73.11.7281-7289.2005

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Review on Stress Tolerance in *Campylobacter jejuni*

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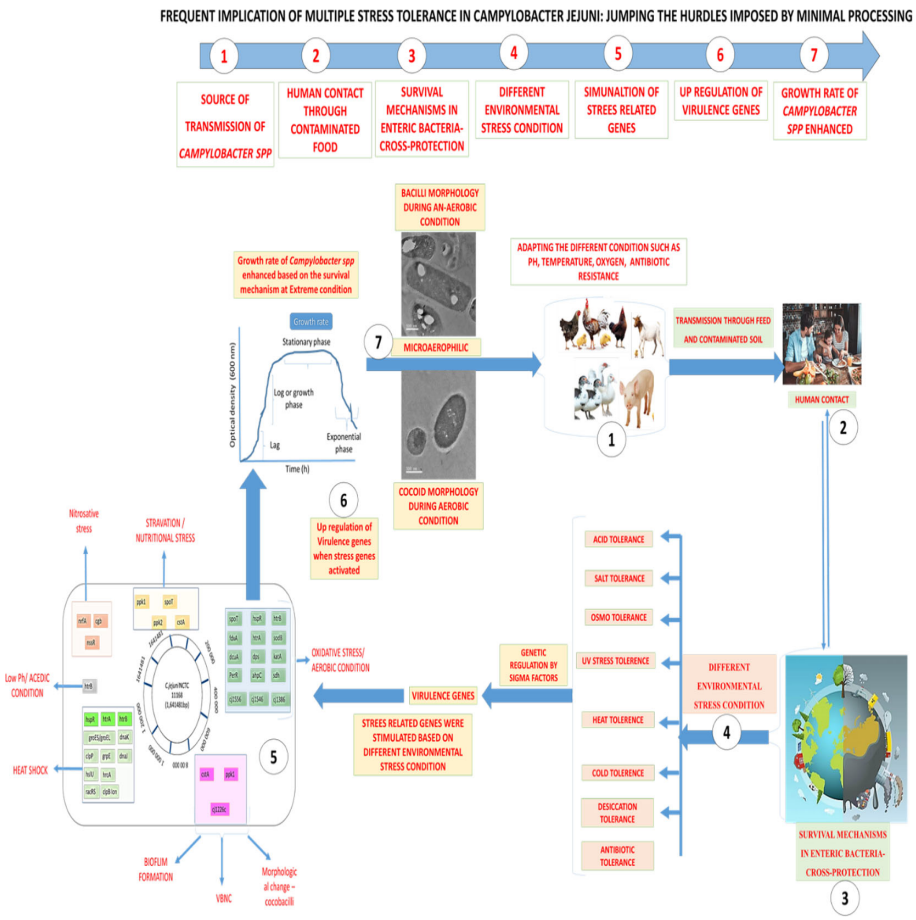
Campylobacter spp. are the leading global cause of bacterial colon infections in humans. Enteropathogens are subjected to several stress conditions in the host colon, food complexes, and the environment. Species of the genus *Campylobacter*, in collective interactions with certain enteropathogens, can manage and survive such stress conditions. The stress-adaptation mechanisms of *Campylobacter* spp. diverge from other enteropathogenic bacteria, such as *Escherichia coli*, *Salmonella enterica* serovar Typhi, *S. enterica* ser. Paratyphi, *S. enterica* ser. Typhimurium, and species of the genera *Klebsiella* and *Shigella*. This review summarizes the different mechanisms of various stress-adaptive factors on the basis of species diversity in *Campylobacter*, including their response to various stress conditions that enhance their ability to survive on different types of food and in adverse environmental conditions. Understanding how these stress adaptation mechanisms in *Campylobacter*, and other enteric bacteria, are used to overcome various challenging environments facilitates the fight against resistance mechanisms in *Campylobacter* spp., and aids the development of novel therapeutics to control *Campylobacter* in both veterinary and human populations.

Keywords: *Campylobacter*, stress, resistance mechanisms, stress adaptation, enteric bacteria



GRAPHICAL ABSTRACT | Overall flow chart on Stress Tolerance in Campylobacter jejuni at different environment condition 1. Source of transmission of Campylobacter species, 2. Human contact and throughout contaminated food, 3. Survival mechanism in entric-bacterial pathogens, 4. Differental environmental adverse condition, 5. Stimulation of stress related genes involved in sustainability, 6. Up regulation (over expression) of virulence genes, 7. Growth rate and survival of Campylobacter species enhanced.

INTRODUCTION

Campylobacter are a Gram-negative, slender, microaerophilic bacteria with a spiral or curved shape (0.2–0.8 mm × 0.5–5 mm). All species of the genus *Campylobacter*, with the exception of *Campylobacter gracilis* (nonmotile) and *Campylobacter showae* (peritrichous flagella), have a single, polar, unsheathed flagellum on one or both sides of the cell. Infection with *Campylobacter* in humans predominantly occurs through handling and ingestion of *Campylobacter*-contaminated raw or undercooked meat, raw milk, tap water, chicken salad, and various chicken-containing dishes (Zhao et al., 2003; Jang et al., 2007; Pedersen et al., 2018; Ovesen et al., 2019; The et al., 2019) as illustrated in **Figure 1**. Most *Campylobacter* infections involve a mild and self-limiting gastroenteritis, with one to three days of fever and vomiting, followed by abdominal pain with watery or bloody diarrhea for three to seven days (Negretti et al., 2019).

The species *Campylobacter jejuni* is a zoonotic pathogen that frequently causes acute gastrointestinal infections in humans when undercooked or raw meat or other products are consumed. Fever, vomiting, abdominal pain, and diarrhea are the prevalent symptoms of campylobacteriosis (Altekruse et al., 1999; Gaynor et al., 2005). In some cases, *C. jejuni* is associated with bacteremia and several post-infectious complications in humans, including immunoreactions and chronic and life-threatening paralysis, such as Guillain–Barré syndrome (GBS) and Miller Fisher syndrome (MFS) (Humphrey et al., 2007; EFSA, 2011).

C. jejuni possesses novel regulatory factors for stress resistance that enable the organism to cause foodborne infections (CDC, 2013). In most pathogens, sigma factor RpoS plays a key role in the stress-resistance mechanisms, but this factor has been reported to be absent in *C. jejuni* (Allen et al., 2018; Cain et al., 2019). *Campylobacter* is a foodborne pathogen with high incidence with norovirus, enteropathogenic *Escherichia coli*, and *Salmonella* in South Korea (Kim et al., 2017; Wang et al., 2020).

The prevalence of thermophilic *Campylobacter* for poultry is *C. jejuni* (6.3%), *C. upsaliensis* (5.9%), and *C. coli* (0.7%). Globaly 20.9% *C. jejuni* are resistant to (fluoro)quinolones. Poultry

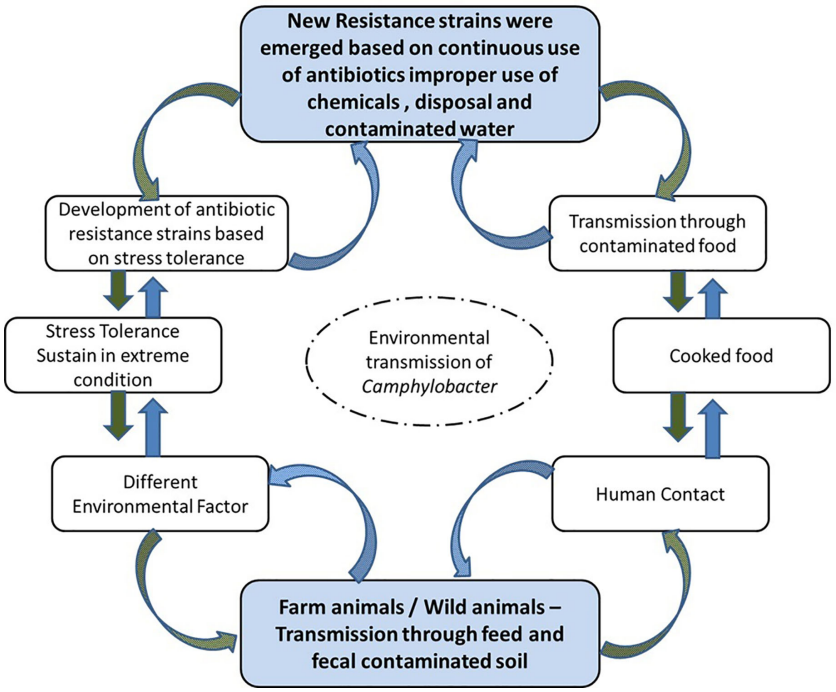


FIGURE 1 | Modes of transmission for *C. jejuni*.

become colonized shortly after birth; commercial broilers are often particularly colonized with *C. jejuni* (EFSA, 2010), the major transmission of *C. jejuni* occurs in small intestinal crypts of poultry within 24 hours (Coward et al., 2008). *Campylobacter* can reach densities as high as 1×10^8 colony-forming units (CFU/g) in the infected bird's intestinal mucosa are asymptomatic (Meade et al., 2009). *C. jejuni* spreads to a small intestine of the gastrointestinal tract, sometimes asymptomatically, after human consumption. The onset of illness is affected by the immune status of the host and the virulence of the *Campylobacter* strain.

The pathogenesis of *C. jejuni* foodborne illness involves adhesions, gut-wall invasion, colonization, and ultimately the release of toxins (Bang et al., 2003; Bolton, 2015; Pedersen et al., 2018). Motility of this pathogen is a key factor influencing colonization and survival in the acidic gut environment (Guerry, 2007; Mehat et al., 2018; Negretti et al., 2019). Flagella-oriented genes such as *flaA* and *flaB*, and *fliF*, *fliM*, and *fliY* are associated with motility-engaged *C. jejuni* (Nachamkin et al., 1993; Wassenaar et al., 1993; Carrillo et al., 2004; Sommerlad and Hendrixson, 2007; Lertsethtakarn et al., 2011). Some Gram-negative bacteria secrete a cytolethal distending toxin (CDT) heat-labile exotoxin and able to induce the distension and death of eukaryotic cells, and this has been demonstrated in *Campylobacter* (Bolton, 2015; Scuron et al., 2016; Pedersen et al., 2018; El-Tawab et al., 2019), which synthesizes this toxin using the genes *cdtA*, *cdtB*, and *cdtC* (Linton et al., 2000; Asakura et al., 2007; Wieczorek et al., 2018). Motility, adherence, invasion, and toxin production are required for cell lysis (Bang et al., 2003). The flagellar guidance of the motility scheme and a chemosensory mechanism that activates flagellar motion result in transmission from the environment to the small bowel (O'Sullivan et al., 2018). *Campylobacter* has extraordinary motility, particularly in gelatinous or viscous material, as indicated by its single or bipolar flagella and helical filamentous structures. The polar flagellum delivers driving torque and rotating metabolic signals, while corkscrew rotation is possible due to the helical form (Ferrero and Lee, 1988). Mucins and glycoproteins, the predominant components of mucus, are the primary chemical attractants during propagation (Hugdahl et al., 1988; Wadhams and Armitage, 2004; Wuichet et al., 2007; Ellström et al., 2016). Iron acquisition also plays a key role in infection with *Campylobacter* (Baillon et al., 1999; Bang et al., 2003; Eucker and Konkel, 2012).

The purpose of this review was to examine the mechanisms that enable *Campylobacter* spp. to survive outside the host environment and remain a threat to public health. A summary of specific survival-based resistance genes is also provided. This information helps identify future pathways to eradicate and control outbreaks of *C. jejuni*.

GENERAL SURVIVAL MECHANISMS IN ENTERIC BACTERIA: MICRO-ORGANISM CROSS-PROTECTION

An extraordinary characteristic of bacteria is their ability to tolerate extreme environmental conditions or stressors. They

not only tolerate ecological stress, but also adapt to different situations such as pressure, temperature, acidity, ultraviolet radiation, dehydration, susceptibility to antibiotics, and salinity. These characteristics raise some questions. Why and how do microbes in these environments survive? What biological mechanisms can we observe from these unique lifestyles? How can we use our understanding or resources to address these conditions, such as pH or temperature, to enhance or slow the growth of microbes?

Micro-organisms commonly face stress or shock during food processing (Ma et al., 2014). Microbes can survive in stressful or adverse environments, and can then tolerate other comparable stressors following the initial stress conditions (Isohanni et al., 2013). Cross-protection capabilities have been identified in *Salmonella* spp., *E. coli*, *Listeria monocytogenes*, and *Cronobacter sakazakii* (Kim et al., 2012; Spector and Kenyon, 2012; Lapierre et al., 2016; Wieczorek et al., 2018). For *C. jejuni*, a higher resistance to stress was observed following exposure to previous stressful environments. *C. jejuni* displayed tolerance or resistance to acid due to acquaintance with acid-aerobic, acidic, and nutrition-deprived stress (Oh et al., 2017), as well as showing oxidative stress cross-protection resulting from acid disturbance (Xu et al., 2019). However, Isohanni and Lyhs (Isohanni et al., 2013) stated that after exposure to heat and cold, *C. jejuni* did not have any cross-protection capacity, as shown in **Figure 2**.

Evidence indicates that antimicrobial agents are not used or are used incorrectly for the production of resistance *Campylobacter* spp. (Pedersen et al., 2018). Patients generally recover from campylobacteriosis without antimicrobial therapy, with treatment based on electrolyte substitution and rehydration. Severe cases can be managed with antibiotics such as tetracycline and macrolides (fluoro) or quinolones, but increases in antibiotic resistance in *C. jejuni* and *C. coli* has jeopardized the effectiveness of these therapeutics (Alfredson and Korolik, 2007; Bolinger et al., 2018).

Early in the food supply chain, *C. jejuni* is exposed to oxidative and desiccation stresses. *Campylobacter* are especially susceptible to the former as a processing technique (Humphrey et al., 1995), and in slaughter facilities, survival of *Campylobacter* in pig, and chicken meat decreases significantly by air-chill-drying the carcass surface (Oosterom et al., 1983). No comparable technique is used during the processing of poultry, and the chilling method initiates the formation of a moist surface that helps bacteria thrive (Butzler and Oosterom, 1991). Due to incomplete oxygen reduction, aerobic respiration generates reactive oxygen species (ROS), including superoxide anions (O_{2-2}) and hydrogen peroxide (H_2O_2), which can lead to the formation of the extremely poisonous hydroxyl radical (HO). *Campylobacter* in the chicken or human body can also be subjected to H_2O_2 or O_2 by the immune system to kill the microbes (Melo et al., 2019). The range of enzymes such as catalase, glutathione, cytochrome, peroxidases, peroxiredoxin alkyl hydroperoxide reductase, superoxide dismutase, and other peroxiredoxins are activated in Campylobacter exposed to ROS and these then facilitate long-term aerobic adaptation of the bacteria (Storz and Imlay, 1999) to facilitate long-term aerobic adaptation (Jones et al., 1993; Klancnik et al., 2009). *C. jejuni* has one catalase, KatA, which supports this process when

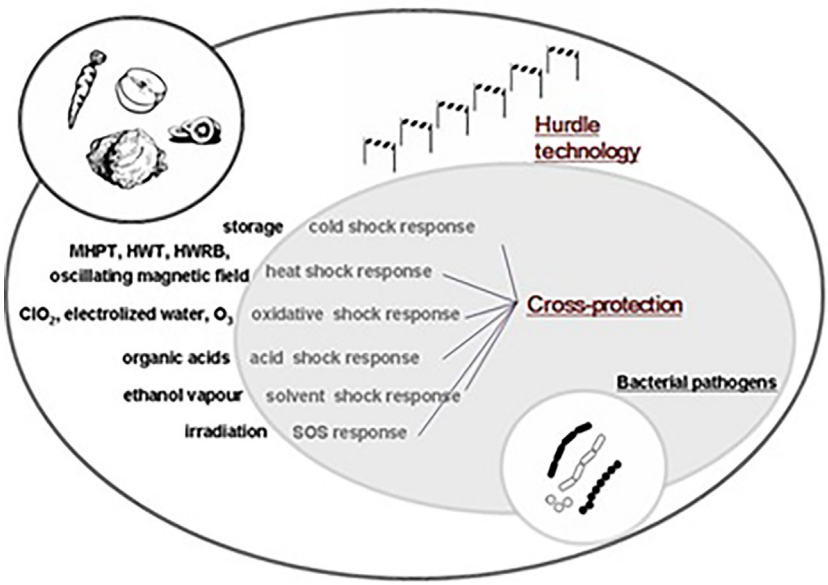


FIGURE 2 | Influencing factors for foodborne pathogens.

the cytoplasmic level of H_2O_2 is high (Bingham-Ramos and Hendrixson, 2008; Melo et al., 2019).

Thermophilic species of *Campylobacter*, like *C. jejuni*, multiply at 37 to 42°CC and are unable to grow at temperatures below 30°CC (optimal growth is at 41.5°CC). At different stages of food processing, *Campylobacter* are exposed to chilled (0–4°CC) and elevated (>37–42°CC) temperatures. Evidence has shown that the response of *Campylobacter* to colder conditions (Hazeleger et al., 1998; Park, 2002) results in the slowest growth at 30°C. Low temperatures, freezing, and thawing impact different kinds of wastewater (particularly those concerning public health) and their long-term survival of enteric microbes (Zhang et al., 2009; Dasti et al., 2010; Hazeleger et al., 1998). Differences in at least 15 distinct genes were recorded between bacterial-cell and human-body temperatures of 37–42°CC, which is within the range of chicken-body temperatures. Around 48.1% of *C. jejuni* isolates showed resistance to tetracycline, and subsequent resistance to nalidixic acid (5.5%), ciprofloxacin (5.5%), azithromycin (1.78%), and erythromycin (1.78%) (Narvaez-Bravo et al., 2017). Dasti et al. (2010) reported ciprofloxacin resistance ranging from 4 μg to 32 μg/ml for the minimal inhibitory concentration. Most ciprofloxacin-resistant strains were divided into three major clonal complexes (ST-21, 48, and 353) by multilocus assessment, whereas both antibiotic-resistant strains were uniquely grouped into ST-45.

OTHER GENERAL SURVIVAL MECHANISMS

The food matrix is one environmental factor that can influence micro-organism survival in the food system (all processes of production, processing, transport, and consumption) (de Oliveira et al., 2019; Farfán et al., 2019). After exposure to stress in the food system, expression of virulence and survival genes increased in *Listeria monocytogenes* (Olesen et al., 2009; Farfán et al., 2019). Day and Hammack (2019), reported enhanced gene expression under stress tolerance in *L. monocytogenes* in processed foods like meat and sausage juices compared with a laboratory setting. In contrast, stress-tolerance genes of *Lactobacillus sakei* were decreased in meat products (Prechtl et al., 2018), chicken meat and juice (Birk et al., 2004). Meat exudate, such as that from poultry meat, contains enzymes, myogens, myoglobin lactic acid, and amino acids (Wang et al., 2013). 'Chicken juice' can be used as a food-based model system for investigation of microbial survivability. Birk et al. (2004) recommended using the system to enhance understanding of *C. jejuni* viability on poultry products. C. jejuni survived longer in chicken juice (due to increased biofilm formation) stored at 5°C and 10°C (Brown et al., 2014). Ligowska et al. (2011) reported that expression of the gene *luxS* was increased in *C. jejuni* cultured in chilled poultry-meat juice. This highly conserved gene encodes the enzyme LuxS (S-ribosylhomocysteine lyase), which forms part of a quorum sensing system with autoinducer-2 (AI-2) and regulates gene expression. Differences in the recovery and identification of *Campylobacter* spp. between meat exudate and carcass rinse sampling methods in poultry have been demonstrated (Simmons et al., 2008; Duffy, 2019), as shown in **Figure 3**.

Previous research has shown that microbes form biofilms during food processing, such as in meat exudate conditions. Species of the genus *Salmonella* created a biofilm on the surface of stainless steel when cultured in laboratory media or meat exudate (Wang et al., 2013). Differences in the shape and cell density of mature biofilms were observed between food processing and laboratory environments. Longo and Spano (2019) reported the formation of biofilm in *L. monocytogenes* and species of the genera *Pseudomonas* and *Staphylococcus* on meat-treated surfaces, such as polyvinyl chloride, polyurethane, and steel. *C. jejuni* was more prone to forming biofilms in chicken juice than in a laboratory environment due to high nutrient availability (Brown et al., 2014). Thus, processed foods that contain many macronutrients are easily contaminated by microbes; these foods include the meat juice of chicken and beef, milk protein, and dairy products (Kusumaningrum et al., 2003; Healy et al., 2010).

VIABLE BUT NON-CULTURABLE (VBNC) STATE

Some microbes can endure unfavorable environments, such as nutrient deprivation, desiccation, inadequate pH, and temperature changes (Blanco-Lizarazo et al., 2018; Jin and Riedel–Kruse, 2018). Few microbes are capable of living in these unfavorable environments, but some organisms may enter a VBNC state for subsistence. Microbes in the VBNC state are unable to multiply, and their morphology is transformed into a coccoid shape (Poursina et al., 2018; Jin and Riedel-Kruse, 2018). Bacteria decrease their metabolism in the VBNC state but may retain the virulence capacity to infect a host and cause disease (Oliver, 2010; Fakruddin et al., 2013; Poursina et al., 2018). The VBNC state has been found in several micro-organisms, such as *C. jejuni*, *V. parahaemolyticus*, *Salmonella* ser. Typhi, and *Helicobacter pylori* (Azevedo et al., 2007; Zeng et al., 2013; Otigbu et al., 2018; Yoon and Lee, 2020). In an unfavorable environment, *C jejuni* can survive by using the VBNC tactic (Gangaiah et al., 2010; Zeng et al., 2013; Otigbu et al., 2018; Yoon and Lee, 2020). *C. jejuni* entered the VBNC state when cultured for 18–28 days at 4°C (Jones et al., 1991). Magajna and Schraft (2015) studied the VBNC status of planktonic cells and biofilm cells at 4°C and found that biofilm cells converted to VBNC status quicker than planktonic cells in nutritionally deprived and hostile-temperature environments. The VBNC form of *C. jejuni* affects CadF expression at 4°C (Otigbu et al., 2018). CadF protein is one of the elements influencing microbial invasion. The VBNC form of Campylobacter has been categorized based on reduced metabolism, augmented production of the degrading enzymes and substrates, and (Chaveerach et al., 2003; Upadhyay et al., 2019). Consequently, microbes can live for longer periods in hostile conditions (Kovacs et al., 2019).

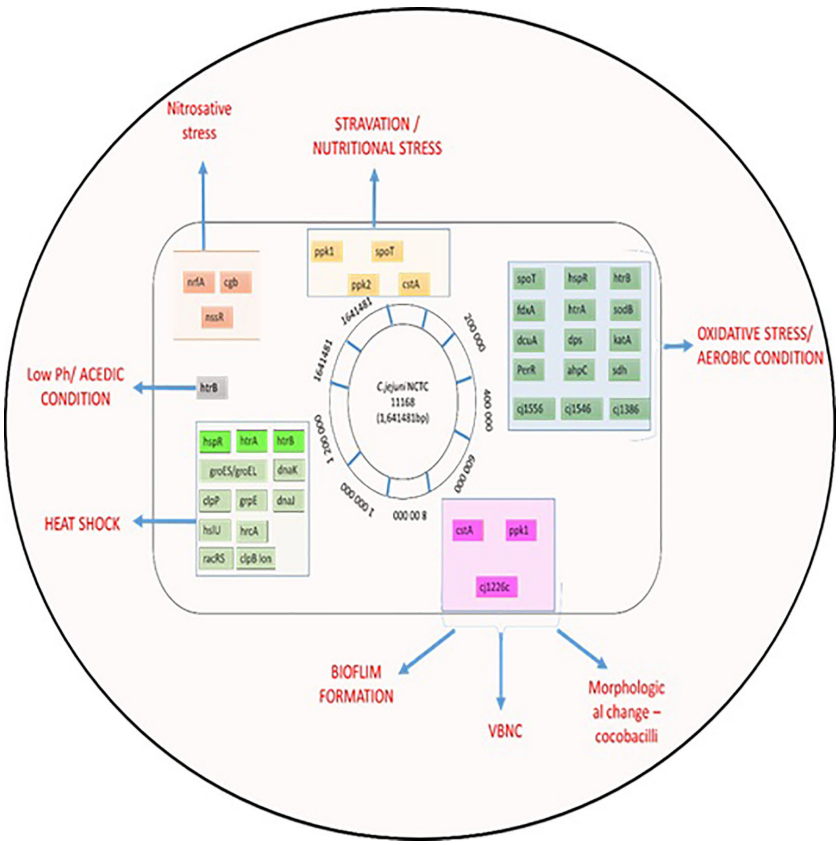


FIGURE 3 | Summary of *C. jejuni* responses to stresses. The chromosome of *C. jejuni* NCTC11168 is represented by a black circle on which the location of genes, involved in different stress responses, are shown as colored lines. Genes are colored according to their role; gene names shaded in grey are involved in multiple stress responses.

ADAPTATION TO MAJOR ENVIRONMENTAL STRESSES BY *CAMPYLOBACTER* SPP.

Adaptation by *Campylobacter* spp. to various stresses such as acidic environment, salt tolerance, thermotolerance (heat and cold), UV stress, osmotolerance, desiccation, biofilm formation, and antibiotic resistance, are explained in detail in **Table 1**.

Genes Involved in Stress Sensing/ Adaptation

Acid-tolerance mechanisms: The adaptive tolerance response (ATR) was identified as the initiator of cross-protection for the survival of microbes under various stressful or unfavorable conditions (Oh et al., 2015), and was also found in foodborne pathogens (Li et al., 2018; Cariri et al., 2019; Mayton et al., 2019). Murphy et al. (2003) discovered an ATR in *C. jejuni* and a comparable result in the initiation of ATR was observed between stress-exposed and nonexposed organisms when the organism at the mid-exponential stage (8 h) was unable to start an ATR under air- and acidic-stress conditions. Conversely, stationary-phase (48 h) organisms could initiate ATR at pH 4.5 under air and acidic status compared to nonexposed organisms. They displayed acidic cross-protection, which initiated ATR under oxygen or air status. In addition, the ATR initiation of microbes at pH 4.5 varies according to the culture media; this might be due to the different nutrient compositions of the various culture media (Kovacs et al., 2019), -. C. *jejuni* demonstrated an ATR capacity at pH 4.5 when exposed to aerobic conditions with acidic and nutritional deprivation (Oh et al., 2017). Acidic stress initiated the upregulation of *perR* genes to counter oxidative disturbance.

Acid shock has a significant biological impact in situations of acidic pH and low (organic) acids. Fatty acids are carboxylic acids generated by fermentation, and include propionate, butyrate, and acetate (Luo et al., 2015; Eguchi and Utsumi, 2016). The fatty acids cause toxicity in their unloaded, protonated form because they may penetrate the plasma membrane, dissociate a proton, and create a lower intracellular pH.

An adaptive tolerance response to aerobic + acid conditions in *C. jejuni* (Oh et al., 2019) was shown to induce a global stress response mechanism (S.H Kim, unpublished data). An adaptive tolerance response (ATR) produced as a result of sub-chronic

TABLE 1 | Cluster of genes involved in the multiple stress responses of *C. jejuni*.

Sr. No	Target Mechanism	Gene	Stress tolerant Gene	Reference
1	Nitric Oxide and Nitrosative Stress in Campylobacter jejuni and Campylobacter coli	nrfA	Nitrite reductase, formate-dependent	Mühlig et al., 2014; Einsle (2011)
		cgb	Single-Domain Hemoglobin in Mediating Resistance to Nitric Oxide and Nitrosative Stress	Elvers et al., 2004; Pittman et al., 2007
		nssR	Single-Domain Hemoglobin in Mediating	Monk et al., 2008; Avila-Ramirez et al., 2013
2	Heat shock efficiency	htrB	Promotes Abiotic and Biotic Stress Tolerance in Transgenic Arabidopsis thaliana	Svensson et al., 2008; Poli et al., 2012
		htrA	high-temperature requirement A (HtrA)-like protease and chaperones in the cell envelope,	Svensson et al., 2008
		groES/groEL	Chaperonin	Laranjo and Oliveira, 2011
		dnaK	Chaperonin	
		clpP	Two promoters; roteolytic component of the Clp or Ti protease	Gerth et al., 1998
		grpE	Nucleotide sequence of a *Bacillus subtilis* gene homologous to the grpE gene	Völker et al., 1992
		dnaJ	Arabidopsis DnaJ (Hsp40) contributes to NaCl-stress tolerance	Zhichang et al., 2010
		hslU	Proteomics Analysis of Drought Stress-Responsive Proteins	Xu et al., 2009
		hrcA	Conserved ATP-dependent proteases of *C. jejuni* to stress tolerance and virulence	Chon et al., 2007
		racRS	Salinity stress tolerance - ascorbate-glutathione	Kang et al., 2013
		clpB lon	Protease ATP-dependent (*E. coli* ClpA) • ATPase activity	Parsell and Lindquist, 1993
3	Nutrition Depletion/Starvation	ppk1	Quorum sensing genes/inhibiting polyphosphate kinase	Sarabhai et al., 2015
		spoT	cytosolic ascorbate peroxidase/peroxiredoxins	Gangaiah et al., 2010
		ppk2	The adenylate cyclase gene MaAC/membrane location of the protein	
		cstA	Arabidopsis genes	Auesukaree et al., 2009
4	Osmotic Tolerance	htrB	ATP binding cassette transporter components PaqP and PaqQ in bacterial salt stress tolerance	Lin et al., 2009a
		ppk1	Inhibiting polyphosphate kinase	Sarabhai et al., 2015
		cj1226c	Influences biofilm formation	Svensson et al., 2008; Svensson et al., 2009
5	Low pH/Acid Tolerance	htrB	ATP binding cassette transporter components PaqP and PaqQ in bacterial salt stress tolerance	Lin et al., 2009b
6	Oxidative Stress/Oxygen Stress	spoT	Quorum sensing genes/inhibiting polyphosphate kinase	Sarabhai et al., 2015
		hspS	Proteomics Analysis of Drought Stress-Responsive	Parsell and Lindquist, 1993
		htrA		
		fdxA	stress-responsive cyclophilin gene	Chen et al., 2007
		sodB	Resistance to peroxynitrite and stage-specific survival in	Master et al., 2002
		dcuA	macrophages	
		dps		
		katA		
		perR		
		ahpC		
		sodB-sdh		
		cj1556	Additionally Influences biofilm formation	Svensson et al., 2009
		cj1546		
		cj1556-cj1386		

stress adaptive response and offers protection against subsequent lethal stress exposure (Noreen, 2019). We have defined an ATR in *C. jejuni* previously. The mediation of acid and oxygen concentration, makes them to adopt improved survival mechanism against lethal pH (Taylor et al., 2017). De novo protein synthesis was necessary for the initiation of ATR in *C.jejuni*, which implies enhanced protein synthesis occurred during the induction phase. During the induction of an ATR to acid stress, analysis of protein expression profiles demonstrated a global cellular response (S.H Kim, unpublished data). Based on MALDI-TOF mass spectrometry different Protein expressed during induction of the ATR in *C.jejuni*, which revealed that the majority of proteins were involved in modification, repair and biosynthesis.

The ATR in *C. jejuni* has been shown to incorporate up-regulation of generic stress proteins involved in protein defense or breakdown, such as the heat-shock response based on universal chaperones DnaK and GroEL, which are among the

most highly conserved protein-coding genes known to be involved (Tang et al., 2017). Chaperone proteins may be involved in aerobic + acid denaturation or damage repair of proteins. Chaperone based GroEL and DnaK heat shock protein (HSPs) have been described as caused under acid conditions in *Salmonella typhimurium* (Ghazaei, 2017), which plays a major role after mild stress, either in the prevention of subsequent DNA damage or in the repair of already damaged DNA. The reported protein response were found to be closely associated with following pathogens such as *S. typhimurium* (Ghazaei, 2017), *Escherichia coli* (Burt et al., 2007) and *Acinetobacter baumannii* (Cardoso et al., 2010). This global reaction, in *C. jejuni*, which induced various mechanisms of survival and offers an initial insight into mechanisms that contribute to resistance of aerobic + acid susceptibility.

ATR-related RpoS: Transcription controller σs, encoded by the *rpoS* gene (RNA polymerase sigma factor), is a replacement sigma factor, the amount of which increases dramatically during any permanent stage of the microbes. The increase in σs concentration and gene expression is known to influence acid-shock proteins, such as high osmolality, low pH, hydration, and oxidation in cell survival (Ferreira et al., 2001). Sudden increases in cell acidification also cause strong increases in *rpoS* levels. Mutants that are defective in *rpoS* or that generate low concentrations of *rpoS* are highly susceptible to acidic conditions.

Salt-Tolerance Mechanisms

Sodium chloride (NaCl) is one of the most used preservatives in the food industry. *C. jejuni* is highly responsive to high osmolarity compared to most other enteric microbes (Feng et al., 2018; Kovacs et al., 2019). *C. jejuni* is unable to multiply with ≥2% NaCl at 42°C, but can multiply in the presence of 0.5% to 1.5% NaCl at 42°C (Gomes et al., 2018). Lake et al. (2019) reported that *C. jejuni* could tolerate 7.5% sodium chloride (NaCl) in media at 4°C better than at 22–30°C as measured using bioluminescence. In microarray analysis, Zhao et al. (2019) found that *C. jejuni* had augmented expression of oxidative-stress genes and heat-shock genes after exposure to hyperosmotic conditions.

Genetic Regulation by Sigma Factors

C. jejuni has a genome size of 1.4 Kbp, coding for approximately 1731 genes. In contrast to other environmental and food pathogens that have several gene-regulation processes occurring *via* sigma factors, *C. jejuni* has only three sigma factors (Wösten et al., 1998; Parkhill et al., 2000; Carrillo et al., 2004), and no recorded extracytoplasmic-function (ECF) sigma factors. The three sigma variables account for most operations related to gene regulation. Sigma 70 or RpoD is the housekeeping sigma factor that regulates most *C. jejuni* promoters. The other two sigma factors, sigma 28 (FilA, Filament A) and sigma 54 (RpoN), regulate 44 different genes that are mostly related to flagellar synthesis and protein secretion (Studholme and Dixon, 2003; Porcelli et al., 2013). The regulatory mechanisms and nucleic-base composition of the sigma-factor promoters were detailed by Petersen et al. (2003). Major promoters recognized by *C. jejuni* sigma subunits have the –10 element, whereas there is no consensus for the –35 element. The regulatory roles of RpoN in *C. jejuni* under various stress conditions were shown were shown using RpoN mutation and complementation in a study by Hwang et al. (2011). FilA is thought to regulate motility as well as the virulence of *C. jejuni* (Carrillo et al., 2004). Thorough genomic research into these mutant strains is required to elucidate the intricacies of gene regulation among the three sigma variables in this uncommon pathogen. Furthermore, how the lack of conservation of the –35 element contributes to optimal transcription *in vivo* remains to be determined. Morphological differences may exist, such as the conversion of a spiral bacterium to a coccus-/rod-shaped bacterium under osmotic and cold stress (Carrillo et al., 2004; Hwang et al., 2011). Even if *C. jejuni* is regarded as a pathogen transmitted *via* meat and poultry, it is not very tolerant to several nonoptimal conditions, particularly desiccation and osmotic stress.

Role of Osmolytes in Cryotolerance

Compared with *Salmonella* spp. and *E. coli*-like enteric bacteria, little is known about the mechanisms that enable survival of *Campylobacter* spp. under various environmental and stress conditions. A previous study found that *C. jejuni's* ability to influence gene expression after exposure to environmental stress was a barrier to comparison with other bacteria (Park, 2002). Rapid temperature decreases cause bacteria to express a distinct set of proteins, and this response is known as cold shock. These proteins are predominantly nucleases, helicases, and ribosome-related elements that communicate with and bind to RNA and DNA. Cold-shock proteins induce a membrane adaptation, cold signal sensing, and translation-device alteration (Ultee et al., 2019). Ultee et al. (2019) reported motility for oxygen consumption, protein synthesis, and *C. jejuni* survival capacity at 4°C. Lu et al. (2011) revealed that *C. jejuni* survive at in low-temperature. This indicates that *C. jejuni* may produce a cold-shock effect that influences low-temperature gene expression to 4°C. CspA is the main cold-shock protein in *C. jejuni*, and functions as an RNA chaperone to enhance more effective cold-shock protein translation (Parkhill et al., 2000; Giuliodori et al., 2010). It is not yet clear how *C. jejuni* respond to or regulate the expression of genes during cold shocks. Based on documented studies, the cold-shock reaction is presented as a complex system of genes that are regulated by the same stimulus, where post-transcriptional conditions are essential. *C. jejuni* poses problems to food security and public health in the food-processing industry, since it survives for several months at 4°C. *C. jejuni* declined by about 1 log cfu/ml when stored at 4°C for seven days (Guévremont et al., 2015; Lake et al., 2019). Oxidative stress can upregulate cold-shock protein expression, which can extend the life span of *C. jejuni* in hypothermal conditions (Karki et al., 2019).

Survival During Ultraviolet (UV) Stress

VBNC refers to a state in which conventional culture on enhanced agar media does not detect microbial cells, although it remains feasible to resuscitate the microbes under preferential circumstances. This unique survival strategy has been shown to exist in nature (Salma et al., 2013). More than 60 different bacterial species have been found to be VBNC, including both Gram-negative (e.g., *E. coli*, *S. enterica*, *C. jejuni*, *H. pylori*, *Pseudomonas*

aeruginosa, and species of the genera *Legionella* and *Vibrio*) and Gram-positive (e.g., species of the genus *Enterococcus*, *Micrococcus luteus*, and *L. monocytogenes*) species (Salma et al., 2013). Following a severe dose of UV (0.192 J/cm2), no viable *Campylobacter* cells were identified from the original level of 7 log cfu/ml in the liquid media (skimmed milk exposed to UV and diluted 1:4 with extreme rehabilitation diluents) (Xiong, 2009). Substantial variability of up to 4 log cfu/ml was observed in the susceptibility of *Campylobacter* isolates following UV treatment. In UV-treated (0.192 J/cm) fresh chicken fillet, *C. jejuni*, was decreased by 0.76 cfu/g, whereas, a reduction in *C. jejuni* of up to 3.97 log cfu/cm was attained with UV treatment of packaging and surface materials. These data indicated that *Campylobacter* is UV-prone, but concerning differentials occurred among the studied isolates. Overall, UV application could help improve the microbiological quality of raw chicken and remove contamination of related surfaces and packaging (Haughton et al., 2011).

Investigations were conducted concerning organism survival in rivers, coastal waters, and sewage to investigate the natural and artificial habitats of *C. jejuni* with UV-B light (280–315 nm) (Hénault-Ethier et al., 2016; García-Peña et al., 2017; Otigbu et al., 2018). Another research project in conjunction with these revealed that *C. jejuni* was susceptible to UV-C light (254 nm). UV sensitivity was greater than that of other microbes (Butler et al., 1987). The application of UV-C radiation to decrease *C. jejuni* in chicken breast also attracted interest (Rodrigues et al., 2019), as well as in broiler meat (Zhuang et al., 2019) and ready-to-eat ham (Yang et al., 2017). UV-light techniques have been extensively explored for reducing micro-organisms, including *Campylobacter*, in foodstuffs (Rodrigues et al., 2019; Zhuang et al., 2019).

UV irradiation achieved a maximal reduction of *C. jejuni* on broiler meat and broiler skin of 0.7 and 0.8 log, respectively. The maximal decrease by UV irradiation on broiler carcasses (254 nm, 32.9 m W/s per square inch) was 0.4 log, and the combination of UV and activated oxygen also achieved a 0.4 log reduction in *C. jejuni*. The primary sanitation method for *C. jejuni* in broiler carcasses cannot rely on UV irradiation alone or in conjunction with activated oxygen. However, application of these methods in conjunction with other sanitization techniques, as well as the adequate processing and sanitation of processing plants, may be more efficient than the use of these processes to reduce *C. jejuni* on broiler carcass surfaces (Isohanni and Lyhs, 2009). UV irradiation was less efficient at removing *C. jejuni* on broiler meat and skin than on agar plates. It reduces *C. jejuni* on grilled skin a little more effectively than on meat. Dry meat undergo ultraviolet radiation has low invasive capacity, and the cutting edges of food perhaps produced shade that interfered with UV irradiation (Rodrigues et al., 2019). The fibers could be isolated by swabbing the surfaces and allowing the swabs to absorb humidity from below the meat layer. After flaming, the skin did not appear to have changed much, and bacteria could not have crossed the threshold skin into the meat. Wong et al. (1998) also indicated that gram positive bacteria were more efficiently reduced by UV irradiation. However, the effects of UV irradiation can differ considerably in *C. jejuni* isolates from different origins and at different growth stages (Yaun et al., 2003).

Oxidative Stress and Aerotolerance

Campylobacter does not usually grow in environments of atmospheric oxygen (air) due to it being microaerophilic and requiring 5–10% carbon dioxide (CO_2) (Frirdich et al., 2019). *Campylobacter* can tolerate oxidative stress even after exposure towards aerobic conditions (Kim et al., 2015). Microaerophilic environment generates favorable growth conditions for *C. jejuni* (Geng et al., 2019). Karki et al. (2019) found that subcultures of *C. jejuni* could develop colonies on blood agar at 4, 37, and 42°C in air conditions. This exposure to aerobic conditions leads to the transformation of both the cell morphology and the pattern of the external membrane proteins. Their results indicated that the bacterial cells had high survivability in aerobic conditions compared to microaerobic conditions. Geng et al. (2019) reported that subcultures of *C. jejuni* from both sterile chicken mince and stream water developed colonies at 5, 25, and 37°C on blood agar, and that cells were more likely to survive when cultured in a microaerophilic than an aerobic environment.

In comparison with microaerobic conditions owing to oxidative pressure, *C. jejuni* showed external structural changes in the form of coccoid morphology (Oh et al., 2015), and the inner ATP synthesis of *C. jejuni* decreased with oxidative stress (Cain et al., 2019). Under microaerophilic environments, *C. jejuni* may develop better than under oxygenic conditions at a cell concentration of $<10^5$ cfu/ml (Kaakoush et al., 2007).

C. jejuni Heat-Shock Response

Heating is one of the sanitizing techniques used for food preservation in the food sector. Heat treatment readily reduces the survival of *C. jejuni* relative to other enteric micro-organisms. For *C. coli*, decimal reduction times (D-values) were 381, 89, 21.9, and 5.7 s at 49.9, 55.4, 60.0, and 62.5°C, respectively, in phosphate buffer saline (PBS) (Habib et al., 2010; Upadhyay et al., 2019). Treatment of *C. jejuni* at 55°C for 3 min, decreased the density by 2–3 log cfu/ml (Kovacs et al., 2019). Heat treatment caused *C. jejuni* to lose its invasion capacity, and upregulate transcriptional factor HrcA for acid shock (Xu et al., 2019).

Desiccation Tolerance

Tolerance to desiccation in *Campylobacter* spp. was first reported by Fernandez et al. (1985) in several biotypes of *C. coli* and *C. jejuni* subjected to 2–8 hours of exposure. The RpoN sigma factor does not significantly contribute to the tolerance to osmotic shock or desiccation, whereas tolerance of cold or refrigeration temperatures can be directly correlated with bacterial survival capacity in cold environments (Burgess et al., 2016). The extreme sensitivity to desiccation and poor tolerance to heat and drying established that blowing hot air was an efficient method to prevent carrying dormant *C. jejuni* from poultry to human hosts in commercial settings (Berrang et al., 2011). Such methods could be applied to farms to prevent pathogenic carriers through poultry.

Biofilm Formation and Stress Adaptation

Extracellular polysaccharide (EPS) accumulation leads to biofilm formation by microbes, biofilm formation could allow additional species to accumulate on surfaces (Simoes and Simões, 2013;

Maes et al., 2019). EPSs compressed of nucleic acids, polysaccharides, proteins, phospholipids, and teichoic acids to form biofilms (Miao et al., 2019). Many factors stimulate biofilm formation, including temperature, NaCl, pH, compounds of food, and type of surface (Arnold and Silvers, 2000; Nguyen et al., 2006; Speranza et al., 2011; Vázquez-Sánchez et al., 2013; Mavri et al., 2016; Whitehouse et al., 2018; Longo and Spano, 2019; Xu et al., 2019). Biofilms can form on dairy-product-handling machinery and nutrition-handling surfaces (Miao et al., 2019), and can therefore contribute to the occurrence of foodborne diseases and create a public health issue (Maes et al., 2019; Miao et al., 2019). There are numerous reports on foodborne diseases in relation to biofilm development (Metselaar et al., 2015; Mavri et al., 2016; Whitehouse et al., 2018; Ma et al., 2019). Microbes in biofilms are more resistant to antibiotics than plankton cells are (Stewart and Costerton, 2001; Olsen, 2015). *C. jejuni* preconditions define their environment for growth, and Surface attachment and biofilm generation are vital tools for environmental stability (Dykes et al., 2003), as shown in **Figure 4**.

C. jejuni can generate biofilms in liquid media as a monospecies (Sałamaszyńska-Guz et al., 2018) in aerobic conditions (Ovesen et al., 2019) *C. jejuni* can form biofilms both as a monospecies and as a combination of microbes (The et al., 2019) and nutritional components (Bronnec et al., 2016). Sałamaszyńska-Guz et al. (2018) showed that the aggregating and pellicle form of *C. jejuni* that forms at 30–37°C in a microaerobic environment allows the bacteria to survive under aerobic conditions. Ovesen et al. (2019) demonstrated that *C. jejuni* easily creates biofilms, and that flagellar motility aggravated biofilm production. It currently reads as though it is the report of Ovesen et al., 2019 stated that *C. jejuni* could acclimate to develop a biofilm linked to CsrA under aerobic conditions (Askoura et al., 2016; Ye et al., 2019). Therefore, CsrA mutation leads to inhibition of biofilm formation (Fields and Thompson, 2008). *C. jejuni* can also contribute to biofilm formation in combination with other microbes under a microaerobic environment, but the combination is specific to the microbes and the environment (The et al., 2019), for example the poultry environment is an example of this specific environment/microbe combination. The biofilm formation capacity of *C. jejuni* depends on culture media, oxidative stress, temperature, and interspecies composition (Bronnec et al., 2016). Protein generation, quorum sensing, and flagellar sensing also influence the capacity of *C. jejuni* to generate biofilms, as shown in **Table 1**.

Antibiotic Susceptibility of *C. jejuni*

Antibiotics are typically used to fight against bacterial infections (Pedersen et al., 2018), and possess different mechanisms to kill or inhibit bacteria. For example, quinolones, such as nalidixic acid, dysregulate DNA synthesis in microbial cells (Jacoby, 2005), whereas macrolides, including erythromycin, bind to ribosomes in the microbes, blocking elongation of the peptide loop (Arsic et al., 2018). Severe cases of campylobacteriosis require adequate treatment with antibiotics (Wieczorek and Osek, 2013), usually a fluoroquinolone and macrolide combination (Devi et al., 2019). Improper and frequent antibiotic use has led to increased antibiotic resistance in *Campylobacter*, which is a public health issue. Consequently, the fluoroquinolone and macrolide efficacy can fail to overcome the antibiotic resistance of *Campylobacter* (Pedersen et al., 2018; Bolinger et al., 2018; Silvan et al., 2018; Devi et al., 2019). The continuous usage of antibiotics such as tetracycline, ciprofloxacin, and erythromycin leads to the development of resistance in enteropathogens; specific resistance genes to these antibiotics were identified in *C. jejuni* isolates (Wirz et al., 2010), and comparable trends in *C. coli* were reported in Canada (Devi et al., 2019). Zwe et al. (2018) found

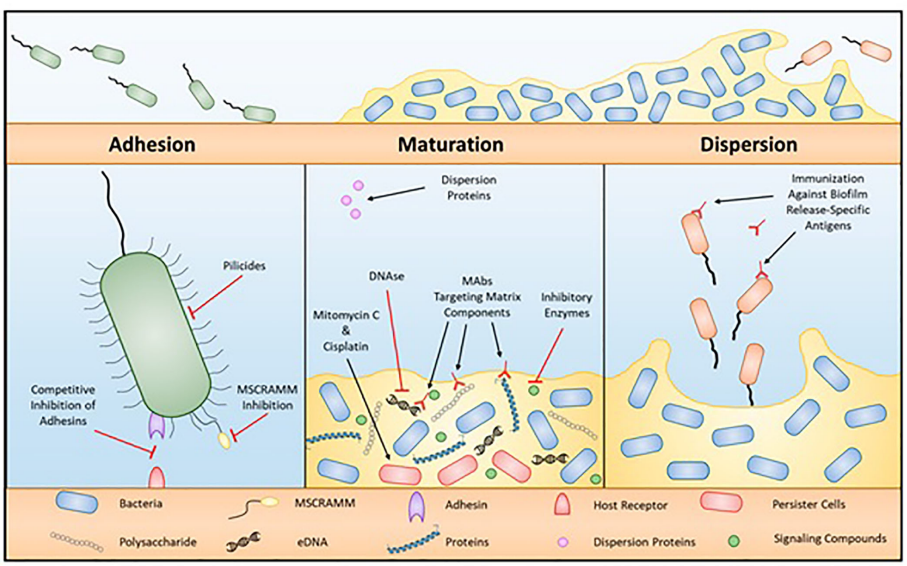


FIGURE 4 | Process of biofilm formation.

that *C. jejuni* isolated from ducks in Singapore was resistant to ciprofloxacin (86.7%), nalidixic acid (84.4%), and erythromycin (11.1%) (Devi et al., 2019). The development of antibiotic resistance in *Campylobacter* means the treatment regime of campylobacteriosis will involve other antibiotics, like gentamycin (Aarestrup and Engberg, 2001; Pedersen et al., 2018).

CONCLUSION

Campylobacter use a range of approaches for environmental and genomic survival, and molecular studies have facilitated a better understanding of these processes. Genetic modifications within the species *C. jejuni* have been significantly targeted, and genome sequencing for this species has been completed. Epidemiological studies and phenotypical analyses found variations in the incidence of strains of *C. jejuni*, or environmental circumstances between strains of *C. jejuni*. It has been easier to understand mechanisms that affect *C. jejuni* persistence by examining the transformation of this important pathogen in natural settings, such as soil and water, and combining connections with environmental changes. However, the reported differences in various strains of *C. jejuni* highlight the constraints of drawing generalized conclusions from individual strain research.

The multiple stress responses of *Campylobacter* spp. may facilitate survival in extreme environmental conditions, in addition to increasing resistance to subsequent traumatic conditions, which might enhance acquisition of virulence genes. Our review demonstrates the contribution of stress-tolerance responses to the resistance and pathogenicity of *C. jejuni*. Minor factors involved in stress management based on stress-responsible protein production are also involved in the activation and up- or down-regulation of virulence genes, and may contribute to the pathogenesis of *C. jejuni*. This finding is based on reported studies validated in different isolates of *C. jejuni* in response to stress adaptation, therefore caution should be taken in segregating and characterizing strains of *C. jejuni*. Gram-negative microaerophilic bacteria like *H. pylori* and *C. jejuni* are extremely common, and are human gastrointestinal pathogens. Only by combining these separate strands can the role of environmental survival in transmitting these important pathogens be fully understood.

Required Future Research to Fill Current Knowledge Gaps

Major gaps in current research on stress responses on *C. jejuni*, so far, researchers have predominantly focused on antibiotic resistance and oxidative stress in *C. jejuni*. However, various other stress conditions and specific survival-mechanism-based evolutionary adaptation methods exist to overcome modern preservative conditions, such as acidity, alkalinity, osmotic imbalance, freezing, high temperatures, UV light, and dryness (reduced water content). Future research should concentrate on understanding the genetic make-up of *C. jejuni* that helps this organism survive various environmental conditions. Identification of these evolutionary adaptive mechanisms and specific signaling pathways will assist future researchers in developing effective methods to overcome the adaptive mechanism(s) of *C. jejuni*. Furthermore, understanding *C. jejuni* stress-oriented genes and their specific expression mechanisms based on environmental stressors have implications in biofilm interactions and their signaling mechanism(s), and in practical terms this could help with current technological hurdles in the food system.

AUTHOR CONTRIBUTIONS

The manuscript was written in detail and sectioned for specialized discussion with the respective authors in the field of research. Designing the outline of the review manuscript (Multiple stress tolerance in *Campylobacter jejuni*), visualization, and conceptualization—S-HK, RC, D-HO. Cross-protection and other general survival mechanisms towards environmental stress—SR. Genes involved in stress sensing/adaptation, acid tolerance mechanisms, protective mechanisms, systems for resistance to acid (AR1) or repressed by oxidants or glucose—AP. System of acid resistance 2 (AR2)/dependent on glutamate, system of acid resistance 3 (AR3)/arginine, ATR-reliant RpoS—EP, HJ, S-BH. Salt tolerance mechanisms—AP, RC. Genetic regulation by sigma factors—RC, S-HK, EB-M. Role of osmolytes in cryotolerance—RC, EB-M, FE, KB. *C. jejuni* heat-shock response—RC, S-HK. Desiccation tolerance—W-SB, AP. Biofilm formation and stress adaptation—RC, SR. Antibiotic susceptibility of *C. jejuni*—S-HK. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aarestrup, F. M., and Engberg, J. (2001). Antimicrobial resistance of thermophilic Campylobacter. *Vet. Res.* 32, 311–321. doi: 10.1051/vetres:2001127

Alfredson, D. A., and Korolik, V. (2007). Antibiotic resistance and resistance mechanisms in Campylobacter jejuni and Campylobacter coli. *FEMS Microbiol. Lett.* 277, 123–132. doi: 10.1111/j.1574-6968.2007.00935.x

Allen, R. C., Angst, D. C., and Hall, A. R. (2018). Resistance gene carriage predicts growth in the absence of antibiotics for natural and clinical Escherichia coli. *Appl. Environ. Microbiol.* 85, 02111–02118. doi: 10.1128/AEM.02111-18

Altekruse, S. F., Stern, N. J., Fields, P.II, and Swerdlow, D. L. (1999). Campylobacter jejuni—an emerging foodborne pathogen. *Emerg. Infect. Dis.* 5, 28–35. doi: 10.3201/eid0501.990104

Arnold, J. W., and Silvers, S. (2000). Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. *Poult. Sci.* 79, 1215–1221. doi: 10.1093/ps/79.8.1215

Arsic, B., Barber, J., Čikoš, A., Mladenovic, M., Stankovic, N., and Novak, P. (2018). 16-membered macrolide antibiotics: a review. *Int. J. Antimicrob. Agents* 51, 283–298. doi: 10.1016/j.ijantimicag.2017.05.020

Asakura, M., Samosornsuk, W., Taguchi, M., Kobayashi, K., Misawa, N., Kusumoto, M., et al. (2007). Comparative analysis of cytolethal distending

toxin (cdt) genes among Campylobacter jejuni, C. coli and C. fetus strains. *Microb. Pathog.* 42, 174–183. doi: 10.1016/j.micpath.2007.01.005

Askoura, M., Sarvan, S., Couture, J.-F., and Stintzi, A. (2016). The Campylobacter jejuni ferric uptake regulator promotes acid survival and cross-protection against oxidative stress. *Infect. Immun.* 84, 1287–1300. doi: 10.1128/IAI.01377-15

Auesukaree, C., Damnernsawad, A., Kruatrachue, M., Pokethitiyook, P., Boonchird, C., Kaneko, Y., et al. (2009). Genome-wide identification of genes involved in tolerance to various environmental stresses in Saccharomyces cerevisiae. *J. Appl. Genet.* 50, 301–310. doi: 10.1007/BF03195688

Avila-Ramirez, C., Tinajero-Trejo, M., Davidge, K. S., Monk, C. E., Kelly, D. J., and Poole, R. K. (2013). Do globins in microaerophilic Campylobacter jejuni confer nitrosative stress tolerance under oxygen limitation? *Antioxid. Redox Signal.* 18, 424–431. doi: 10.1089/ars.2012.4750

Azevedo, N. F., Almeida, C., Cerqueira, L., Dias, S., Keevil, C. W., and Vieira, M. J. (2007). Coccoid form of Helicobacter pylori as a morphological manifestation of cell adaptation to the environment. *Appl. Environ. Microbiol.* 73, 3423–3427. doi: 10.1128/AEM.00047-07

Baillon, M. L. A., van Vliet, A. H., Ketley, J. M., Constantinidou, C., and Penn, C. W. (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen Campylobacter jejuni. *J. Bacteriol.* 181, 4798–4804. doi: 10.1128/JB.181.16.4798-4804.1999

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

Berrang, M. E., Hofacre, C. L., and Meinersmann, R. J. (2011). Forced hot air to dry feces and kill bacteria on transport cage flooring1. *J. Appl. Poult. Res.* 20, 567–572. doi: 10.3382/japr.2011-00391

Bingham-Ramos, L. K., and Hendrixson, D. R. (2008). Characterization of two putative cytochrome c peroxidases of Campylobacter jejuni involved in promoting commensal colonization of poultry. *Infect. Immun.* 76, 1105–1114. doi: 10.1128/IAI.01430-07

Birk, T., Ingmer, H., Andersen, M. T., Jørgensen, K., and Brøndsted, L. (2004). Chicken juice, a food based model system suitable to study survival of Campylobacter jejuni. *Lett. Appl. Microbiol.* 38, 66–71. doi: 10.1046/j.1472-765x.2003.01446.x

Blanco-Lizarazo, C. M., Sotelo-Díaz, I., Arjona-Roman, J. L., Llorente-Bousquets, A., and Miranda-Ruvalcaba, R. (2018). Effect of starter culture and low concentrations of sodium nitrite on fatty acids, color, and Escherichia coli behavior during salami processing. *Int. J. Food Sci.* 2018, 5934305. doi: 10.1155/2018/5934305

Bolinger, H. K., Zhang, Q., Miller, W. G., and Kathariou, S. (2018). Lack of evidence for erm (B) infiltration into erythromycin-resistant Campylobacter coli and Campylobacter jejuni from commercial turkey production in Eastern North Carolina: a major turkey-growing region in the United States. *Foodborne Pathog. Dis.* 15, 698–700. doi: 10.1089/fpd.2018.2477

Bolton, D. J. (2015). Campylobacter virulence and survival factors. *Food Microbiol.* 48, 99–108. doi: 10.1016/j.fm.2014.11.017

Bronnec, V., Turoňová, H., Bouju, A., Cruveiller, S., Rodrigues, R., Demnerova, K., et al. (2016). Adhesion, biofilm formation, and genomic features of Campylobacter jejuni Bf, an atypical strain able to grow under aerobic conditions. *Front. Microbiol.* 7, 1002. doi: 10.3389/fmicb.2016.01002

Brown, H. L., Reuter, M., Salt, L. J., Cross, K. L., and Betts, R. P. (2014). A.H.M. van Vliet, Chicken juice enhances surface attachment and biofilm formation of Campylobacter jejuni. *Appl. Environ. Microbiol.* 80, 7053–7060. doi: 10.1128/AEM.02614-14

Burgess, C. M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., et al. (2016). The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. *Int. J. Food Microbiol.* 221, 37–53. doi: 10.1016/j.ijfoodmicro.2015.12.014

Burt, S. A., van der Zee, R., Koets, A. P., de Graaff, A. M., van Knapen, F., Gaastra, W., et al. (2007). Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in Escherichia coli O157: H7. *Appl. Environ. Microbiol.* 73 (14), 4484–4490. doi: 10.1128/AEM.00340-07

Butler, R. C., Lund, V., and Carlson, D. A. (1987). Susceptibility of Campylobacter jejuni and Yersinia enterocolitica to UV radiation. *Appl. Environ. Microbiol.* 53, 375–378. doi: 10.1128/AEM.53.2.375-378.1987

Butzler, J. P., and Oosterom, J. (1991). Campylobacter: pathogenicity and significance in foods. *Int. J. Food Microbiol.* 12, 1–8. doi: 10.1016/0168-1605(91)90043-O

Cain, J. A., Dale, A. L., Niewold, P., Klare, W. P., Man, L., White, M. Y., et al. (2019). Proteomics reveals multiple phenotypes associated with N-linked glycosylation in Campylobacter jejuni. *Mol. Cell. Proteom.* 18, 715–734. doi: 10.1074/mcp.RA118.001199

Cardoso, K., Gandra, R. F., Wisniewski, E. S., Osaku, C. A., Kadowaki, M. K., Felipach-Neto, V., et al. (2010). DnaK and GroEL are induced in response to antibiotic and heat shock in Acinetobacter baumannii. *J. Med. Microbiol.* 59 (9), 1061–1068. doi: 10.1099/jmm.0.020339-0

Cariri, M. L., de Melo, A. N. F., Mizzi, L., Ritter, A. C., Tondo, E., de Souza, E. L., et al. (2019). Quantitative assessment of tolerance response to stress after exposure to oregano and rosemary essential oils, carvacrol and 1, 8-cineole in Salmonella Enteritidis 86 and its isogenic deletion mutants Δdps, ΔrpoS and ΔompR. *Food Res. Int.* 122, 679–687. doi: 10.1016/j.foodres.2019.01.046

Carrillo, C. D., Taboada, E., Nash, J. H., Lanthier, P., Kelly, J., Lau, P. C., et al. (2004). Genome-wide expression analyses of Campylobacter jejuni NCTC11168 reveals coordinate regulation of motility and virulence by flhA. *J. Biol. Chem.* 279, 20327–20338. doi: 10.1074/jbc.M401134200

Centers for Disease Control and Prevention(CDC) (2013). Multistate outbreak of Campylobacter jejuni infections associated with undercooked chicken livers—northeastern United State. *MMWR Morb. Mortal. Wkly. Rep.* 62, 874–876.

Chaveerach, P., ter Huurne, A. A. H. M., Lipman, L. J. A., and van Knapen, F. (2003). Survival and resuscitation of ten strains of Campylobacter jejuni and Campylobacter coli under acid conditions. *Appl. Environ. Microbiol.* 69, 711–714. doi: 10.1128/AEM.69.1.711-714.2003

Chen, A. P., Wang, G. L., Qu, Z. L., Lu, C. X., Liu, N., Wang, F., et al. (2007). Ectopic expression of ThCYP1, a stress-responsive cyclophilin gene from Thellungiella halophila, confers salt tolerance in fission yeast and tobacco cells. *Plant Cell Rep.* 26, 237–245. doi: 10.1007/s00299-006-0238-y

Chon, M. T., Ingmer, H., Mulholland, F., Jørgensen, K., Wells, J. M., and Brøndsted, L. (2007). Contribution of conserved ATP-dependent proteases of Campylobacter jejuni to stress tolerance and virulence. *Appl. Environ. Microbiol.* 73, 7803–7813. doi: 10.1128/AEM.00698-07

Coward, C., van Diemen, P. M., Conlan, A. J., Gog, J. R., Stevens, M. P., Jones, M. A., et al. (2008). Competing isogenic Campylobacter strains exhibit variable population structures in vivo. *Appl. Environ. Microbiol.* 74, 3857–3867. doi: 10.1128/AEM.02835-07

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

Day, J. B., and Hammack, T. S. (2019). Bio-Plex suspension array immunodetection of Listeria monocytogenes from cantaloupe and packaged salad using virulence protein inducing activated charcoal enrichment media. *Food Microbiol.* 84, 103225. doi: 10.1016/j.fm.2019.05.009

de Oliveira, M. G., Rizzi, C., Galli, V., Lopes, G. V., Haubert, L., Dellagostin, O. A., et al. (2019). Presence of genes associated with adhesion, invasion, and toxin production in Campylobacter jejuni isolates and effect of temperature on their expression. *Can. J. Microbiol.* 65, 253–260. doi: 10.1139/cjm-2018-0539

Devi, A., Mahony, T. J., Wilkinson, J. M., and Vanniasinkam, T. (2019). Antimicrobial susceptibility of clinical isolates of Campylobacter jejuni from New South Wales, Australia. *J. Glob. Antimicrob. Resist.* 16, 76–80. doi: 10.1016/j.jgar.2018.09.011

Duffy, L. (2019). *Campylobacter through poultry processing: selection and survival*. The University of Queensland, PhD Thesis, pp. 1–331. doi: /10.14264/uql.2019.26

Dykes, G. A., Sampathkumar, B., and Korber, D. R. (2003). Planktonic or biofilm growth affects survival, hydrophobicity and protein expression patterns of a pathogenic Campylobacter jejuni strain. *Int. J. Food Microbiol.* 89, 1–10. doi: 10.1016/S0168-1605(03)00123-5

EFSA (2010). Scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA J.* 8, 200. doi: 10.3389/fmicb.2011.00200

EFSA (2011). Scientific opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA J.* 9, 2105–2246. doi: 10.2903/j.efsa.2011.2105

Eguchi, Y., and Utsumi, R. (2016). "Two component Systems in Sensing and Adapting to Acid Stress in Escherichia Coli," in *Stress and environmental regulation of gene expression and adaptation in bacteria*. Ed. F. J. de Brujin (Hoboken, NJ: Wiley), 927–934.

Einsle, O. (2011). Structure and function of formate-dependent cytochrome c nitrite reductase, NrfA. *Methods Enzymol.* 496, 399–422. doi: 10.1016/B978-0-12-386489-5.00016-6

Ellström, P., Hansson, I., Nilsson, A., Rautelin, H., and Olsson Engvall, E. (2016). Lipooligosaccharide locus classes and putative virulence genes among chicken and human Campylobacter jejuni isolates. *BMC Microbiol.* 16, 116. doi: 10.1186/s12866-016-0740-5

Elvers, K. T., Wu, G., Gilberthorpe, N. J., Poole, R. K., and Park, S. F. (2004). Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in Campylobacter jejuni and Campylobacter coli. *J. Bacteriol.* 186, 5332–5341. doi: 10.1128/JB.186.16.5332-5341.2004

El-Tawab, A., Awad, A., Elhofy, F., Hotzel, H., and Sobhy, M. (2019). Phenotypic and genotypic chactracterization of Campylobacter isolated from poultry. *Benha V. M. J.* 37 (2), 33–36.

Eucker, T. P., and Konkel, M. E. (2012). The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal Campylobacter jejuni invasion of host cells by stimulating membrane ruffling. *Cell. Microbiol.* 14, 226–238. doi: 10.1111/j.1462-5822.2011.01714.x

Fakruddin, M., Mannan, K. S. B., and Andrews, S. (2013). Viable but nonculturable bacteria: food safety and public health perspective. *ISRN Microbiol.* 2013, 703813. doi: 10.1155/2013/703813

Farfán, M., Lártiga, N., Benavides, M. B., Alegria-Morán, R., Sáenz, L., Salcedo, C., et al. (2019). Capacity to adhere to and invade human epithelial cells, as related to the presence of virulence genes in, motility of, and biofilm formation of Campylobacter jejuni strains isolated from chicken and cattle. *Can. J. Microbiol.* 65, 126–134. doi: 10.1139/cjm-2018-0503

Feng, J., Ma, L., Nie, J., Konkel, M. E., and Lu, X. (2018). Environmental stress-induced bacterial lysis and extracellular DNA release contribute to Campylobacter jejuni biofilm formation. *Appl. Environ. Microbiol.* 84, e02068–e02017. doi: 10.1128/AEM.02068-17

Fernández, H., Vergara, M., and Tapia, F. (1985). Dessication resistance in thermotolerant Campylobacter species. *Infection* 13, 197. doi: 10.1007/BF01642813

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

Ferrero, R. L., and Lee, A. (1988). Motility of Campylobacter jejuni in a viscous environment: comparison with conventional rod-shaped bacteria. *J. Gen. Microbiol.* 134, 53–59. doi: 10.1099/00221287-134-1-53

Fields, J. A., and Thompson, S. A. (2008). Campylobacter jejuni CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. *J. Bacteriol.* 190, 3411–3416. doi: 10.1128/JB.01928-07

Frirdich, E., Biboy, J., Pryjma, M., Lee, J., Huynh, S., Parker, C. T., et al. (2019). The Campylobacter jejuni helical to coccoid transition involves changes to peptidoglycan and the ability to elicit an immune response. *Mol. Microbiol.* 112, 280–301. doi: 10.1111/mmi.14269

Gangaiah, D., Liu, Z., Arcos, J., Kassem, I. I., Sanad, Y., Torrelles, J. B., et al. (2010). Polyphosphate kinase 2: a novel determinant of stress responses and pathogenesis in Campylobacter jejuni. *PloS One* 5, e12142. doi: 10.1371/journal.pone.0012142

García-Peña, F. J., Llorente, M. T., Serrano, T., Ruano, M. J., Belliure, J., Benzal, J., et al. (2017). Isolation of Campylobacter spp. from three species of antarctic penguins in different geographic locations. *EcoHealth* 14, 78–87. doi: 10.1007/s10393-016-1203-z

Gaynor, E. C., Wells, D. H., MacKichan, J. K., and Falkow, S. (2005). The Campylobacter jejuni stringent response controls specific stress survival and virulence-associated phenotypes. *Mol. Microbiol.* 56, 8–27. doi: 10.1111/j.1365-2958.2005.04525.x

Geng, Y., Liu, G., Liu, L., Deng, Q., Zhao, L., Sun, X. X., et al. (2019). Real-time recombinase polymerase amplification assay for the rapid and sensitive detection of Campylobacter jejuni in food samples. *J. Microbiol. Methods* 157, 31–36. doi: 10.1016/j.mimet.2018.12.017

Gerth, U., Krüger, E., Derré, I., Msadek, T., and Hecker, M. (1998). Stress induction of the Bacillus subilis clpP gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. *Mol. Microbiol.* 28, 787–802. doi: 10.1046/j.1365-2958.1998.00840.x

Ghazaei, C. (2017). Role and mechanism of the Hsp70 molecular chaperone machines in bacterial pathogens. *J. Med. Microbiol.* 66 (3), 259–265. doi: 10.1099/jmm.0.000429

Giuliodori, A. M., di Pietro, F., Marzi, S., Masquida, B., Wagner, R., Romby, P., et al. (2010). The cspA mRNA is a thermosensor that modulates translation of the cold-shock protein CspA. *Mol. Cell* 37, 21–33. doi: 10.1016/j.molcel.2009.11.033

Gomes, C. N., Passaglia, J., Vilela, F. P., da Silva, F. M. P., Duque, S. S., and Falcao, J. P. (2018). High survival rates of Campylobacter coli under different stress conditions suggest that more rigorous food control measures might be needed in Brazil. *Food Microbiol.* 73, 327–333. doi: 10.1016/j.fm.2018.02.014

Graillot, V., Dormoy, I., Dupuy, J., Shay, J. W., Huc, L., Mirey, G., et al. (2016). Genotoxicity of cytolethal distending toxin (CDT) on isogenic human colorectal cell lines: potential promoting effects for colorectal carcinogenesis. *Front. Cell. Infect. Microbiol.* 6, 34. doi: 10.3389/fcimb.2016.00034

Guerry, P. (2007). Campylobacter flagella: not just for motility. *Trends Microbiol.* 15, 456–461. doi: 10.1016/j.tim.2007.09.0

Guévremont, E., Lamoureux, L., Ward, P., and Villeneuve, S. (2015). Survival of Campylobacter jejuni on fresh spinach stored at 4 °C or 12 °C. *Food Control* 50, 736–739. doi: 10.1016/j.foodcont.2014.10.023

Habib, I., Uyttendaele, M., and de Zutter, L. (2010). Survival of poultry-derived Campylobacter jejuni of multilocus sequence type clonal complexes 21 and 45 under freeze, chill, oxidative, acid and heat stresses. *Food Microbiol.* 27, 829–834. doi: 10.1016/j.fm.2010.04.009

Haughton, P. N., Lyng, J. G., Morgan, D. J., Cronin, D. A., Fanning, S., and Whyte, P. (2011). Efficacy of high-intensity pulsed light for the microbiological decontamination of chicken, associated packaging, and contact surfaces. *Foodborne Pathog. Dis.* 8, 109–117. doi: 10.1089/fpd.2010.0640

Hazeleger, W. C., Wouters, J. A., Rombouts, F. M., and Abee, T. (1998). Physiological activity of Campylobacter jejuni far below the minimal growth temperature. *Appl. Environ. Microbiol.* 64, 3917–3922. doi: 10.1128/AEM.64.10.3917-3922.1998

Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., et al. (2010). Cronobacter (Enterobacter sakazakii): an opportunistic foodborne pathogen. *Foodborne Pathog. Dis.* 7, 339–350. doi: 10.1089/fpd.2009.0379

Hénault-Ethier, L., Martin, V. J., and Gélinas, Y. (2016). Persistence of Escherichia coli in batch and continuous vermicomposting systems. *Waste Manage.* 56, 88–99. doi: 10.1016/j.wasman.2016.07.033

Hugdahl, M. B., Beery, J. T., and Doyle, M. P. (1988). Chemotactic behavior of Campylobacter jejuni. *Infect. Immun.* 56, 1560–1566. doi: 10.1128/IAI.56.6.1560-1566.1988

Humphrey, T., Mason, M., and Martin, K. (1995). The isolation of Campylobacter jejuni from contaminated surfaces and its survival in diluents. *Int. J. Food Microbiol.* 26, 295–303. doi: 10.1016/0168-1605(94)00135-s

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

Hwang, S., Jeon, B., Yun, J., and Ryu, S. (2011). Roles of RpoN in the resistance of Campylobacter jejuni under various stress conditions. *BMC Microbiol.* 11, 207. doi: 10.1186/1471-2180-11-207

Isohanni, P. M., and Lyhs, U. (2009). Use of ultraviolet irradiation to reduce Campylobacter jejuni on broiler meat. *Poult. Sci.* 88, 661–668. doi: 10.3382/ps.2008-00259

Isohanni, P., Huehn, S., Aho, T., Alter, T., and Lyhs, U. (2013). Heat stress adaptation induces cross-protection against lethal acid stress conditions in Arcobacter butzleri but not in Campylobacter jejuni. *Food Microbiol.* 34, 431–435. doi: 10.1016/j.fm.2013.02.001

Jacoby, G. A. (2005). Mechanisms of resistance to quinolones. *Clin. Infect. Dis.* 41, S120–S126. doi: 10.1086/428052

Jang, K. I., Kim, M. G., Ha, S. D., Kim, K. S., Lee, K. H., Chung, D. H., et al. (2007). Morphology and adhesion of Campylobacter jejuni to chicken skin under varying conditions. *J. Microbiol. Biotechnol.* 17, 202–206.

Jin, X., and Riedel-Kruse, I. H. (2018). Biofilm Lithography enables high-resolution cell patterning via optogenetic adhesin expression. *Proc. Natl. Acad. Sci. U. S. A.* 115, 3698–3703. doi: 10.1073/pnas.1720676115

Jones, D. M., Sutcliffe, E. M., and Curry, A. (1991). Recovery of viable but non-culturable Campylobacter jejuni. *J. Gen. Microbiol.* 137, 2477–2482. doi: 10.1099/00221287-137-10-2477

Jones, D. M., Sutcliffe, E. M., Rios, R., Fox, A. J., and Curry, A. (1993). Campylobacter jejuni adapts to aerobic metabolism in the environment. *J. Med. Microbiol.* 38, 145–150. doi: 10.1099/00222615-38-2-145

Kaakoush, N. O., Miller, W. G., de Reuse, H., and Mendz, G. L. (2007). Oxygen requirement and tolerance of Campylobacter jejuni. *Res. Microbiol.* 158, 644–650. doi: 10.1016/j.resmic.2007.07.009

Kang, G. Z., Li, G. Z., Liu, G. Q., Xu, W., Peng, X. Q., Wang, C. Y., et al. (2013). Exogenous salicylic acid enhances wheat drought tolerance by influence on the expression of genes related to ascorbate-glutathione cycle. *Biol. Plantarum* 57 (4), 718–724. doi: 10.1007/s10535-013-0335-z

Karki, A. B., Wells, H., and Fakhr, M. K. (2019). Retail liver juices enhance the survivability of Campylobacter jejuni and Campylobacter coli at low temperatures. *Sci. Rep.* 9, 2733. doi: 10.1038/s41598-018-35820-7

Kim, J. S., Artymovich, K. A., Hall, D. F., Smith, E. J., Fulton, R., Bell, J., et al. (2012). Passage of Campylobacter jejuni through the chicken reservoir or mice promotes phase variation in contingency genes Cj0045 and Cj0170 that strongly associates with colonization and disease in a mouse model. *Microbiology* 158, 1304–1316. doi: 10.1099/mic.0.057158-0

Kim, J. C., Oh, E., Kim, J., and Jeon, B. (2015). Regulation of oxidative stress resistance in Campylobacter jejuni, a microaerophilic foodborne pathogen. *Front. Microbiol.* 6, 751. doi: 10.3389/fmicb.2015.00751

Kim, S. H., Park, C., Lee, E. J., Bang, W. S., Kim, Y. J., and Kim, J. S. (2017). Biofilm formation of Campylobacter strains isolated from raw chickens and its reduction with DNase I treatment. *Food Control.* 71, 94–100. doi: 10.1016/j.foodcont.2016.06.038

Klancnik, A., Guzej, B., Jamnik, P., Vuckovic, D., Abram, M., and Mozina, S. S. (2009). Stress response and pathogenic potential of Campylobacter jejuni cells exposed to starvation. *Res. Microbiol.* 160, 345–352. doi: 10.1016/j.resmic.2009.05.002

Kovacs, J. K., Felso, P., Horvath, G., Schmidt, J., Dorn, A., Abraham, H., et al. (2019). Stress response and virulence potential modulating effect of peppermint essential oil in Campylobacter jejuni. *BioMed. Res. Int.* 2019, 2971741. doi: 10.1155/2019/2971741

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C., and Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *Int. J. Food Microbiol.* 85, 227–236. doi: 10.1016/S0168-1605(02)00540-8

Lake, I. R., Colón-González, F. J., Takkinen, J., Rossi, M., Sudre, B., Dias, J. G., et al. (2019). Exploring Campylobacter seasonality across Europe using The European Surveillance System (TESSy), 2008 to 2016. *Euro Surveill.* 24, 1800028. doi: 10.2807/1560-7917.ES.2019.24.13.180028

Lapierre, L., Gatica, M. A., Riquelme, V., Vergara, C., Yanez, J. M., San Martin, B., et al. (2016). Characterization of antimicrobial susceptibility and its association with virulence genes related to adherence, invasion, and cytotoxicity in Campylobacter jejuni and Campylobacter coli isolates from animals, meat, and humans. *Microb. Drug Resist.* 22, 432–444. doi: 10.1089/mdr.2015.0055

Laranjo, M., and Oliveira, S. (2011). Tolerance of Mesorhizobium type strains to different environmental stresses. *Antonie Van Leeuwenhoek.* 99, 651–662. doi: 10.1007/s10482-010-9539-9

Lertsethtakarn, P., Ottemann, K. M., and Hendrixson, D. R. (2011). Motility and chemotaxis in Campylobacter and Helicobacter. *Annu. Rev. Microbiol.* 65, 389–410. doi: 10.1146/annurev-micro-090110-102908

Li, X., Kim, M.-J., and Yuk, H.-G. (2018). Influence of 405 nm light-emitting diode illumination on the inactivation of Listeria monocytogenes and Salmonella spp. on ready-to-eat fresh salmon surface at chilling storage for 8 h and their susceptibility to simulated gastric fluid. *Food Control* 88, 61–68. doi: 10.1016/j.foodcont.2018.01.002

Ligowska, M., Cohn, M. T., Stabler, R. A., Wren, B. W., and Brondsted, L. (2011). Effect of chicken meat environment on gene expression of Campylobacter jejuni and its relevance to survival in food. *Int. J. Food Microbiol.* 145, S111–S115. doi: 10.1016/j.ijfoodmicro.2010.08.027

Lin, A. E., Krastel, K., Hobb, R.II, Thompson, S. A., Cvitkovitch, D. G., and Gaynor, E. C. (2009a). Atypical roles for Campylobacter jejuni amino acid ATP binding cassette transporter components PaqP and PaqQ in bacterial stress tolerance and pathogen-host cell dynamics. *Infect. Immun.* 77 (11), 4912–4924. doi: 10.1128/IAI.00571-08

Lin, A. E., Krastel, K., Hobb, R.II, Thompson, S. A., Cvitkovitch, D. G., and Gaynor, E. C. (2009b). Atypical roles for Campylobacter jejuni AA-ABC transporter components PaqP and PaqQ in bacterial stress tolerance and pathogen-host cell dynamics. *Infect. Immun.* 7 (11). doi: 10.1128/IAI.00571-08

Linton, D., Gilbert, M., Hitchen, P. G., Dell, A., Morris, H. R., Wakarchuk, W. W., et al. (2000). Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of Campylobacter jejuni. *Mol. Microbiol.* 37, 501–514. doi: 10.1046/j.1365-2958.2000.02020.x

Longo, A., and Spano, G. (2019). "Stress responses of LAB," in *Food molecular microbiology*. Eds. S. Paramithiotis and J. K. Patra (UK: CRC Press), 164–182.

Lu, X., Liu, Q., Wu, D., Al-Qadiri, H. M., Al-Alami, N. I., Kang, D. H., et al. (2011). Using of infrared spectroscopy to study the survival and injury of Escherichia coli O157:H7, Campylobacter jejuni and Pseudomonas aeruginosa under cold stress in low nutrient media. *Food Microbiol.* 28, 537–546. doi: 10.1016/j.fm.2010.11.002

Luo, C., Lü, F., Shao, L., and He, P. (2015). Application of eco-compatible biochar in anaerobic digestion to relieve acid stress and promote the selective colonization of functional microbes. *Water Res.* 68, 710–718. doi: 10.1016/j.watres.2014.10.052

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

Ma, Z., Bumunang, E. W., Stanford, K., Bie, X., Niu, Y. D., and McAllister, T. A. (2019). Biofilm formation by shiga toxin-producing Escherichia coli on stainless steel coupons as affected by temperature and incubation time. *Microorganisms* 7:95. doi: 10.3390/microorganisms7040095

Maes, S., Heyndrickx, M., Vackier, T., Steenackers, H., Verplaetse, A., and Reu, K. (2019). Identification and spoilage potential of the remaining dominant microbiota on food contact surfaces after cleaning and disinfection in different food industries. *J. Food Prot.* 82, 262–275. doi: 10.4315/0362-028X.JFP-18-226

Magajna, B. A., and Schraft, H. (2015). Campylobacter jejuni biofilm cells become viable but non-culturable (VBNC) in low nutrient conditions at 4 °C more quickly than their planktonic counterparts. *Food Control.* 50, 45–50. doi: 10.1016/j.foodcont.2014.08.022

Master, S. S., Springer, B., Sander, P., Boettger, E. C., Deretic, V., and Timmins, G. S. (2002). Oxidative stress response genes in Mycobacterium tuberculosis: role of ahpC in resistance to peroxynitrite and stage-specific survival in macrophages. *Microbiology* 148, 3139–3144. doi: 10.1099/00221287-148-10-3139

Mavri, A., Ribič, U., and Možina, S. S. (2016). "The biocide and antibiotic resistance in Campylobacter jejuni and Campylobacter coli," in *Emerging and traditional technologies for safe, healthy and quality food*. Eds. V. Nedovič, P. Raspor, J. Levič, V. T. Šaponjac and G. V. Barbosa-Cánovas (Springer, Cham: International Publishing Switzerland), 269–283.

Mayton, H. M., Marcus, I. M., and Walker, S. L. (2019). Escherichia coli O157:H7 and Salmonella Typhimurium adhesion to spinach leaf surfaces: Sensitivity to water chemistry and nutrient availability. *Food Microbiol.* 78, 134–142. doi: 10.1016/j.fm.2018.10.002

Meade, K. G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B., and O'Farrelly, C. (2009). Comparative in vivo infection models yield insights on early host immune response to Campylobacter in chickens. *Immunogenetics* 61, 101–110. doi: 10.1007/s00251-008-0346-7

Mehat, J. W., Park, S. F., van Vliet, A. H. M., and La Ragione, R. M. (2018). CapC, a novel autotransporter and virulence factor of Campylobacter jejuni. *Appl. Environ. Microbiol.* 84, e01032–e01018. doi: 10.1128/AEM.01032-18

Melo, R. T., Grazziotin, A. L., Valadares Júnior, E. C., Prado, R. R., Mendonça, E. P., Monteiro, G. P., et al. (2019). Evolution of Campylobacter jejuni of poultry origin in Brazil. *Food Microbiol.* 82, 489–496. doi: 10.1016/j.fm.2019.03.009

Metselaar, K. I., Ibusquiza, P. S., Camargo, A. R. O., Krieg, M., Zwietering, M. H., den Besten, H. M. W., et al. (2015). Performance of stress resistant variants of Listeria monocytogenes in mixed species biofilms with Lactobacillus plantarum. *Int. J. Food Microbiol.* 213, 24–30. doi: 10.1016/j.ijfoodmicro.2015.04.021

Miao, J., Lin, S., Soteyome, T., Peters, B. M., Li, Y., Chen, H., et al. (2019). Biofilm formation of Staphylococcus aureus under food heat processing conditions: first report on CML production within biofilm. *Sci. Rep.* 9, 1312. doi: 10.1038/s41598-018-35558-2

Monk, C. E., Pearson, B. M., Mulholland, F., Smith, H. K., and Poole, R. K. (2008). Oxygen- and NssR-dependent globin expression and enhanced iron acquisition in the response of Campylobacter to nitrosative stress. *J. Biol. Chem.* 283, 28413–28425. doi: 10.1074/jbc.M801016200

Mühlig, A., Behr, J., Scherer, S., and Müller-Herbst, S. (2014). Stress response of Salmonella enterica serovar typhimurium to acidified nitrite. *Appl. Environ. Microbiol.* 80, 6373–6382. doi: 10.1128/AEM.01696-14

Murphy, C., Carroll, C., and Jordan, K. N. (2003). Induction of an adaptive tolerance response in the foodborne pathogen, Campylobacter jejuni. *FEMS Microbiol. Lett.* 223, 89–93. doi: 10.1016/S0378-1097(03)00348-3

Nachamkin, I., Yang, X. H., and Stern, N. J. (1993). Role of Campylobacter jejuni flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. *Appl. Environ. Microbiol.* 59, 1269–1273. doi: 10.1128/AEM.59.5.1269-1273.1993

Narvaez-Bravo, C., Taboada, E. N., Mutschall, S. K., and Aslam, M. (2017). Epidemiology of antimicrobial resistant Campylobacter spp. isolated from retail meats in Canada. *Int. J. Food Microbiol.* 253, 43–47.

Negretti, N. M., Clair, G., Talukdar, P. K., Gourley, C. R., Huynh, S., Adkins, J. N., et al. (2019). Campylobacter jejuni demonstrates conserved proteomic and transcriptomic responses when co-cultured with human INT 407 and Caco-2 epithelial cells. *Front. Microbiol.* 10, 755. doi: 10.3389/fmicb.2019.00755

Nguyen, H. T. T., Corry, J. E. L., and Miles, C. A. (2006). Heat resistance and mechanism of heat inactivation in thermophilic campylobacters. *Appl. Environ. Microbiol.* 72, 908–913. doi: 10.1128/AEM.72.1.908-913.2006

Noreen, Z. (2019). *Role of Type VI Secretion System in Stress Adaptations and Developing Control Strategies for Campylobacter jejuni (Doctoral dissertation* (Islamabad: COMSATS University).

Oh, E., McMullen, L., and Jeon, B. (2015). Impact of oxidative stress defense on bacterial survival and morphological change in Campylobacter jejuni under aerobic conditions. *Front. Microbiol.* 6, 295. doi: 10.3389/fmicb.2015.00295

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

Oh, E., Andrews, K. J., McMullen, L. M., and Jeon, B. (2019). Tolerance to stress conditions associated with food safety in Campylobacter jejuni strains isolated from retail raw chicken. *Sci. Rep.* 9 (1), 1–9. doi: 10.1038/s41598-019-48373-0

Olesen, I., Vogensen, F. K., and Jespersen, L. (2009). Gene transcription and virulence potential of Listeria monocytogenes strains after exposure to acidic and NaCl stress. *Foodborne Pathog. Dis.* 6, 669–680. doi: 10.1089/fpd.2008.0243

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34, 415–425. doi: 10.1111/j.1574-6976.2009.00200.x

Olsen, I. (2015). Biofilm-specific antibiotic tolerance and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 877–886. doi: 10.1007/s10096-015-2323-z

Oosterom, J., de Wilde, G. J. A., de Boer, E., de Blaauw, L. H., and Karman, H. (1983). Survival of Campylobacter jejuni during poultry processing and pig slaughtering. *J. Food Prot.* 46, 702–706. doi: 10.4315/0362-028X-46.8.702

O'Sullivan, L., Lucid, A., Neve, H., Franz, C. M., Bolton, D., McAuliffe, O., et al. (2018). Comparative genomics of Cp8viruses with special reference to Campylobacter phage vB_CjeM_los1, isolated from a slaughterhouse in Ireland. *Arch. Virol.* 163 (8), 2139–2154.

Otigbu, A. C., Clarke, A. M., Fri, J., Akanbi, E. O., and Njom, H. A. (2018). Antibiotic sensitivity profiling and virulence potential of Campylobacter jejuni isolates from estuarine water in the Eastern Cape Province, South Africa. *Int. J. Environ. Res. Public Health* 15, 925. doi: 10.3390/ijerph15050925

Ovesen, S., Durack, J., Kirk, K. F., Nielsen, H. L., Nielsen, H., and Lynch, S. V. (2019). Motility and biofilm formation of the emerging gastrointestinal pathogen Campylobacter concisus differs under microaerophilic and anaerobic environments. *Gut Microbes.* 10, 34–44. doi: 10.1080/19490976.2018.1472201

Park, S. F. (2002). The physiology of Campylobacter species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol.* 74, 177–188. doi: 10.1016/s0168-1605(01)00678-x

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Parsell, D. A., and Lindquist, S. (1993). The function of heat-shock proteins stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27, 437–496. doi: 10.1146/annurev.ge.27.120193.002253

Pedersen, S. K., Wagenaar, J. A., Vigre, H., Roer, L., Mikoleit, M., Aidara-Kane, A., et al. (2018). Proficiency of WHO global foodborne infections network external quality assurance system participants in identification and susceptibility testing of thermotolerant Campylobacter spp. from 2003 to 2012. *J. Clin. Microbiol.* 56, e01066–e01018. doi: 10.1128/JCM.01066-18

Petersen, L., Larsen, T. S., Ussery, D. W., On, S. L., and Krogh, A. (2003). RpoD promoters in Campylobacter jejuni exhibit a strong periodic signal instead of a -35 box. *J. Mol. Biol.* 326, 1361–1372. doi: 10.1016/S0022-2836(03)00034-2

Pittman, M. S., Elvers, K. T., Lee, L., Jones, M. A., Poole, R. K., Park, S. F., et al. (2007). Growth of Campylobacter jejuni on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. *Mol. Microbiol.* 63, 575–590. doi: 10.1111/j.1365-2958.2006.05532.x

Poli, V. F., Thorsen, L., Olesen, I., Wik, M. T., and Jespersen, L. (2012). Differentiation of the virulence potential of Campylobacter jejuni strains by use of gene transcription analysis and a Caco-2 assay. *Int. J. Food Microbiol.* 155, 60–68. doi: 10.1016/j.ijfoodmicro.2012.01.019

Porcelli, I., Reuter, M., Pearson, B. M., Wilhelm, T., and van Vliet, A. H. M. (2013). Parallel evolution of genome structure and transcriptional landscape in the Epsilonproteobacteria. *BMC Genomics* 14, 616. doi: 10.1186/1471-2164-14-616

Poursina, F., Fagri, J., Mirzaei, N., and Safaei, H. G. (2018). Overexpression of spoT gene in coccoid forms of clinical Helicobacter pylori isolates. *Folia Microbiol.* 63, 459–465. doi: 10.1007/s12223-017-0557-0

Prechtl, R. M., Janßen, D., Behr, J., Ludwig, C., Küster, B., Vogel, R. F., et al. (2018). Sucrose-induced proteomic response and carbohydrate utilization of Lactobacillus sakei TMW 1.411 during dextran formation. *Front. Microbiol.* 9, 2796. doi: 10.3389/fmicb.2018.02796

Rodrigues, B., Alvares, T., Costa, M., Sampaio, G., de L. Torre, C. A. L., Panzenhagen, P., et al (2019). Combined effect of modified atmosphere package and short-wave ultraviolet does not affect Proteus mirabilis growth on Rainbow Trout Fillets (Oncorhynchus mykiss). *J. Food Nutr. Res.* 7, 342–346. doi: 10.12691/jfnr-7-5-2

Sałamaszyńska-Guz, A., Rose, S., Lykkebo, C. A., Taciak, B., Bącal, P., Uśpieński, T., et al. (2018). Biofilm formation and motility are promoted by Cj0588-directed methylation of rRNA in Campylobacter jejuni. *Front. Cell. Infect. Microbiol.* 7, 533. doi: 10.3389/fcimb.2017.00533

Salma, M., Rousseaux, S., Sequeira-Le Grand, A., Divol, B., and Alexandre, H. (2013). Characterization of the viable but nonculturable (VBNC) state in Saccharomyces cerevisiae. *PloS One* 8, e77600. doi: 10.1371/journal.pone.0077600

Sarabhai, S., Harjai, K., Sharma, P., and Capalash, N. (2015). Ellagic acid derivatives from Terminalia chebula Retz. increase the susceptibility of Pseudomonas aeruginosa to stress by inhibiting polyphosphate kinase. *J. Appl. Microbiol.* 118, 817–825. doi: 10.1111/jam.12733

Scuron, M. D., Boesze-Battaglia, K., Dlakic, M., and Shenker, B. J. (2016). The cytolethal distending toxin contributes to microbial virulence and disease pathogenesis by acting as a tri-perditious toxin. *Front. Cell. Infect. Microbiol.* 6, 168. doi: 10.3389/fcimb.2016.00168

Silvan, J. M., Zorraquin-Pena, I., de Llano, D. G., Moreno-Arribas, M. V., and Martinez-Rodriguez, A. J. (2018). Antibacterial activity of glutathione-stabilized silver nanoparticles against Campylobacter multidrug-resistant strains. *Front. Microbiol.* 9, 458. doi: 10.3389/fmicb.2018.00458

Simmons, M., Hiett, K. L., Stern, N. J., and Frank, J. F. (2008). Comparison of poultry exudate and carcass rinse sampling methods for the recovery of Campylobacter spp. subtypes demonstrates unique subtypes recovered from exudate. *J. Microbiol. Methods* 74, 89–93. doi: 10.1016/j.mimet.2008.03.007

Simões, L. C., and Simões, M. (2013). Biofilms in drinking water: problems and solutions. *RSC Adv.* 3, 2520–2533. doi: 10.1039/c2ra22243d

Sommerlad, S. M., and Hendrixson, D. R. (2007). Analysis of the roles of FlgP and FlgQ in flagellar motility of Campylobacter jejuni. *J. Bacteriol.* 189, 179–186. doi: 10.1128/JB.01199-06

Spector, M. P., and Kenyon, W. J. (2012). Resistance and survival strategies of Salmonella enterica to environmental stresses. *Food Res. Int.* 45, 455–481. doi: 10.1016/j.foodres.2011.06.056

Speranza, B., Corbo, M. R., and Sinigaglia, M. (2011). Effects of nutritional and environmental conditions on Salmonella sp. biofilm formation. *J. Food Sci.* 76, M12–M16. doi: 10.1111/j.1750-3841.2010.01936.x

Stewart, P. S., and Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138. doi: 10.1016/S0140-6736(01)05321-1

Storz, G., and Imlay, J. A. (1999). Oxidative stress. *Curr. Opin. Microbiol.* 2, 188–194. doi: 10.1016/s1369-5274(99)80033-2

Studholme, D. J., and Dixon, R. (2003). Domain architectures of σ54-dependent transcriptional activators. *J. Bacteriol.* 185, 1757–1767. doi: 10.1128/JB.185.6.1757-1767.2003

Svensson, S. L., Frirdich, E., and Gaynor, E. C. (2008). "Chapter 32: Survival strategies of Campylobacter jejuni: stress responses, the viable but nonculturable state, and biofilms," in *Campylobacter, 3rd ed.* Eds. I. Nachamkin, C. M. Szymanski and M. J. Blaser (Washington, DC: ASM Press), 571–590.

Svensson, S. L., Davis, L. M., Mackichan, J. K., Allan, B. J., Pajaniappan, M., Thompson, S. A., et al. (2009). The CprS sensor kinase of the zoonotic pathogen Campylobacter jejuni influences biofilm formation and is required for optimal chick colonization. *Mol. Microbiol.* 71, 253–272. doi: 10.1111/j.1365-2958.2008.06534.x

Tang, Y., Cawthraw, S., Bagnall, M. C., Gielbert, A. J., Woodward, M. J., and Petrovska, L. (2017). Identification of temperature regulated factors of Campylobacter jejuni and their potential roles in virulence. *AIMS Microbiol.* 3 (4), 885. doi: 10.3934/microbiol.2017.4.885

Taylor, A. J., Zakai, S. A., and Kelly, D. J. (2017). The periplasmic chaperone network of Campylobacter jejuni: evidence that SalC (Cj1289) and PpiD (Cj0694) are involved in maintaining outer membrane integrity. *Front. Microbiol.* 8, 531. doi: 10.3389/fmicb.2017.00531

The, A. H. T., Lee, S. M., and Dykes, G. A. (2019). Association of some Campylobacter jejuni with Pseudomonas aeruginosa biofilms increases attachment under conditions mimicking those in the environment. *PloS One* 14, e0215275. doi: 10.1371/journal.pone.0215275

Ultee, E., Ramijan, K., Dame, R. T., Briegel, A., and Claessen, D. (2019). Stress-induced adaptive morphogenesis in bacteria. *Adv. Microb. Physiol.* 74, 97–141. doi: 10.1016/bs.ampbs.2019.02.001

Upadhyay, A., Arsi, K., Upadhyaya, I., Donoghue, A. M., and Donoghue, D. J. (2019). "Natural and environmentally friendly strategies for controlling Campylobacter jejuni colonization in poultry, survival in poultry products and infection in humans," in *Food safety in poultry meat production*. Eds. K. Venkitanarayanan, S. Thakur and S. C. Ricke (CT, USA: Springer Nature Switzerland), 67–93.

Vázquez-Sánchez, D., Habimana, O., and Holck, A. (2013). Impact of food-related environmental factors on the adherence and biofilm formation of natural Staphylococcus aureus isolates. *Curr. Microbiol.* 66, 110–121. doi: 10.1007/s00284-012-0247-8

Völker, U., Mach, H., Schmid, R., and Hecker, M. (1992). Stress proteins and cross-protection by heat shock and salt stress in Bacillus subtilis. *J. Gen. Microbiol.* 138, 2125–2135. doi: 10.1099/00221287-138-10-2125

Wadhams, G. H., and Armitage, J. P. (2004). Making sense of it all: bacterial chemotaxis. *Nat. Rev. Mol. Cell Biol.* 5, 1024–1037. doi: 10.1038/nrm1524

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., et al. (2002). Colony multiplex PCR assay for identification and differentiation of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, and C. fetus subsp. fetus. *J. Clin. Microbiol.* 40, 4744–4747. doi: 10.1128/JCM.40.12.4744-4747.2002

Wang, H., Ding, S., Wang, G., Xu, X., and Zhou, G. (2013). In situ characterization and analysis of Salmonella biofilm formation under meat processing environments using a combined microscopic and spectroscopic approach. *Int. J. Food Microbiol.* 167, 293–302. doi: 10.1016/j.ijfoodmicro.2013.10.005

Wang, C., Zhou, H., Guo, F., Yang, B., Su, X., Lin, J., et al. (2020). Oral immunization of chickens with Lactococcus lactis expressing cjaA temporarily reduces Campylobacter jejuni colonization. *Foodborne Pathog. Dis.* 17 (6), 366–372.

Wassenaar, T. M., van der Zeijst, B. A., Ayling, R., and Newell, D. G. (1993). Colonization of chicks by motility mutants of Campylobacter jejuni demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* 139, 1171–1175. doi: 10.1099/00221287-139-6-1171

Whitehouse, C. A., Zhao, S., and Tate, H. (2018). Antimicrobial resistance in Campylobacter species: mechanisms and genomic epidemiology. *Adv. Appl. Microbiol.* 103, 1–47. doi: 10.1016/bs.aambs.2018.01.001

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

Wieczorek, K., Wolkowicz, T., and Osek, J. (2018). Antimicrobial resistance and virulence-associated traits of Campylobacter jejuni isolated from poultry food chain and humans with diarrhea. *Front. Microbiol.* 9, 1508. doi: 10.3389/fmicb.2018.01508

Wirz, S. E., Overesch, G., Kuhnert, P., and Korczak, B. M. (2010). Genotype and antibiotic resistance analyses of Campylobacter isolates from ceca and carcasses of slaughtered broiler flocks. *Appl. Environ. Microbiol.* 76 (19), 6377–6386. doi: 10.1128/AEM.00813-10

Wong, E., Linton, R. H., and Gerrard, D. E. (1998). Reduction of Escherichia coli and Salmonella senftenbergon pork skin and pork muscle using ultraviolet light. *Food Microbiol.* 15, 415–423. doi: 10.1006/fmic.1998.0185

Wösten, M. M. S. M., Boeve, M., Koot, M. G. A., van Nuenen, A. C., and van der Zeijst, B. A. M. (1998). Identification of Campylobacter jejuni promoter sequences. *J. Bacteriol.* 180, 594–599. doi: 10.1128/JB.180.3.594-599.1998

Wuichet, K., Alexander, R. P., and Zhulin, I. B. (2007). Comparative genomic and protein sequence analyses of a complex system controlling bacterial chemotaxis. *Methods Enzymol.* 422, 1–31. doi: 10.1016/S0076-6879(06)22001-9

Xiong, J. (2009). Survival of Animal-derived Campylobacter Strains in Raw and Pasteurized Milk, and the Roles of Capsule in Campylobacter Survival in vitro, and in Chick Colonization. MS Thesis, 2009-09-10, University of North Carolina. Available at: http://www.lib.ncsu.edu/resolver/1840.16/1045

Xu, G., Li, C., and Yao, Y. (2009). Proteomics analysis of drought stress-responsive proteins in Hippophae rhamnoides L. *Plant Mol. Biol. Rep.* 27 (2), 153–161. doi: 10.1007/s11105-008-0067-y

Xu, T., Yu, M., Liu, J., Lin, H., Liang, J., and Zhang, X. H. (2019). Role of RpoN from Labrenzia aggregata LZB033 (Rhodobacteraceae) in formation of flagella and biofilms, motility, and environmental adaptation. *Appl. Environ. Microbiol.* 85, e02844–e02818. doi: 10.1128/AEM.02844-18

Yang, S., Sadekuzzaman, M., and Ha, S.-D. (2017). Reduction of Listeria monocytogenes on chicken breasts by combined treatment with UV-C light and bacteriophage ListShield. *LWT* 86, 193–200. doi: 10.1016/j.lwt.2017.07.060

Yaun, B. R., Sumner, S. S., Eifert, J. D., and Marcy, J. E. (2003). Response of Salmonella and Escherichia coli O157:H7 to UV energy. *J. Food Prot.* 66, 1071–1073. doi: 10.4315/0362-028x-66.6.1071

Ye, B., He, S., Zhou, X., Cui, Y., Zhou, M., and Shi, X. (2019). Response to acid adaptation in Salmonella enterica serovar enteritidis. *J. Food Sci.* 84, 599–605. doi: 10.1111/1750-3841.14465

Yoon, J. H., and Lee, S. Y. (2020). Characteristics of viable-but-nonculturable Vibrio parahaemolyticus induced by nutrient-deficiency at cold temperature. *Crit. Rev. Food Sci. Nutr.* 60, 1302–1320. doi: 10.1080/10408398.2019.1570076

Zeng, X., Xu, F., and Lin, J. (2013). Specific TonB-ExbB-ExbD energy transduction systems required for ferric enterobactin acquisition in Campylobacter. *FEMS Microbiol. Lett.* 347, 83–91. doi: 10.1111/1574-6968.12221

Zhang, L., Man, S. M., Day, A. S., Leach, S. T., Lemberg, D. A., Dutt, S., et al. (2009). Detection and isolation of Campylobacter species other than C. jejuni from children with Crohn's disease. *J. Clin. Microbiol.* 47, 453–455. doi: 10.1128/JCM.01949-08

Zhao, T., Ezeike, G. O., Doyle, M. P., Hung, Y. C., and Howell, R. S. (2003). Reduction of Campylobacter jejuni on poultry by low-temperature treatment. *J. Food Prot.* 66, 652–655. doi: 10.4315/0362-028x-66.4.652

Zhao, Z., Peng, T., Oh, J. I., Glaeser, J., Weber, L., Li, Q., et al. (2019). A response regulator of the OmpR family is part of the regulatory network controlling the oxidative stress response of Rhodobacter sphaeroides. *Environ. Microbiol. Rep.* 11, 118–128. doi: 10.1111/1758-2229.12718

Zhichang, Z., Wanrong, Z., Jinping, Y., Jianjun, Z., Xufeng, L. Z. L., and Yang, Y. (2010). Over-expression of Arabidopsis DnaJ (Hsp40) contributes to NaCl-stress tolerance. *Afr. J. Biotechnol.* 9, 972–978. doi: 10.5897/AJB09.1450

Zhuang, H., Rothrock, M. J. Jr., Hiett, K. L., Lawrence, K. C., Gamble, G. R., Bowker, B. C., et al. (2019). In-package air cold plasma treatment of chicken breast meat: treatment time effect. *J. Food Qual.* 2019, 1837351. doi: 10.1155/2019/1837351

Zwe, Y. H., Tang, V. C. Y., Aung, K. T., Gutiérrez, R. A., Ng, L. C., and Yuk, H.-G. (2018). Prevalence, sequence types, antibiotic resistance and, gyrA mutations of Salmonella isolated from retail fresh chicken meat in Singapore. *Food Control* 90, 233–240. doi: 10.1016/j.foodcont.2018.03.004

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Genomic Characterization of *Campylobacter jejuni* Adapted to the Guinea Pig (*Cavia porcellus*) Host

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Campylobacter jejuni is the leading bacterial cause of gastroenteritis worldwide with excessive incidence in low-and middle-income countries (LMIC). During a survey for *C. jejuni* from putative animal hosts in a town in the Peruvian Amazon, we were able to isolate and whole genome sequence two *C. jejuni* strains from domesticated guinea pigs (*Cavia porcellus*). The *C. jejuni* isolated from guinea pigs had a novel multilocus sequence type that shared some alleles with other *C. jejuni* collected from guinea pigs. Average nucleotide identity and phylogenetic analysis with a collection of *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* suggest that the guinea pig isolates are distinct. Genomic comparisons demonstrated gene gain and loss that could be associated with guinea pig host specialization related to guinea pig diet, anatomy, and physiology including the deletion of genes involved with selenium metabolism, including genes encoding the selenocysteine insertion machinery and selenocysteine-containing proteins.

Keywords: gastroenteritis, campylobacteriosis, *Campylobacter jejuni*, selenocysteine, source attribution

INTRODUCTION

The Gram-negative zoonotic bacteria *Campylobacter jejuni* is a principal cause of bacterial foodborne illness worldwide. *C. jejuni* is separated into two different sub-species, *C. jejuni* subsp. *jejuni* (*Cjj*) and *C. jejuni* subsp. *doylei* (*Cjd*). Of these *Cjj* is the most widely distributed subspecies commonly isolated from poultry and ruminants (Nauta et al., 2009; Kaakoush et al., 2015; Skarp et al., 2016; Mourkas et al., 2020). Contrarily, *Cjd* is infrequently isolated, and then, only directly from human bacteremia and gastroenteritis cases (Kasper and Dickgiesser, 1985; Steele et al., 1985).

Abbreviations: LMIC, low- to middle- income country; MLST, multi-locus sequence typing; ST, sequence type; CC, clonal complex; WGS, whole genome sequencing.

These two sub-species are phenotypically distinguishable by the inability of *Cjd* to reduce nitrate and growth instability at 42°C, and they are genetically distinct by several consistently conserved genomic features (Lastovica and Skirrow, 2000; Miller et al., 2007; Parker et al., 2007).

In high income countries, such as the United States, the most common source of *C. jejuni* infection are undercooked poultry products, but other sources include raw milk, contaminated water, and most recently, juvenile canines (Friedman et al., 2000; Centers for Disease C, Prevention, 2013; Pitkanen, 2013; Eurosurveillance Editorial Team, 2015; Kaakoush et al., 2015; Burakoff et al., 2018; Montgomery et al., 2018). In low- and middle-income countries (LMICs), such as Peru, where this pathogen remains one of the leading causes of bacterial gastroenteritis in children under the age of five, the epidemiology of *Campylobacter* remains understudied (Platts-Mills and Kosek, 2014; Platts-Mills et al., 2015). Although recent genomic studies have begun to unfold the relationship between poultry sources and human infections, this analysis is restricted by the number of *Campylobacter* genomes available for comparison (Pascoe et al., 2020). As a result, the exploration of additional *Campylobacter* hosts in LMICs is a pre-requisite for future disease control interventions.

Whole genome sequencing of *C. jejuni* has revealed evidence for lineages isolated from multiple species (generalists) and for lineages isolated from predominantly single host species (specialists) (Sheppard et al., 2011; Sheppard et al., 2014; Mourkas et al., 2020). For example, *C. jejuni* isolated from cattle predominately contained genes encoding vitamin B5 biosynthesis, while the genes were frequently absent from strains isolated from poultry (Sheppard et al., 2013). Aside from poultry and ruminants, *C. jejuni* has been detected and isolated from numerous different birds (Cody et al., 2015; Atterby et al., 2018; Lawton et al., 2018) and a variety of mammalian species, including but not limited to raccoons, rodents and lagomorphs (Graham et al., 2016; Mutschall et al., 2020). Among these host, wild birds are also often colonized by host specialist *C. jejuni* (Cody et al., 2015; Lawton et al., 2018; Atterby et al., 2018). Studies in Ecuador have found *C. jejuni* in domestic guinea pigs (*Cavia porcellus*) (Lowenstein et al., 2016; Toledo et al., 2017), and multilocus sequence typing (MLST) analysis of these isolates showed the presence of many unique sequence types (Graham et al., 2016). Among rural communities across Ecuador and Peru, guinea pigs are a substantial source of animal protein and are almost ubiquitously bred within communities located at high altitudes, however they are easily raised and commercialized throughout these countries.

In this study, we exploited whole genome sequencing (WGS) and comparative genomic analysis of *C. jejuni* isolates collected from guinea pigs, chickens and dogs in Santo Tomas, a town in the Peruvian Amazon, in order to ascertain possible sources of human infection. The isolates from the dogs and chickens were similar to *Cjj* isolated from human stool in this area of Peru. However, the analysis of genomes of *C. jejuni* strains isolated from guinea pigs provided evidence of considerable novel alterations including to gene gain and loss that presumably have allowed adaption to the guinea pig host.

MATERIALS AND METHODS

Sampling and Culture

Guinea pig fecal samples were obtained from a guinea pig breeder located in Santo Tomas, Iquitos, Peru in the Peruvian Amazon. This is a peri-urban community of approximately 1,500 households made-up of ~5,000 individuals. Guinea pigs are a common source of animal protein in other areas of country, such as the highlands of Peru, yet not the Peruvian Amazon. Household rearing of guinea pigs in these communities are unusual, and this specific collection site was part of a multi-species farm individually rearing guinea pigs, goats and pigs for commercial activities. Approximately 150 guinea pigs were housed in a single pen without any other animal species.

Fecal pellets were taken and placed in Cary Blair transport medium and processed within 24 hours. Stools were inoculated on *Campylobacter* Blood Free Selective agar base (Oxoid, Lenexa, KS, USA) without any supplementation. Plates were incubated for 48 to 72 hours at 37°C at 5% O_2, 10% CO_2, 85% N_2. Colonies demonstrating typical *Campylobacter* morphology were assessed using oxidase and catalase tests, as well as Gram staining. DNA was extracted from all bacterial cultures using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) as specified by manufacturer's instructions. A duplex qPCR targeting a 16S rRNA and the *Campylobacter* adhesion to fibronectin (*cadF)* genes was performed to confirm all bacterial cultures as *Campylobacter* spp. or *C. jejuni*/*C. coli.*

Bacterial Isolate Genome Sequencing

DNA was extracted from *C. jejuni* isolates (**Table 1**) and sequenced using an Illumina MiSeq platform. Sequencing libraries were prepared with the Nextera XT kit according to manufacturer's instructions (Illumina, San Diego, CA), and batches of 24 isolate gDNA were barcoded and sequenced in multiplex to achieve 80-120x coverage. The pooled libraries were loaded into a MiSeq system and sequenced using a MiSeq reagent kit (v2, 500 cycle; Illumina). The sequence reads were trimmed and assembled using the SPAdes assembler (ver. 3.13.0) (Bankevich et al., 2012) within Geneious Prime 2020.2.1. The average number of contigs was 64 (range: 43–101) for an average total assembled sequence size of 1.67 Mbp (range: 1.61–1.74). The average N50 contig length (L50) was 14,577 bp (range: 3,794-55,912 bp) and the average GC content was 30.8% (range: 30.5-31.6). Short read data and assembled WGS are available on the NCBI SRA and NCBI WGS and are associated with BioProjects PRJNA658163, PRJNA658164, PRJNA658165, PRJNA658166, PRJNA658168, PRJNA658171, PRJNA658172, and PRJNA658173.

Molecular Typing and Comparison

The isolate genomes were submitted to the pubMLST database (https://pubmlst.org/campylobacter/) for curation and analysis. MLST sequence types (STs) as described previously were assigned (Dingle et al., 2001; Jolley et al., 2018). The assigned *C. jejuni* STs were used to search the complete *C. jejuni* database (98,275 profiles present on August 1, 2020). Among the isolates in the database, isolates from a dataset used in a previous study in

TABLE 1 | *C. jejuni* strains collected in this study.

		Allele[a]									
Strain	**Genome size**	**aspA**	**glnA**	**gltA**	**glyA**	**pgm**	**tkt**	**uncA**	**ST**	**CC**	**Genome[b]**
Guineapig012	1.679	544	**538**	638	**601**	**730**	816	464	10316		JACRSF000000000
Guineapig013	1.681	544	**538**	638	**601**	**730**	816	464	10316		JACRSG000000000
Dog014	1.737	7	84	5	10	11	3	6	1036	ST-353	JACRSH000000000
Dog015	1.734	7	84	5	10	11	3	6	1036	ST-353	JACRSI000000000
Dog017	1.637	2	4	5	25	1057	203	5	10311		JACRSJ000000000
Chick018	1.618	2	17	5	10	11	3	6	8741	ST-353	JACRSK000000000
Chick019	1.620	2	17	5	10	11	3	6	8741	ST-353	JACRSL000000000
Chick020	1.620	2	17	5	10	11	3	6	8741	ST-353	JACRSM000000000

[a]*Allele numbers in bold were observed in guinea pig isolates from Ecuador column Genome[b].*

Ecuador were compared to the MLST data set that was determined here and initial relationships of MLST data were visualized as a minimum spanning tree using the GrapeTree plugin of pubMLST (Zhou et al., 2018) (data not shown).

Phylogenetic Analysis

A selection of MLST alleles from 82 *Campylobacter* (**Table 1**, **Supplementary Table 1**), including the eight isolates sequenced in this study, 44 isolated from animals in Ecuador (Graham et al., 2016), 18 additional Peruvian clinical isolates (Pascoe et al., 2020), eight from *Cjj* isolates, three from *Cjd* isolates, and one from *C. coli* were examined to determine the phylogenetic relationship. The MLST allele sequences were concatenated, and then alignments of the concatenated sequences were performed using the MAFFT (Katoh and Standley, 2013) plugin of Geneious Prime 2020.2.1. The neighbor-joining dendrogram and phylogenetic analyses were performed using MEGA version 7 (Kumar et al., 2016). Briefly, the dendrogram was constructed using the neighbor-joining method and Poisson correction. Bootstraps were conducted with 500 replicates. Similarly, for isolates with WGS data, the sequences for 62 core genes (see **Figure 2**) were concatenated, and then alignments of the concatenated sequences were performed using the MAFFT. Thirty-eight *Campylobacter* genomes, including the eight isolates from this study, 18 additional Peruvian clinical isolates (Pascoe et al., 2020), eight complete genome *Cjj* strains, three from *Cjd* strains, and one from *C. coli* were examined to determine the phylogenetic relationship in more detail. For each genome, the sequences for 62 core genes (see **Figure 2**) were concatenated, and then alignments of the concatenated sequences were performed using the MAFFT (Katoh and Standley, 2013) plugin of Geneious Prime 2020.2.1. The neighbor-joining dendrogram and phylogenetic analyses were performed using MEGA version 7 (Kumar et al., 2016). Briefly, the dendrogram was constructed using the neighbor-joining method and Poisson correction. Bootstraps were conducted with 500 replicates.

Pangenome Analysis

The thirty-eight *Campylobacter* strains selected for phylogenetic analysis were also utilized for the initial pangenome analysis to generate and visualize the Peruvian pangenome of *C. jejuni* compared to other well characterized *Campylobacter* strains using Anvi'o software (v6.2) (Eren et al., 2015) with the pangenomics workflow (Delmont and Eren, 2018). The species, subspecies and source for each of the strains was imported as additional layers in the database, and the average nucleotide identity (ANI) was generated using fastANI software (v1.31) (Jain et al., 2018). To visualize the different aspects of the pangenome, the gene clusters were binned using the search filters in the Anvi'o interactive interface using the following search conditions: (1) core genes – gene clusters present in minimum of 36 genomes (95%); (2) accessory genes – gene clusters present in minimum of 2 genomes and maximum of 35 genomes; (3) singleton genes – gene clusters present in a maximum of 1 genome using Anvi'o analysis blastp with a minimum bit score of 0.5 for gene clustering

Core Genome Alignment Analysis

The thirty-eight *Campylobacter* strains described previously were also used to determine the core genome alignment using the Harvest software suite (Treangen et al., 2014). The core genome alignment was determined using Parsnp software (v1.1) with the -c flag (ignore MUMi) and the *C. jejuni* str. Guineapig012 as the reference genome. The Parsnp output was visualized with Gingr software (v1.2), and the newick file visualized with MEGA X software (Kumar et al., 2018) for final figure preparation. Additionally, the newick file was visualized with Anvi'o software with the following strain information added as an additional layer: (1) species; (2) subspecies; (3) source; (4) sequence type (ST); (5) clonal complex; (6) cgMLST; (7) percentage of CJIE1 present in genome; (8) percentage of CJIE2 present in genome; (9) percentage of CJIE3 present in genome; (10) percentage of CJIE4 present in genome. The sequence type (ST), clonal complex, and cgMLST for each of the strains was determined by screening each of the genomes against the pubMLST database (https://pubmlst.org/campylobacter/). The percentage of *Campylobacter jejuni* integrated elements (CJIE) 1-4 (CJIE1, CJIE2, CJIE3, and CJIE4) present in each of the genomes was determined by extracting the DNA sequence of each of the four CJIEs from *Campylobacter jejuni* subsp. *jejuni* str. RM1221 and conducting a BLASTn search against each of the genomes using Geneious Prime (v2020.1.2). Percentage represents total percentage of the particular CJIE present in the genome of that strain and is represented in the figure by intensity.

Core and Accessory Genome Analysis

The initial pangenome analysis results generated by Anvi'o were confirmed using Roary and Scoary software. The core and accessory genome of 37 C. *jejuni* strains (the *C. coli* str. RM5611 was excluded from this analysis) were determined at 90% identity using Roary software (v3.12.0) (Page et al., 2015) with the following flags: -e (create a multiFASTA alignment of core genes using PRANK); -n (fast core gene alignment with MAFFT); -v (verbose output to STDOUT); -i 90 (minimum percentage identity for blastp; 90%). The Roary analysis was repeated at the 95% and 85% identity cutoffs to check for any major variations in the core and accessory genomes at the different percentages. The number of core, soft-core, shell and cloud genes as well as the overall core and accessory genome determined by the Roary analysis were visualized using the roary_plots.py script. Genes statistically unique to the two guinea pig associated *C. jejuni* genomes and those genes statistically significantly missing in the two genomes compared to the other 35 C. *jejuni* genomes were identified using the Scoary program (v1.6.16) (Brynildsrud et al., 2016) with the following command: scoary -g 90_gene_presence_absence. csv -t Guinea_pig_traits.csv -o Scoary/90_percent.

RESULTS

C. jejuni Isolates From Guinea Pig Cluster With Others From the Same Host

We collected and sequenced *C. jejuni* isolates from domestic guinea pigs (n=2), domestic chickens (n=3) and domestic dogs (n=3) within Santo Tomas, Peru. The *C. jejuni* isolates were assigned four sequence types (ST) and 5 isolates shared a clonal complex (CC-353) (**Table 1**). The STs of the isolates from the dogs (ST-1036 and ST-10311) and chickens (ST-8741) were identified among several *C. jejuni* in the pubMLST database. Both guinea pig isolates possessed the same novel ST-10316, but some of its rare alleles (glnA538, glyA601 and pgm730) were present in other STs found in a group of guinea pigs isolates from Ecuador (**Supplementary Table 1**). We examined the relationship between these strains, along with a collection of Peruvian clinical *Cjj* strains (Pascoe et al., 2020), and well-studied *Cjj* strains with complete genomes, three *Cjd* strains, and a *C. coli* strain (**Supplementary Table 1**). As the Ecuadorian strains only had data for the 7 MLST gene alleles, the analysis only used the MLST allele sequences from all strains to create a neighbor joining dendrogram (**Figure 1**). The results clearly indicated that there were two large clusters of guinea pig *C. jejuni* isolates. The first cluster was within the *Cjj* clade, and the other cluster that contains the isolates from Peru formed a clade distinct from the other *C. jejuni* subspecies. Moreover, this clade was further away from the *Cjj* clade than the *Cjd* clade (**Figure 1**).

Phylogenetic and ANI Analysis Support a New Subspecies for the Guinea Pig Isolates

To better understand the relationship of the guinea pig isolates with other *C. jejuni*, phylogenetic analysis of those strains with WGS was performed using a set of 62 C. *jejuni/C. coli* core genes that were fairly evenly spaced around the chromosome. As previously mentioned, the genomic data for *C. jejuni* isolates from Ecuador only included the MLST alleles (Graham et al., 2016), and so, these samples were removed from further analysis. Again, the two guinea pig isolates from Peru formed a clade distinct from the other *C. jejuni* subspecies (**Figure 2**). The *C. jejuni* isolates collected from dogs and chickens, and all of the *C. jejuni* Peruvian clinical isolates examined were within the large *Cjj* clade. Average nucleotide identity (ANI) analysis of these genome sequences also provides evidence that the isolates from guinea pig were distinct with ANI values of ∼94-95% with both *Cjj* and *Cjd* genomes. Additionally, the ANI values between the two historic subspecies, *Cjj* and *Cjd*, is ∼95-96% (**Figure 3**).

Genomic Features and Distinctions of the *C. jejuni* Isolated From Guinea Pig

The calculated genome sizes for the two isolates from guinea pig was ~ 1.68 Mb. This genome size is slightly larger than the mean genome size of 1.66 Mb (ranging from 1.61-1.79 Mb) for *Cjj* used in this study. In contrast, the genomes from guinea pig isolates were smaller than the three *Cjd* genomes that had a mean size of 1.79 Mb (ranging from 1.73-1.89 Mb).

We determined the pangenome using Anvi'o software for the 38 *Campylobacter* genomes and found a total of 3,395 gene clusters, where a cluster is a coding gene or a group of paralogous genes. The pangenome consisted of 1,354 clusters as part of the core genome (≥ 95% of genomes), 1,344 gene clusters in the accessory genome, and 697 gene clusters unique to a single genome (singleton). The composition of the gene clusters in the core genome were composed predominately of single copy genes compared to the vast majority of the gene clusters in the accessory genome containing paralogs. As a different species of *Campylobacter*, *C. coli* contained the largest amount of singleton gene clusters followed by *C. jejuni* subsp. *jejuni* str. 1336 (CM000854) that was isolated from a wild bird. Neither of the guinea pig isolates contained a large amount of singleton gene clusters particularly compared to *C. jejuni* subsp *doylei* strains and the *C. coli* strains, which is due to their similarity to each other. In fact, the two guinea pig isolates share a large unique portion of their genomes that are not found in the other 36 genomes in the analysis. Further examination of these guinea pig unique gene clusters shows that there are very few paralogs in the clusters, but there is a high level of geometric and functional homogeneity among the genes in the clusters. Although very few of the genes in these guinea pig unique gene clusters have an actual assigned COG function (**Figure 3**).

To characterize genomic differences between the guinea pig isolates and the other *Campylobacter* genomes used in this study, we determined the presence/absence of the four *Campylobacter jejuni* integrated elements (CJIEs) and the genomes on an individual nucleotide level. Conducting core genome single nucleotide polymorphism (SNP) analysis using one of the guinea pig isolates as the reference demonstrates the two isolates from guinea pigs are very closely related. Furthermore, there are significant SNP differences across the entire guinea pig isolate genome compared to the other *Campylobacter* genomes that further supports these strains are distinct to the guinea pig

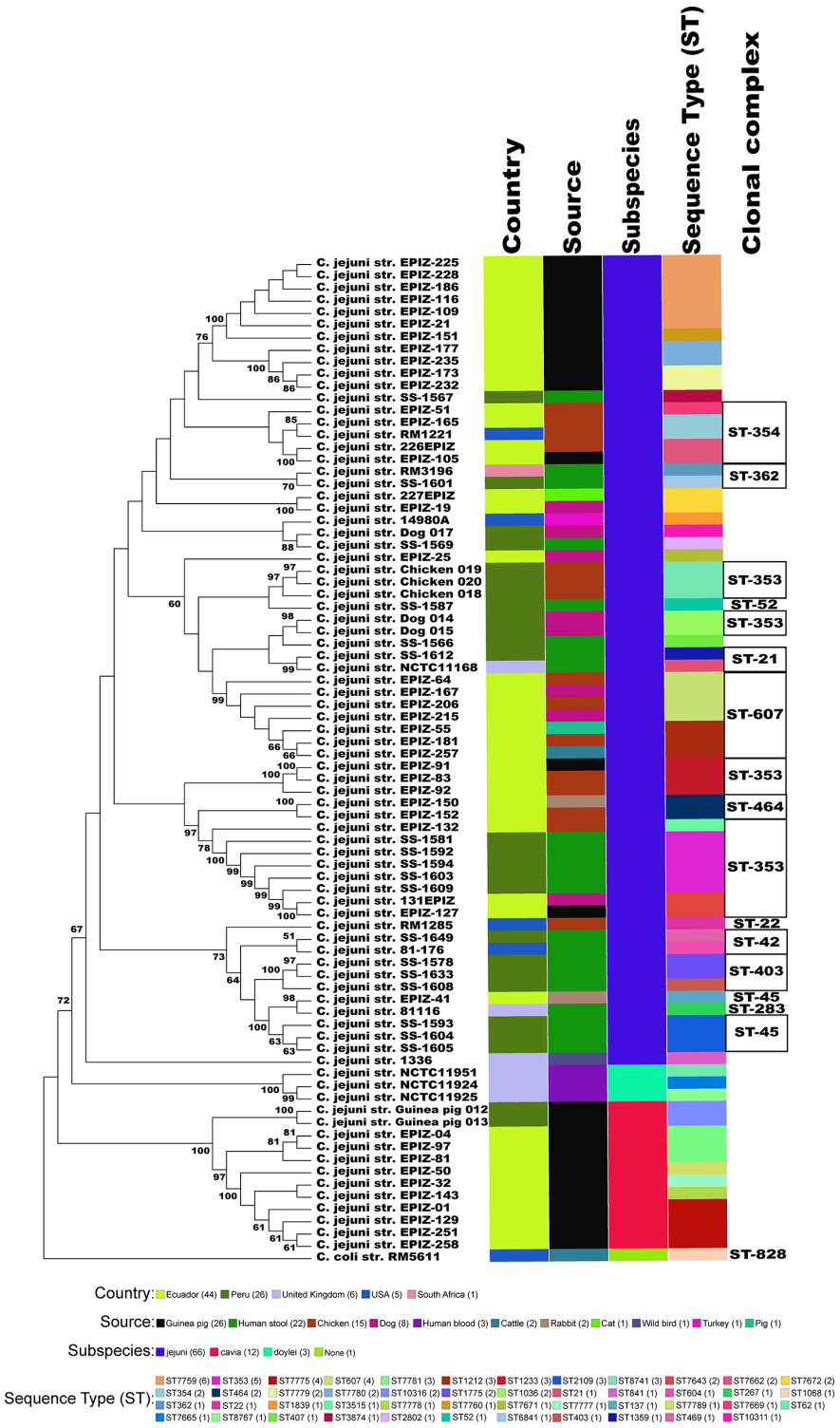

FIGURE 1 | Minimum spanning tree of the MLST results. The concatenated sequences of the 7 MLST alleles (*aspA, glnA, gltA, glyA, pgm, tkt*, and *uncA*) from 82 Campylobacter strains were aligned with MAFFT and a dendrogam was created using neighbor-joining algorithm and the Kimura 2-parameter distance correction model. The concatenated profile sequence for the *C. coli* strain RM5611 (CP007179) was included for comparison. The topology only is shown in the figure. Metadata for isolates including country, isolate source, subspecies and sequence type are color-coded and noted in the key associated with the figure. Clonal complex is also noted in the figure.

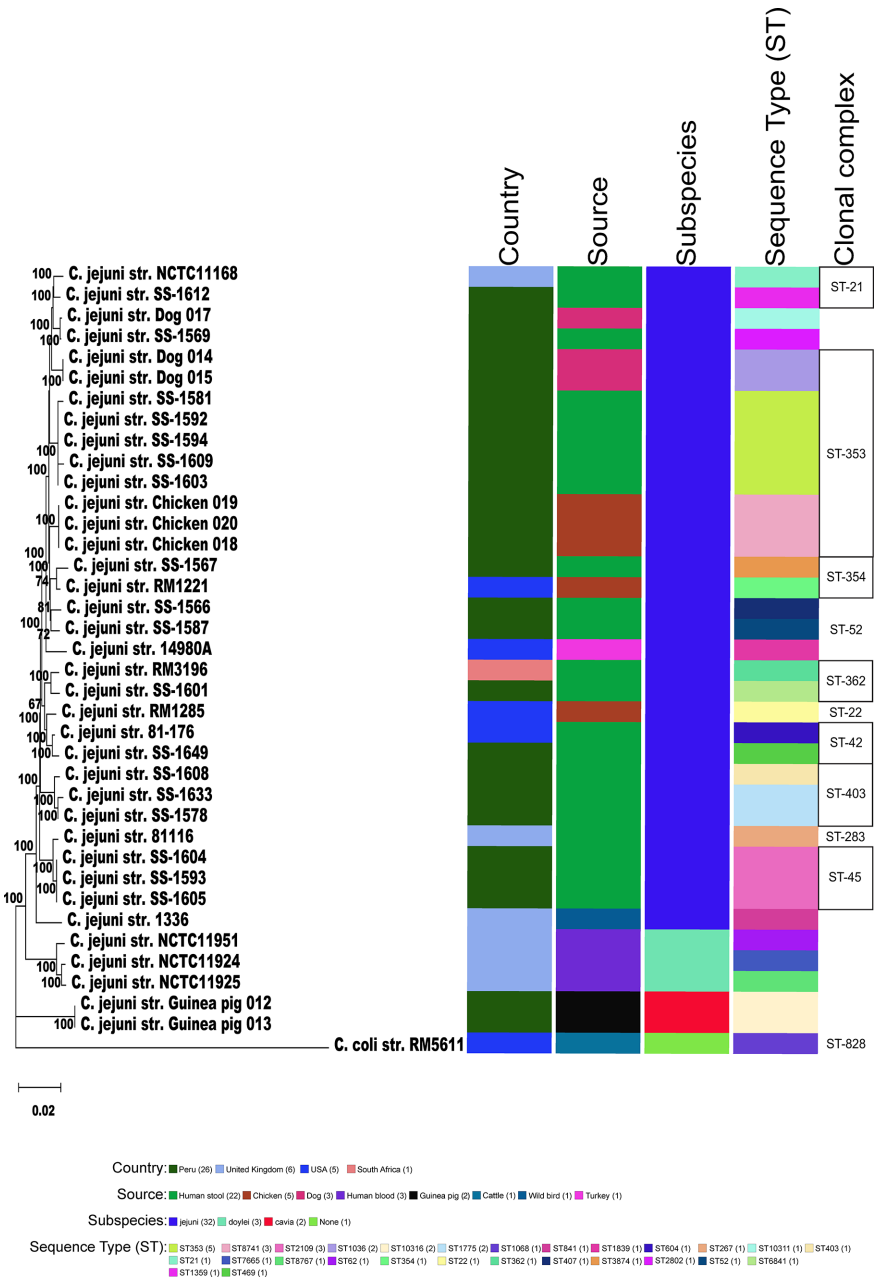

FIGURE 2 | Phylogeny of Campylobacters. A) Sixty-two concatenated core genes (concatenated in the order gyrB, pyrG, aspA, atpA, infB, nrdB, lpxB, fabH, glmM, glyA, pgm, nusA, mqnC, clpB, tatC, kdsC, uvrB, glnA, dnaE, dnaK, msbA, dapA, fliP, trmA, folD, aroA, cheR, purH, argF, livM, cmeD, folC, pssA, waaC, dnaX, cfa, ftsY, groEL, pdxA, pnp, hydA, spoT, rodA, mobA, ppk, fumC, katA, fabI, kpsD, flgI, flgK, cadF, addB, putA, acs, nuoB, rplQ, tkt, recA, murB, gltA, secY) within the genomes of 38 genomic sequences were aligned with MAFFT. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura 2-parameter distance correction model. The topology only is shown in the figure. Bootstrap values of ≥50%, generated from 500 replicates, are shown at the nodes. The concatenated profile sequence for the *C. coli* strain RM5611 (CP007179) was included for comparison. Metadata for isolates including country, isolate source, subspecies and sequence type are color-coded and noted in the key within the figure. Clonal complex is also noted in the figure.

host (**Figure 4**). Additionally, the core and accessory genome mapping further supports the similarity between the two guinea pig isolates and the difference compared to the other 36 *Campylobacter* genomes. Finally, examination of the four CJIEs among the Peruvian strains reveals that CJIE3 is the most common of the elements while CJIE4 is not really found in any of the isolates except *C. jejuni* subsp. *jejuni* str. RM1221. The only one of the four CJIEs that was slightly present in the guinea pig associated isolates were CJIE1, but less than 35% of the integrated element was present in the isolates.

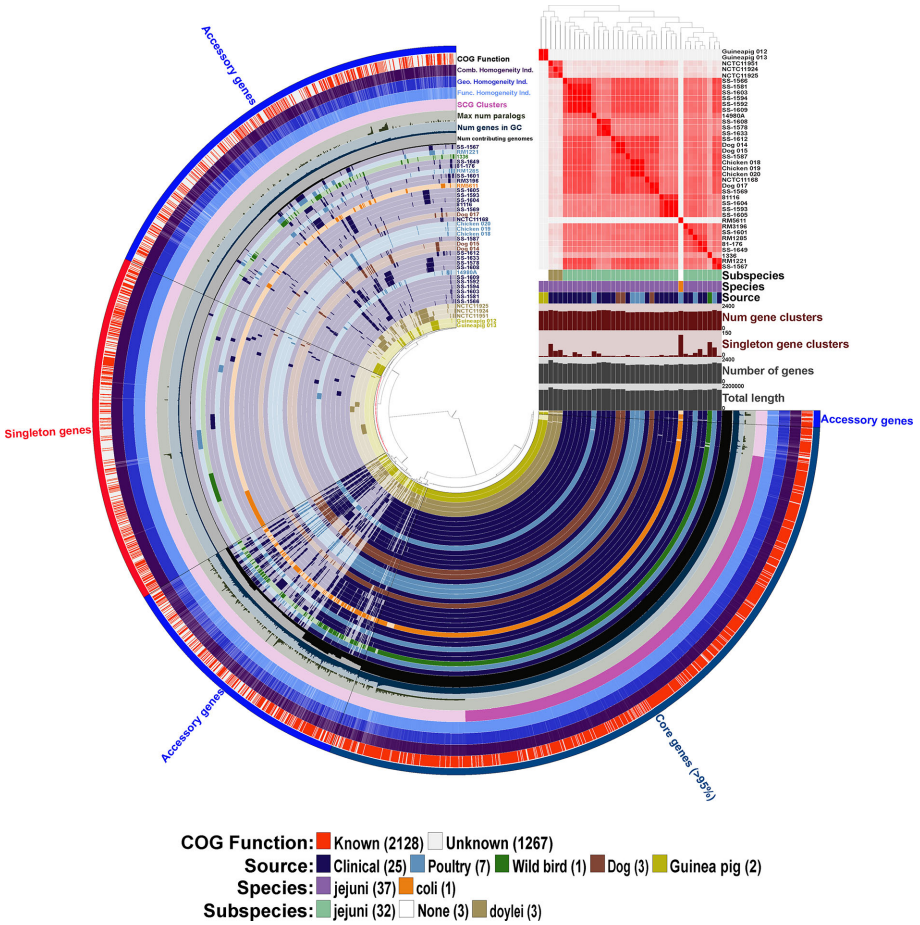

FIGURE 3 | Initial pangenome analysis of *Campylobacter* strains isolated from various Peruvian sources and other previously characterized strains using Anvi'o software. Sample information includes the average nucleotide identity (ANI) for the 38 *Campylobacter* strains using percent identity for the intensity of each square with a >95% cutoff. Subspecies, species and source of isolation for each of the *Campylobacter* strains is included with the color key presented in the legend at the bottom of the figure, additionally the 38 strain layers or rings are colored corresponding to the source of isolation with the exception of the *C. coli* str. RM5611 (CP007179) (colored based on species) and the three *C. jejuni* subsp. *doylei* strains (colored based on subspecies). Sample information also includes for each individual strain the number of gene clusters, number of singleton gene clusters, total number of genes, and total length. The 38 inner rings represent the 38 *Campylobacter* strains and the presence/absence of a particular gene cluster with the dark colored bar representing the gene cluster is present versus the light-colored bar representing the gene cluster is absent from the strain. Dendrogram at the top of the figure organizes the 38 strain rings based on the frequency of 3,395 gene clusters determined in the pangenome analysis, whereas the inner dendrogram represents the relationship of the strains based on the presence/absence of the gene clusters. Each of the 3,395 gene clusters is binned into one of three following categories: (1) core genes (1,354 gene clusters; present in >95% of genomes); (2) accessory genes (1,344 gene clusters; present in 2 < genomes > 35); (3) singleton genes (697 gene cluster; present in only a single genome). The layer or ring immediately after the 38 *Campylobacter* strain layers represents the number of genomes contributing to each of the gene cluster bars in the 38 strain layers (ranging from 1 to 38 genomes). The next layer up signifies the number of genes in each of the gene clusters that the bars in the 38 strain layers represents, and the next two layers above is the maximum number of paralogs for each of the gene clusters and single copy gene clusters, respectively. The next three layers represent the homogeneity of the gene clusters including the functional homogeneity, geometric homogeneity and the combined homogeneity, respectively. The final outer layer displays if the cluster of orthologous groups (COG) function for the gene cluster is known or not, red representing a known function and white an unknown function. The figure and all described analysis was conducted using Anvi'o software (v6.2).

Core Genes and Genes Unique to Guinea Pig Isolates

An additional pangenome analysis was conducted to further characterize these genomes including confirming the Anvi'o results from the initial pangenome analysis, determining a detailed list of specific core and accessory genes, and identifying those genes unique to the distinct *C. jejuni* strains from guinea pigs. The additional pangenome analysis was conducted using the software programs Roary and Scoary on all the *C. jejuni* genomes (*C. coli* was excluded for this analysis) to overall gain a better appreciation of the significant genomic differences of the strains at the individual gene level. Roary analysis using different percentages (85%-95%) of protein identity cutoff found a range of 1,122 – 918 genes in the core genome (≥95% of the genomes) and accessory genomes ranging from 3,676 – 4,909 genes (**Table 2**). Examination of the gene

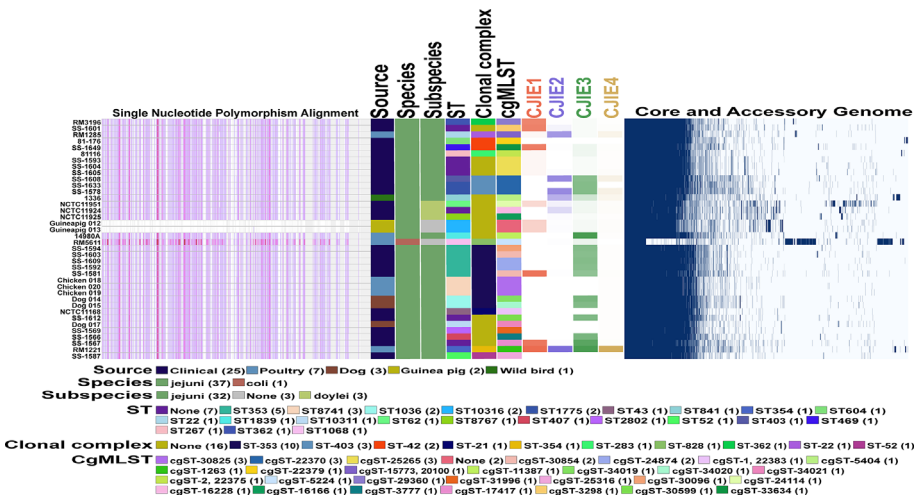

FIGURE 4 | Comparative genomics of Peruvian *Campylobacter jejuni* strains against each other and other *Campylobacter* strains. Core genome phylogeny and single nucleotide polymorphism (SNP) alignments of 38 *Campylobacter* strains using the Harvest software suite. *C. jejuni* str. Guineapig012 was used as the reference strain for analysis. Different characteristics of each of the 38 strains including sequence type (ST) and clonal complex (CC) based on 7 gene multilocus sequence typing (MLST), and the core genome MLST (cgMLST) both using PubMLST database (https://pubmlst.org/) for analysis are shown as follows. Color legend for each section is in the legend in the figure. Intensity mapping of amount of four *Campylobacter jejuni* integrative elements (CJIEs) present in each of the Campylobacter genomes. Percentage of each CJIE present in genome based on nucleotide BLAST (blastn) search against the four full CJIEs present in *Cjj* str. RM1221 using Geneious PRIME software. Visualized comparison of all the core and accessory genes for each of the *Campylobacter* genomes against each other using Roary software.

content differences of the guinea pig distinct isolates with the other *C. jejuni* based on Roary/Scoary analysis demonstrated that there were over 50 genes absent from these isolates, however 55.6% are uncharacterized genes, but does include genes involved in transport, membrane structure, metabolic processes, and translation. Moreover, there were 169 genes that were present only in the guinea pig distinct isolates, but again 84.6% were hypothetical proteins. These Roary/Scoary pangenome results (**Table 3**) were similar to Anvi'o pangenome results (**Figure 3**) that a majority of genes associated only with the guinea pig isolates lacked COG function. However, among genes associated only with the guinea pig isolates there were a few associated with metabolic processes, conjugation, type IV secretion, DNA/nucleotide binding, translation and transmembrane transport (**Table 3**).

Absence of Selenium Utilization Genes Within *C. jejuni* Isolated From Guinea Pig

Initially, the *selA* gene that encodes the selenocysteine synthase was among the 62 C. *jejuni/C. coli* core genes. However, we were unable to find *selA* in either guinea pig isolate. Further analysis demonstrated that these two genomes were devoid of most of the selenium utilization genes at several locations around the genome including the complete absence of *selAB* (**Figure 5A**), *selD* and *yefD* (**Figure 5B**), and a partial deletion within *selU*. Each of these gene products are believed to play a role in the translational incorporation of selenocysteine into certain proteins. The *fdhA* gene that encodes a subunit of formate dehydrogenase is one such selenoprotein, but this gene was also completely absent from the *C. jejuni* genomes isolated from guinea pigs (**Figure 5B**). Moreover, these genomes were also missing other genes that are associated with formate dehydrogenase including *fdhB*, *fdhC*, *fdhD*, *fdhM*, *fdhT*. and *fdhU* (**Figure 5B**). It should be noted that these additional formate dehydrogenase genes are not selenoproteins.

TABLE 2 | Campylobacter jejuni core genes*.

	95% Identity	90% Identity	85% Identity
Core genes[1]	918	1,098	1,122
Soft core genes[2]	62	68	61
Shell genes[3]	1,134	969	941
Cloud genes[4]	3,713	2,875	2,674

[1]*Present in 36 ≤ genomes ≤ 37.*
[2]*Present in 35 ≤ genomes ≤ 36.*
[3]*Present in 5 ≤ genomes ≤ 35.*
[4]*Present in < 5 genomes.*
**Core genome analysis conducted by Roary software.*

Acquisition of the Entner-Doudoroff Pathway

The genomes of the guinea pig distinct isolates both possessed genes for the Entner-Doudoroff pathway, which catalyze the conversion of glucose to pyruvate. Previously, these genes have been identified within certain strains of *Cjj*, *Cjd* and *C. coli* (Vegge et al., 2016). These genes were not among the group of unique genes within the guinea pig *C. jejuni* isolates, as they are also present in the *Cjd* genomes that we examined, but absent from the particular the *Cjj* and *C. coli* in this study.

DISCUSSION

Although there is a high incidence of campylobacteriosis in LMICs, there is limited understanding about the zoonotic

TABLE 3 | *Campylobacter jejuni* strains associated with guinea pig related genes*.

Genes present only in C. jejuni strains associated with guinea pig (169)	
GO Function/Category	
Hypothetical protein (143)	Tricarboxylic acid cycle (1)
Propionate catabolic process (1)	Oxidation-reduction process (2)
Conjugation (3)	Nucleotide binding (1)
Oxygen transport (1)	Membrane (2)
Carbohydrate metabolic process (1)	Transcription regulation (1)
Transmembrane transport (1)	DNA binding (1)
Transferase activity (2)	Isomerase activity (2)
DNA repair (1)	Translation (2)
Type IV secretion system (3)	N-acetyltransferase activity (1)
Genes absent in C. jejuni strains associated with guinea pig (54)	
GO Function/Category	
Hypothetical protein (30)	Translation (2)
Signal transduction (3)	C4-dicarboxylate transport (1)
Oxygen transport (1)	Oxidation-reduction process (4)
Membrane (5)	Siderophore-dependent iron import (1)
Transmembrane transport (2)	DNA repair (1)
Spermidine transport (2)	Transcription regulation (1)
M-molybdopterin cofactor biosynthetic process (1)	

*Based on 90% amino acid identity using Roary/Scoary software for analysis.

sources of *Campylobacter*. During a collection of samples from possible host animals in the town of Santo Tomas in Amazonian Peru, we were able to isolate *C. jejuni* strains from guinea pig, chicken and dog samples. By analyzing and comparing the WGS of these *C. jejuni* strains with other genomes, we provided evidence that the two strains isolated from guinea pigs from Peru were distinct to that particular host.

The Peruvian Amazon guinea pig isolates had a new unique ST among the more than 95,000 pubMLST samples but shared several MLST alleles with other *C. jejuni* strains previously isolated from guinea pigs in Ecuador (Graham et al., 2016; Vasco et al., 2016). From a neighbor-joining tree based on MLST alleles, most of the guinea pig isolates clustered into two distinct guinea pig specific lineages (**Figure 1**). The cluster that contained the *C. jejuni* strains from guinea pigs isolated in this study and several other guinea pig specific *C. jejuni* strains (Graham et al., 2016; Vasco et al., 2016), is distinct from both *Cjj* and *Cjd* clusters. The guinea pig specific *C. jejuni* lineage is also supported by phylogenetic analysis of 62 core genes and ANI analysis. Again, the results from these two distinct analyses positions the two *C. jejuni* strains from guinea pigs away from other *C. jejuni* strains.

The guinea pig isolates that we sequenced showed evidence of both gene loss and gene addition that help support host adaption. The deletion of multiple genes involved in biosynthesis and utilization of selenocysteine is the most apparent difference when compared to other *C. jejuni* strains. The absence of genes involved in selenium utilization and formate dehydrogenase (a selenocysteine containing enzyme) are not novel occurrences among *Campylobacter*. In fact, the many genomes of the *C. lanienae* clade within the *C. fetus* group are missing many of these same genes (Miller et al., 2017). Guinea pigs are known to have a low selenium dietary requirement with poor selenium dietary reserve. It has been hypothesized that microbial strains that colonize guinea pigs may have selective pressure to favor enzyme pathways that are not selenium dependent (Jensen and Pallauf, 2008). Genes also absent were the *panBCD* genes encoding the vitamin B5 biosynthesis pathway. that are associated with *Cjj* strains that are adapted to cattle (Sheppard et al., 2013). It was suggested that higher levels of vitamin B5 in the diet of poultry, as compared to grass-fed cattle, has made the *panBCD* genes dispensable in poultry specific *Cjj* strains (Sheppard et al., 2013), and this appears to also be true for the isolates from guinea pigs. Finally, the presence of the Entner-Douderoff pathway genes suggest that glucose may be utilized as a carbon source and may also provide fitness advantages to the guinea pig specific *C. jejuni* strains as was observed by Vegge et al. for other *Campylobacters* (Vegge et al., 2016).

Zoonotic host adaption and restriction among *Campylobacter* strains is important to characterize since it aids in the determination of the origin of human infections in settings where there are multiple putative sources of infection. This is important not only in the US and Europe, but across a wider geography and host range in order to inform strategies of disease control in diverse settings. Although this is not a genome-wide association study, the analysis provides initial evidence for genetic factors that lead to the development of a host-specific *C. jejuni*. Microbial adaptation to zoonotic hosts leading to host specialism has been described for the *Campylobacter* isolates from cattle, poultry, and wild birds (Sheppard et al., 2010; Sheppard et al., 2011; Griekspoor et al., 2013; Sheppard et al., 2013; Mourkas et al., 2020). In this study, we identified several host specializing genomic determinants within *C. jejuni* isolates from guinea pigs that may help in source

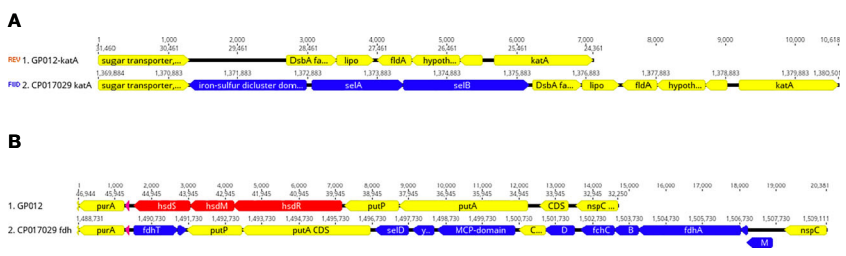

FIGURE 5 | Deletion of selenium utilization and formate dehydrogenase genes. **(A)** Schematic representation demonstrating the deletion of *selAB* from the genomic region adjacent *katA* within the guinea pig isolate and this same region from the genome of *Cjj* strain 14980A that possesses *selAB*. **(B)** Schematic representation demonstrating the deletion of three gene clusters: 1) *fdhTU*, 2) *selDyefD*, and 3) *fdhDCBA* and *fdhM* in a genomic region between *purA* and *nspC* within the guinea pig isolate and this same region in *Cjj* strain 14980A.

attribution. It appears that the sequestration of *C. jejuni* within the guinea pig host has provided an appropriate barrier and created a particular niche to proliferate this lineage. We only report the genome sequences of two guinea pig host-associated strains, thus, sequencing and phenotypic analysis of additional C. jejuni isolates from guinea pigs will be necessary to verify these strains as host specialists and to determine if they form a new subspecies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JACRSF000000000 JACRSG000000000 JACRSH000000000 JACRSI000000000 JACRSJ000000000 JACRSK000000000 JACRSL000000000 JACRSM000000000.

AUTHOR CONTRIBUTIONS

Conceptualization: CP and MK. FS and MK led collection of the isolates. HG sequenced the isolates. Formal Analysis: KC, CP, WM, SH, FS, DT, PB, and P-PY. Original Draft Preparation: CP, KC, FS, and MK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.607747/full#supplementary-material

REFERENCES

Atterby, C., Mourkas, E., Meric, G., Pascoe, B., Wang, H., Waldenstrom, J., et al. (2018). The Potential of Isolation Source to Predict Colonization in Avian Hosts: A Case Study in *Campylobacter jejuni* Strains From Three Bird Species. *Front. Microbiol.* 9, 591. doi: 10.3389/fmicb.2018.00591

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Brynildsrud, O., Bohlin, J., Scheffer, L., and Eldholm, V. (2016). Rapid scoring of genes in microbial pan-genome-wide association studies with Scoary. *Genome Biol.* 17, 238. doi: 10.1186/s13059-016-1108-8

Burakoff, A., Brown, K., Knutsen, J., Hopewell, C., Rowe, S., Bennett, C., et al. (2018). Outbreak of Fluoroquinolone-Resistant *Campylobacter jejuni* Infections Associated with Raw Milk Consumption from a Herdshare Dairy - Colorado, 2016. *MMWR Morb. Mortal. Wkly. Rep.* 67, 146–148. doi: 10.15585/mmwr.mm6705a2

Centers for Disease C, Prevention (2013). Incidence and trends of infection with pathogens transmitted commonly through food - foodborne diseases active surveillance network, 10 U.S. sites, 1996-2012. *MMWR Morb. Mortal. Wkly. Rep.* 62, 283–287.

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

Delmont, T. O., and Eren, A. M. (2018). Linking pangenomes and metagenomes: the Prochlorococcus metapangenome. *PeerJ* 6, e4320. doi: 10.7717/peerj.4320

Dingle, K. E., Colles, F. M., Wareing, D. R., Ure, R., Fox, A. J., Bolton, F. E., et al. (2001). Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* 39, 14–23. doi: 10.1128/JCM.39.1.14-23.2001

Eren, A. M., Esen, O. C., Quince, C., Vineis, J. H., Morrison, H. G., Sogin, M. L., et al. (2015). Anvi'o: an advanced analysis and visualization platform for 'omics data. *PeerJ* 3, e1319. doi: 10.7717/peerj.1319

Eurosurveillance Editorial Team (2015). The 2013 joint ECDC/EFSA report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks published. *Euro. Surveill.* 20, 1–23. doi: 10.2807/ese.20.04.21021-en

Friedman, C. R., Neiman, J., Wegener, H. C., and Tauxe, R. V. (2000). "Epidemiology of Campylobacter jejuni infections in the United States and other industrialised nations," in *Campylobacter*. Eds. I. Nachamkin and M. J. Blaser (Washington, DC: ASM Press).

Graham, J. P., Vasco, K., and Trueba, G. (2016). Hyperendemic *Campylobacter jejuni* in guinea pigs (*Cavia porcellus*) raised for food in a semi-rural community of Quito, Ecuador. *Environ. Microbiol. Rep.* 8, 382–387. doi: 10.1111/1758-2229.12396

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

Jain, C., Rodriguez, R. L., Phillippy, A. M., Konstantinidis, K. T., and Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* 9, 5114. doi: 10.1038/s41467-018-07641-9

Jensen, C., and Pallauf, J. (2008). Estimation of the selenium requirement of growing guinea pigs (*Cavia porcellus*). *J. Anim. Physiol. Anim. Nutr. (Berl).* 92, 481–491. doi: 10.1111/j.1439-0396.2007.00738.x

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* 3, 124. doi: 10.12688/wellcomeopenres.14826.1

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

Kasper, G., and Dickgiesser, N. (1985). Isolation from gastric epithelium of Campylobacter-like bacteria that are distinct from "Campylobacter pyloridis". *Lancet* 1, 111–112. doi: 10.1016/S0140-6736(85)92005-7

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Lastovica, A. J., and Skirrow, M. B. (2000). "Clinical significance of Campylobacter and related species other than Campylobacter jejuni and C. coli," in *Campylobacter*. Eds. I. Nachamkin and M. J. Blaser (Washington, DC: ASM Press), 89–120.

Lawton, S. J., Weis, A. M., Byrne, B. A., Fritz, H., Taff, C. C., Townsend, A. K., et al. (2018). Comparative analysis of *Campylobacter* isolates from wild birds and chickens using MALDI-TOF MS, biochemical testing, and DNA sequencing. *J. Vet. Diagn. Invest.* 30, 354–361. doi: 10.1177/1040638718762562

Lowenstein, C., Waters, W. F., Roess, A., Leibler, J. H., and Graham, J. P. (2016). Animal Husbandry Practices and Perceptions of Zoonotic Infectious Disease Risks Among Livestock Keepers in a Rural Parish of Quito, Ecuador. *Am. J. Trop. Med. Hyg.* 95, 1450–1458. doi: 10.4269/ajtmh.16-0485

Miller, W. G., Parker, C. T., Heath, S., and Lastovica, A. J. (2007). Identification of genomic differences between *Campylobacter jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* at the nap locus leads to the development of a *C. jejuni* subspeciation multiplex PCR method. *BMC Microbiol.* 7, 11 doi: 10.1186/1471-2180-7-11

Miller, W. G., Yee, E., Lopes, B. S., Chapman, M. H., Huynh, S., Bono, J. L., et al. (2017). Comparative Genomic Analysis Identifies a *Campylobacter* Clade Deficient in Selenium Metabolism. *Genome Biol. Evol.* 9, 1843–1858. doi: 10.1093/gbe/evx093

Montgomery, M. P., Robertson, S., Koski, L., Salehi, E., Stevenson, L. M., Silver, R., et al. (2018). Multidrug-Resistant *Campylobacter jejuni* Outbreak Linked to Puppy Exposure - United States, 2016-2018. *MMWR Morb. Mortal. Wkly. Rep.* 67, 1032–1035. doi: 10.15585/mmwr.mm6737a3

Mourkas, E., Taylor, A. J., Meric, G., Bayliss, S. C., Pascoe, B., Mageiros, L., et al. (2020). Agricultural intensification and the evolution of host specialism in the enteric pathogen *Campylobacter jejuni*. *Proc. Natl. Acad. Sci. U.S.A.* 117, 11018–11028. doi: 10.1073/pnas.1917168117

Mutschall, S. K., Hetman, B. M., Bondo, K. J., Gannon, V. P. J., Jardine, C. M., and Taboada, E. N. (2020). *Campylobacter jejuni* Strain Dynamics in a Raccoon (*Procyon lotor*) Population in Southern Ontario, Canada: High Prevalence and Rapid Subtype Turnover. *Front. Vet. Sci.* 7, 27. doi: 10.3389/fvets.2020.00027

Nauta, M., Hill, A., Rosenquist, H., Brynestad, S., Fetsch, A., van der Logt, P., et al. (2009). A comparison of risk assessments on *Campylobacter* in broiler meat. *Int. J. Food Microbiol.* 129, 107–123. doi: 10.1016/j.ijfoodmicro.2008.12.001

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Parker, C. T., Miller, W. G., Horn, S. T., and Lastovica, A. J. (2007). Common genomic features of *Campylobacter jejuni* subsp. *doylei* strains distinguish them from *C. jejuni* subsp. *jejuni*. *BMC Microbiol.* 7, 50. doi: 10.1186/1471-2180-7-50

Pascoe, B., Schiaffino, F., Murray, S., Meric, G., Bayliss, S. C., Hitchings, M. D., et al. (2020). Genomic epidemiology of *Campylobacter jejuni* associated with asymptomatic pediatric infection in the Peruvian Amazon. *PloS Negl. Trop. Dis.* 14, e0008533. doi: 10.1371/journal.pntd.0008533

Pitkanen, T. (2013). Review of *Campylobacter* spp. in drinking and environmental waters. *J. Microbiol. Methods* 95, 39–47. doi: 10.1016/j.mimet.2013.06.008

Platts-Mills, J. A., and Kosek, M. (2014). Update on the burden of *Campylobacter* in developing countries. *Curr. Opin. Infect. Dis.* 27, 444–450. doi: 10.1097/QCO.0000000000000091

Platts-Mills, J. A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havt, A., et al. (2015). Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *Lancet Glob. Health* 3, e564–e575. doi: 10.1016/S2214-109X(15)00151-5

Sheppard, S. K., Colles, F., Richardson, J., Cody, A. J., Elson, R., Lawson, A., et al. (2010). Host association of *Campylobacter* genotypes transcends geographic variation. *Appl. Environ. Microbiol.* 76, 5269–5277. doi: 10.1128/AEM.00124-10

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

Sheppard, S. K., Didelot, X., Meric, G., Torralbo, A., Jolley, K. A., Kelly, D. J., et al. (2013). Genome-wide association study identifies vitamin B5 biosynthesis as a host specificity factor in *Campylobacter*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11923–11927. doi: 10.1073/pnas.1305559110

Sheppard, S. K., Cheng, L., Meric, G., de Haan, C. P., Llarena, A. K., Marttinen, P., et al. (2014). Cryptic ecology among host generalist *Campylobacter jejuni* in domestic animals. *Mol. Ecol.* 23, 2442–2451. doi: 10.1111/mec.12742

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

Steele, T. W., Lanser, J. A., and Sangster, N. (1985). Nitrate-negative campylobacter-like organisms. *Lancet* 1, 394. doi: 10.1016/S0140-6736(85)91410-2

Toledo, Z., Simaluiza, R. J., Astudillo, X., and Fernandez, H. (2017). Occurrence and antimicrobial susceptibility of thermophilic *Campylobacter* species isolated from healthy children attending municipal care centers in Southern Ecuador. *Rev. Inst. Med. Trop. Sao Paulo* 59, e77. doi: 10.1590/s1678-9946201759077

Treangen, T. J., Ondov, B. D., Koren, S., and Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 15, 524. doi: 10.1186/s13059-014-0524-x

Vasco, K., Graham, J. P., and Trueba, G. (2016). Detection of Zoonotic Enteropathogens in Children and Domestic Animals in a Semirural Community in Ecuador. *Appl. Environ. Microbiol.* 82, 4218–4224. doi: 10.1128/AEM.00795-16

Vegge, C. S., Jansen van Rensburg, M. J., Rasmussen, J. J., Maiden, M. C., Johnsen, L. G., Danielsen, M., et al. (2016). Glucose Metabolism *via the* Entner-Doudoroff Pathway in *Campylobacter*: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates. *Front. Microbiol.* 7, 1877. doi: 10.3389/fmicb.2016.01877

Zhou, Z., Alikhan, N. F., Sergeant, M. J., Luhmann, N., Vaz, C., Francisco, A. P., et al. (2018). GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. *Genome Res.* 28, 1395–1404. doi: 10.1101/gr.232397.117

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